# ATTEMPTED PRODUCTION OF MYASTHENIA GRAVIS IN THE RAT

J. D. PARKES

*Department of Physiology, St. Bartholomew's Hospital Medical College, London*

Received for publication May 4, 1966.

THE defect in myasthenia gravis is primarily neuromuscular in site. This is shown by the ameliorating effect of drugs that act at the neuromuscular junction, and suggested by the clinical features of fatigue of skeletal muscle with recovery following rest. Motor end-plate abnormalities are seen microscopically (Coers and Desmedt, 1959 ; McDermot, 1960). End-plate disfunction has been shown by Dahlback, Elmquist, Johns, Radner and Thesleff, (1961), who demonstrated a reduction in frequency of discharge of spontaneous miniature end-plate potentials. This suggests that the abnormality is at a presynaptic level. Alajouanine, Scherrer and Bourguignon (1959), recording ulnar nerve potentials in human myasthenic subjects showed that there was no failure of the motor nerve trunk.

It has been suggested that myasthenia gravis results from an abnormality of the immune mechanism, and is auto-immune in nature (Simpson, 1960 ; Burnet, 1962). The defect being neuromuscular in site, so any theoretical antibody should be directed against specific neuromuscular junction region antigens other than against those of nerve or muscle. If this is so it should be possible to provoke myasthenia experimentally by antigenic stimulation using motor end-plate region antigens. Witebsky, Rose, Terplan, Paine and Egan, (1957) require such an experimental demonstration as part of the proof that a disease is auto-immune in nature.

Thus animal responses to an antigen rich in muscle end-plates were studied. The precise specificity of mammalian presynaptic neuromuscular junction region proteins is not at present known and a convenient method for their preparation from mammalian species, not available. Thus a mixed muscle antigen was used being prepared from muscle regions rich in end-plates. Animals were injected, not with their own muscle, but with that from the same species. This, therefore, formed an immune rather than an auto-immune study. An attempt was made to assess to what degree any changes occurring in the experimental animal resembled those of human myasthenia gravis.

## MATERIALS AND METHODS

*Antigen preparation.*—Fragments of muscle from Wistar albino rats of both sexes were used. The gracilis muscle of the rat shows 2 regions of localized end-plate concentration. These are situated $\frac{1}{3}$ and $\frac{2}{3}$ along its length. Here the end-plates are grouped into narrow bands with a width of about 1 mm. Small junctional portions of muscle from these regions, 3–4 mm. wide (in the resting state) were removed. To check the area removed, end-plate staining by the method of Coers and Woolf (1959) was done using part of the sample. These muscle fragments were then emulsified in 5 times their own volume of sterile distilled water. This homogenation was done using firstly a rotating cutting machine, and then a grinding piston. One portion of this muscle mash was used in this state. To a second portion, half its own volume of finely precipitated hydrated aluminium phosphate was added to act as an adjuvant.

Present Address:—Department of Neurosurgery and Neurology, Addenbrooke's Hospital, Cambridge.

*Immunization schedule.*—Wistar albino rats of both sexes, aged 3–6 months were used. They were given weekly injections on 4 occasions of 0·5 ml. of muscle mash, beneath the dorsal skin. A total of 20 animals received muscle with no adjuvant, 8 being given the adjuvant compound. Rats of the same species were used as controls, being of about the same weight at time of test. They received no injections of muscle mash. Three animals were given injections of hydrated aluminium phosphate alone.

*Test procedure.*—During the 8th week following the initial injection, the neuromuscular responses of the sensitized animals were studied. The rats were anaesthetized with ether and pentobarbitone. The tension developing in the left tibialis anterior as a result of supramaximal nerve stimulation was then measured, using a recording spring lever, directly calibrated with weights. A resting tension of 30–70 g. was applied to the muscle. The nerve was stimulated by a square wave pulse of 45 micro sec. duration at a frequency of 15 cycles/sec.; giving a rapid single twitch response; and with a frequency of 63 cycles/sec. resulting in a completely fused tetanus. Stimulation at these rates was done for a period of 5 min. Following this a concentric needle electrode was implanted in the tibialis anterior muscle, and the electro-myogram recorded during nerve stimulation, using a cathode ray oscilloscope.

Following these initial groups of stimuli, the left common carotid artery was cannulated in a retrograde direction. Artificial ventilation was commenced, and the nerve stimulated at a frequency of 60 cycles/min. D-tubo-curare (5$\mu$g./ml.) was then injected over the course of 30 sec. until a 50 per cent decrease in contraction height was obtained. The sensitized rats were thus tested, and an analysis made of the greatest initial muscular tension, and the degree of fatigue of this with time. These results were compared with those from the control animals, and those animals that had received adjuvant alone.

Specimens of skeletal muscle for histological examination were then taken from the right tibialis anterior (that muscle which had not been stimulated) and from the lateral thigh muscles. After formalin fixation, sections for microscopy were prepared by staining with haematoxylin and eosin. Sections of cardiac muscle, skin and thymus were also prepared.

## RESULTS

The sensitized animals at the 8th week showed no demonstrable weakness during eating and playing. The skin hairs had a coarser texture than those of normal rats. There was a slight and patchy loss of abdominal and dorsal hair in some animals. All rats increased in weight during this 8 week period. They were as active in their cages in the evening as in the morning and no obvious signs of muscle fatigue were seen.

*Tests of muscle power.*—Results are given in terms of the ratio of maximal muscle tension developing (to nerve stimulation), to the total body weight of the animal. There was not a close correlation between tension developed by rats of approximately the same weight in either control or sensitized groups—the scatter was wider, however, in the sensitized group. The mean initial tension developing, and its decline with time, is shown in Figs. 1 and 2. The results with single and tetanic rates of stimuli were similar in both groups. The maximal muscle tension exerted by the sensitized series expressed in these terms is lower than the tension developing in the control group. There is no significant difference, however, in the rate of decline of tension over a 5 min. period, in the 2 groups. The ratio of percentage decline of muscle tension, with time, between control and sensitized animals, remains close to unity. This indicates the similarity of muscle fatigue in both cases.

In 3 of the sensitized animals a rapid decline in tetanic tension was seen. This was never apparent in the control group. This is illustrated in Fig. 3.

A very rapid nerve stimulus rate of 1184 cycles/sec. was used in some cases. This is at about the maximum speed at which the nerve can convey separate

impulses. In both groups, a maximum tetanic tension was produced, which fell over the ensuing 45 sec. to the resting level. In both control and sensitized series, this resting level was reached after the same time interval; showing no abnormality of fatigue with this abnormal method of stimulation.

No abnormal insertion activity was seen in the anaesthetized animal, with the emplacement of a concentric needle electrode. The changes in muscle action potential voltage with continued nerve stimulation at 15 cycles/sec., are shown in Fig. 4. There is a rapid initial decline in amplitude of action potential voltage in both groups; this voltage then remains constant following about 30 sec.

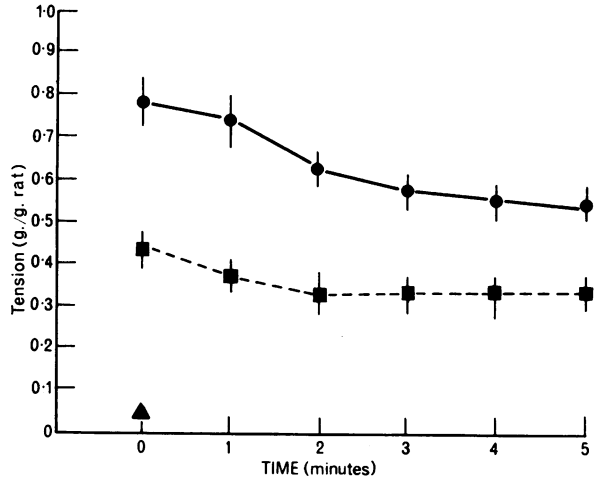



FIG. 1.—Graph showing the mean decline (± S.D.) in tetanic tension with time in the tibialis anterior muscle of sensitized and control rats.

● — control ■ — sensitised

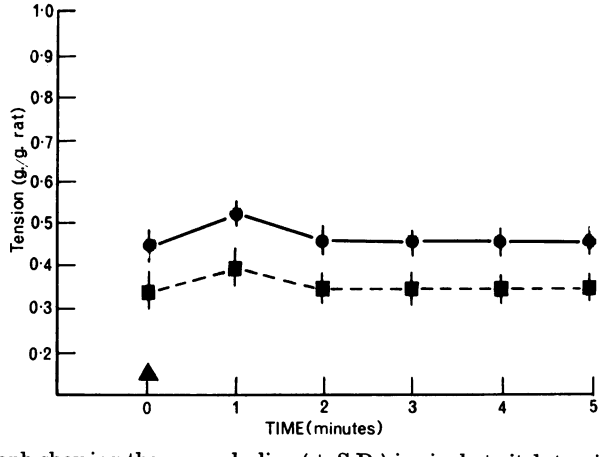



FIG. 2.—Graph showing the mean decline (± S.D.) in single twitch tension with time in the tibialis anterior muscle of sensitized and control rats.

● — control ■ — sensitised

initial stimulus. In the later period of stimulation, there was no demonstrable fatigue of muscle action potential, in the sensitized series.

*Response to curare.*—All the rats had received the same quantity of stimuli before curare was given. There was no difference between the rate of production

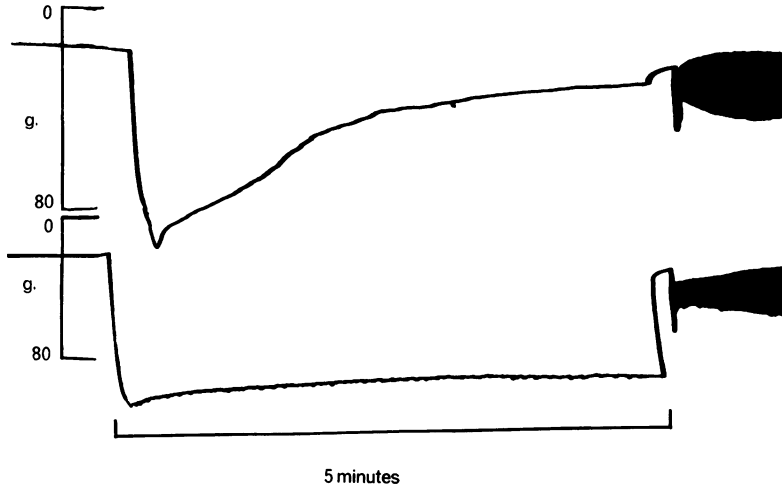



FIG. 3.—Kymograph tracing of tibialis anterior contraction with nerve stimulation (rate = 63 cycles/sec) Upper trace muscle—sensitized, lower trace control rat. This rapid fatigue of tetanus was only seen in 3 experiments with sensitized rats.

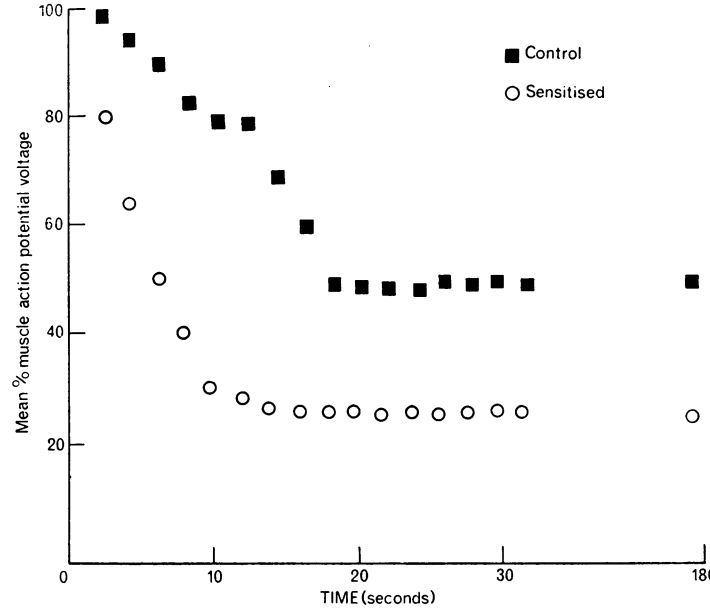



FIG. 4.—Graph showing the mean per cent decline in muscle action potential voltage (produced by nerve stimulation), with time, in sensitized and control rats. Stimulus rate = 15 cycles/second.

or the degree of muscular paresis, produced by a given dose of curare, in control and sensitized groups. Twenty sensitized rats all showed a 50 per cent or greater, decline in contraction height following the injection of between 3–5 $\mu$g. curare. Eighteen controls showed similar results. The remaining 2 required 7·5 $\mu$g. of curare to effect a similar degree of paresis. Thus there was no abnormal sensitivity to curare shown. The weights of rats in both groups were closely comparable.

The aminals which had received aluminium phosphate injections alone, showed no difference in behaviour from those in the control group ; whilst those receiving both muscle and adjuvant, showed similar changes to those occurring in animals that had received muscle alone.

To study any possible change provoked at the neuromuscular junction during antigen-antibody reaction (of an acute nature) *in vivo*, rats sensitized to egg albumin were studied. During the course of nerve stimulation, 1 ml. of a 1/1000 dilution of egg albumin in normal saline, was injected intraarterially. Throughout the experiments, the tension of muscle contraction remained constant. This would indicate that a (non-muscle specific) antibody, reacting with corresponding antigen, does not cause disturbance of neuromuscular function.

*Histological changes in the sensitized animals.*—There was no obvious macroscopic abnormality of the skeletal muscle of the sensitized animals.

The main skeletal muscle changes seen microscopically were localized and generalized muscular atrophy ; variation in muscle fibre diameter ; occasional and diffuse calcification ; with some evidence of fibre regeneration. Cellular infiltration was not a prominent feature.

The most striking change was one of a localized and generalized muscle fibre degeneration. There was a slight to moderate muscle fibre degeneration in all the skeletal muscles examined. The degree of muscle degeneration varied considerably from one specimen to another—both in the same, and from different animals. The earliest change was one of loss of fibre striation, the sarcoplasm having a hyaline appearance, and being more refractile than normal muscle tissue. In these areas, the muscle nuclei were pyknotic, with a dense chromatin network. Many areas showed more striking changes of diffuse fibre vacuolation, extending for either a short distance, or more rarely for a greater length of the fibre. These changes were widespread, and scattered at random between many areas of relatively normal looking fibres. The sarcolemmal sheath appeared swollen in areas, with underlying muscle tissue showing multiple vacuoli. Occasionally, an isolated bundle of muscle fibres all showing hyaline degeneration, was seen. Some specimens of muscle showed changes of greater severity with a considerable loss of muscle fibre over a wide area.

There was a considerable variation in muscle fibre diameter. This change again appeared of random distribution throughout the specimen. The diameter variation exceeded that seen in the normal rat tibialis. There was a little relationship between fibre enlargement, and accompanying atrophy. The distribution of these changes can be interpreted as showing widespread primary muscle disease, rather than individual motor-unit involvement.

Several fibres throughout each section showed increased basophilism. These fibres were mainly small in diameter, and the basophilic colouration associated with some increase in small densely staining, sub-sarcolemmal nuclei. This probably forms evidence of fibre regeneration. These changes were not marked. Several

fibres appeared to show longitudinal splitting; it was difficult to be sure of this without serial sections.

Intramuscular calcification was a not uncommon finding. The calcium was deposited in small plaques with no obvious central nidus of degenerate fibre. Calcification occurred diffusely throughout the specimen. It was not localized to any one area, and occurred both in relationship to small blood vessels, and also in the muscle fibre plane, between fibres and apparently in relationship to a necrotic fibre. There was no, or little, cellular reaction. The presence of calcium was confirmed by staining with alizarin.

There was a very little evidence of cellular infiltration. No isolated islets of small round cell infiltration, corresponding to a "lymphorrhage" were seen. Occasional cellular cuffs around small arterioles and capillaries were present, and these collections were formed of small round cells, the rare plasma cell and eosinophil. There was some evidence of atrophic fibre phagocytosis, with an occasional polymorph or macrophage seen bordering areas of degenerate fibre. An increase in connective tissue was seen only in those muscles showing the greatest degree of fibre degeneration. In local areas of such degeneration there was present more collagen than would result from empty endomysial sheaths. There was no obvious attempt at organization in these areas. There was no evident alteration in muscle vascular supply.

Sections of cardiac muscle showed no departure from the normal. There was no suggestion of fibre atrophy, nor of any cellular infiltration.

There were no obvious histological changes in the thymus. The thymic gland of the normal rat gives an appearance of considerable activity with several germinal centres. The incidence of secondary centres in the sensitized animals' thymus was not significantly greater than that found in normal controls. There was no obvious difference in the histological structures of the skin.

## DISCUSSION

Myasthenia is not known to occur naturally in any animal species other than man (apart from 2 suggestive descriptions of myasthenia in dogs: Ormrod, 1961; Hall and Walker, 1962). However, the abnormality of muscle fatigue and the histological changes seen in (although not necessarily characteristic of) the skeletal muscle of human myasthenia were not produced in these experiments in rats.

Russell (1953), described the microscopic changes commonly seen in the skeletal muscle in myasthenia gravis. She described muscle fibres showing acute coagulative necrosis with sometimes eosinophilic change and loss of cross striation.

## EXPLANATION OF PLATES

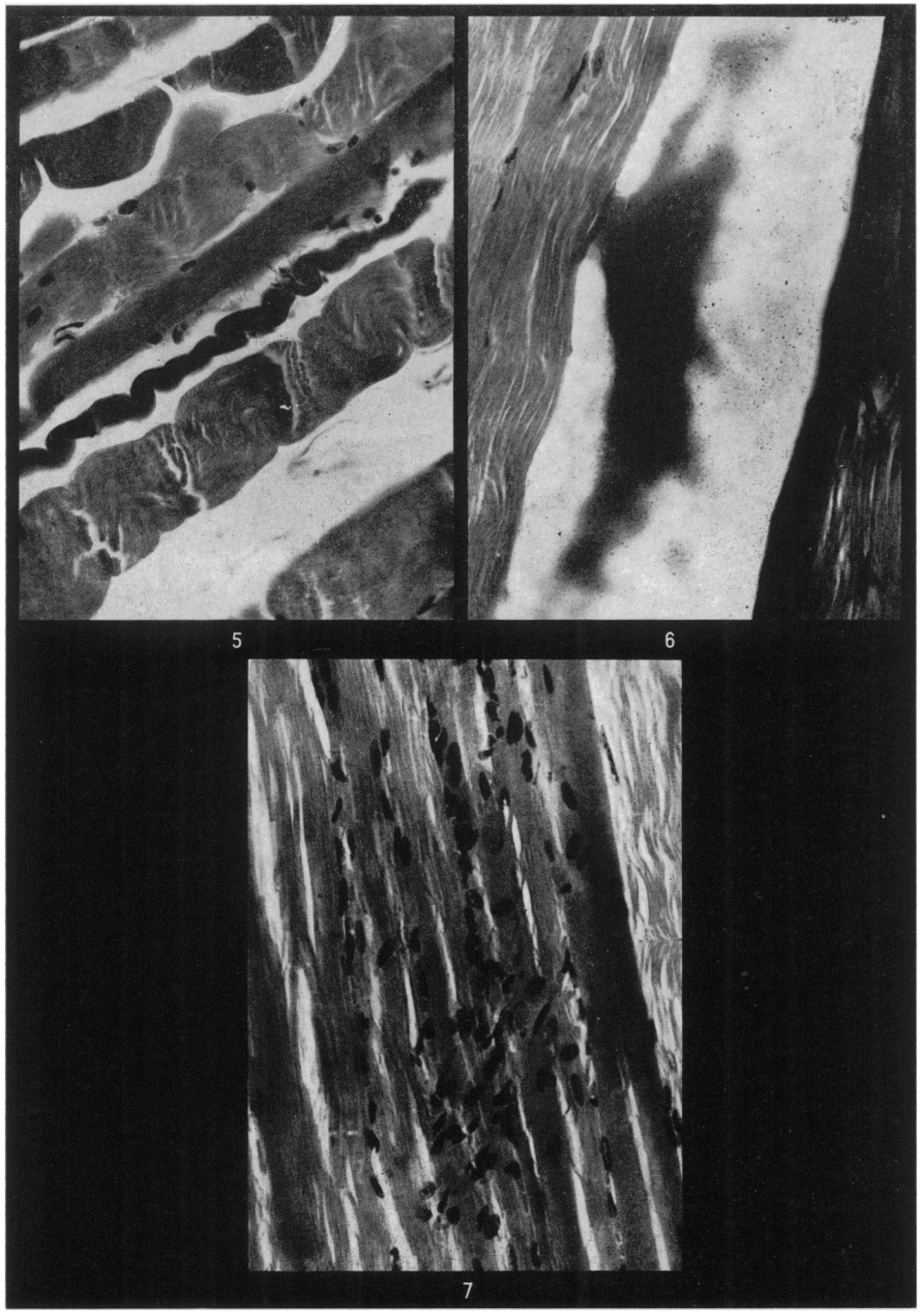
FIG. 5.—Skeletal muscle from sensitized rat showing varying degrees of loss of cross striation; variation in fibre diameter and one smaller, more basophilic fibre with several densely staining sub-sarcolemmal nuclei.

FIG. 6.—Isolated plaque of calcification lying between two skeletal muscle fibres. There is here no cellular reaction.

FIG. 7.—Rat tibialis anterior from muscle sensitized animal, showing increased cellular infitraltion.

FIG. 8.—Skeletal muscle fibres showing regional loss of cross striation with vacuolation and variation in fibre size.

FIG. 9.—The thymic gland from a sensitized rat. Photomicrograph: ×33. No increase in germinal centre formation as compared with the normal rat thymus, is seen.



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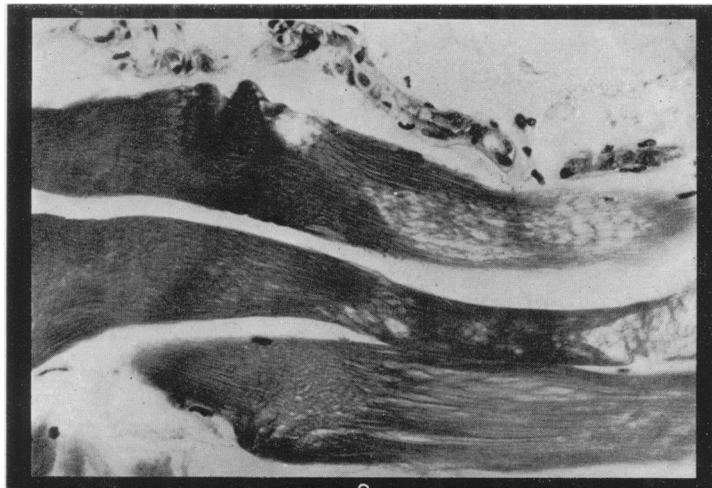

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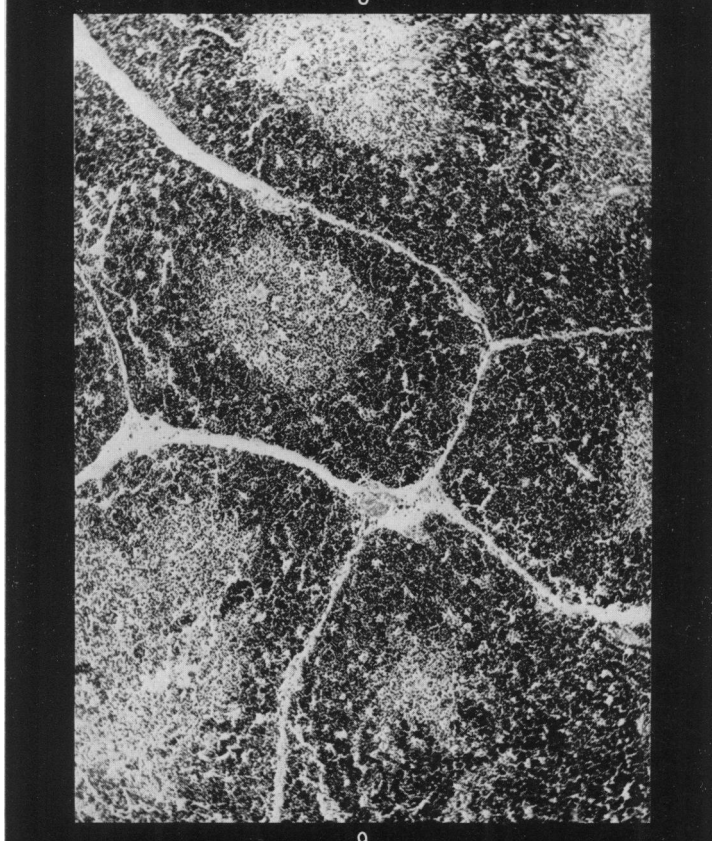

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There may be a surrounding inflammatory cellular reaction with phagocytosis of inflamed muscle fibres. This change may be localized to one fibre, or may be generalized. The second type of abnormality is the formation of small intramuscular collections of lymphocytes (lymphorrhage). In addition there is the least specific type of change with simple focal lesions with eosinophilia and some fibre swelling ; the striation being well retained with no inflammatory reaction. These changes do not closely resemble those described here.

No evidence as to the presence of circulating or fixed antibodies to the antigen mixture used is presented here. However, it seems probable that the changes resulting of widespread extent, resulted directly from antigen stimulation, and were provoked by an antibody antigen reaction. If this is so and also myasthenia an auto-immune disease, it is strange that features resembling those of myasthenia are not produced ; and the absence of these features must be interpreted as evidence against an auto-immune cause of myasthenia. The weakness seen can be explained purely on the basis of the histological changes of muscle disease, and resembles the muscle weakness, accompanying muscle dystrophies or polymyositis. The increased sensitivity of human myasthenics to the blocking action of curare (Bergh, 1953) was not mirrored by an increased sensitivity of the muscle injected rat.

There is no good present evidence for myasthenia gravis being caused by (as opposed to being accompanied by) an auto-immune mechanism. A specific antigen has not as yet been demonstrated. Antibodies shown by fluorescent techniques have been directed against muscle fibres, rather than against the end-plate muscle region. (Strauss, Seegal, Hsu, Burkholder, Nastuk and Osserman, 1960). Neonatal myasthenia may be cited as evidence for the passive transfer of some agent from mother to foetus ; there is no evidence that this is an antibody. Myasthenia has not been provoked in the experimental animal. The histological changes provoked in this series did not closely resemble those of human myasthenia gravis, and no thymic abnormalities were seen. Thus none of the essential criteria suggested by Witebsky for considering the disease auto-immune in nature, seem to have been, as yet, fulfilled.

Smithers (1959) studying the role of thymus and lymphocytes in disease concluded that they were implicated in auto-immunity and that the thymic changes of myasthenia gravis were strongly suggestive of an auto-immune process. Smithers described the occurrence of thymic tumours in man in relation to other diseases with possible immunological implications, such as Hodgkin's desease (Thomson, 1955), Cushing's syndrome (Thorne, 1952) and hypoplastic anaemia (Matras and Priesel, 1928). Marshall and White (1961) similarly concluded that the thymic lesions in myasthenia reflected an immune response. They argued that between 60–70 per cent of cases of myasthenia show characteristic thymic lesions, consisting of hyperplasia of the gland, with the formation of germinal centres in the medulla. These structures are histologically identical with those seen in lymph nodes after antigenic stimulation. These lesions suggested to Marshall and White (1961) an immune response, but previous workers (Harris, Rhoads and Stokes, 1948 ; Askonas and White, 1956) had failed to demonstrate histological changes in the thymic glands of immunized animals, or to obtain evidence of antibody formation in this organ. To study the point further, Marshall and White (1961) injected typhoid antigens directly into the thymus of guinea pigs. The injection areas later showed generalized germinal centre

activity, and much plasma cell formation. They concluded that the direct injection of antigens into the thymus leads to histological changes usually associated with antibody formation in other lymphoid organs—and in contrast to the complete lack of response observed by others after antigen introduction into the blood stream. The failure of reaction to circulating antigens suggested to Marshall and White (1961) the existence of a barrier against antigen entry into the thymus.

Holding this view it is possible that direct motor end-plate fraction introduction into the thymus would result in a differing response to that provoked by subcutaneous injection, and indeed result in features resembling those of myasthenia gravis.

The association of a secondary myasthenic muscle reaction with certain diseases has been observed. This has been described in bronchial carcinoma (Heathfield and Williams, 1954) and with polymyositis. Somewhat similar defects have been described in amyotrophic lateral sclerosis (Lambert and Mulder, 1957), syringomyelia (Kugelberg and Taverner, 1950) and in poliomyelitis (Pinelli and Buchthal, 1951). This phenomenon may perhaps explain the excessive fatigue accompanying tetanic stimuli, in 3 animals of the sensitized series.

The changes seen more closely resemble those of primary muscle disease rather than myasthenia. The features of muscle fibre atrophy, hypertrophy, calcification and slight cellular infiltration are those common to a number of muscular disorders both in animal and man, and these experiments raise the possibility of certain muscle disorders resulting from an abnormality of the immune mechanism. Muscle weakness rather than abnormal fatigue accompanies these disorders.

Klinge (1932) repeatedly injected horse serum into rabbits. In one rabbit a generalized myopathy developed with focal intra muscular deposits of giant cells, mononuclear cells, histiocytes and fibroblasts. There was a waxy degeneration of muscle fibres. Kallos and Pagel (1937) made a study of injection of muscle anti serum into rabbit muscle, and described changes comparable to those of polymyositis. More recently Tal and Liban (1962) produced lesions in the muscle of rabbits and guinea pigs by injection of muscle mash and Freund's adjuvant. The changes resulting were those of fibre atrophy and some hypertrophy, with disappearance of muscle cross striation. Marked fibre necrosis, leucocytic infiltration and intra muscular calcification were not seen in these animals. However, they were visibly weak. The cellular infiltration in many of these experimental dystrophies may represent some aspect of the Arthus phenomenon (Opie, 1924) rather than represent a specific attempt at necrotic muscle fibre phagocytosis and repair.

A normal pattern of muscle fatigue is preserved in the presence of histological abnormalities of muscle fibre and muscle weakness. This emphasises the fact that motor end plate malfunction is responsible for abnormal muscle fatigue in myasthenia. Under physiological conditions muscle fatigue is dependant upon events occurring within the muscle, and nerve and nerve muscle junctions do not show fatigue. Adams, Denny-Brown and Pearson (1953) stress this misuse of the word fatigue for a description of a myasthenic phenomenon. The experience of an exhausted athlete and the myasthenic patient may be momentarily similar, but are not closely comparable. Those muscles showing the most abnormal histology in myasthenia gravis are not necessarily the weakest, but the possibility

is raised, that muscle weakness in myasthenia gravis is correlated to skeletal muscle abnormality, and abnormal muscle fatigue, with neuromuscular junction malfunction.

SUMMARY

Following antigenic stimulation with motor end-plate rich muscle antigen histological changes suggestive of muscle destruction occur in the rat.

This histological picture is accompanied by abnormal muscle weakness, but no abnormality of muscle fatigue.

These changes can be interpreted as those of muscle disease with no features suggestive of an end-plate abnormality of behaviour.

These changes are readily provoked. The absence of features of myasthenia can be interpreted as evidence against myasthenia resulting from an abnormality of the immune mechanism.

Part of this work is contained in a thesis accepted for the degree of M.D. (Cantab.). I am indebted to Mr. D. C. Moore and Miss W. Morgan for their technical help.

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