Electrophysiological changes similar to those of myasthenia gravis in rats with experimental autoimmune thymitis

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Myasthenia gravis is a clinical disorder of neuromuscular conduction, the definitive study of which has been hampered by the lack of a suitable animal model. The exact nature of the defect is uncertain but there is typically a progressive reduction in the number of contracting muscle fibres during repetitive motor nerve stimulation and a less marked, but demonstrable, reduction in the response to even the first stimulus in some cases (Johns, Grob, and Harvey, 1956). In the whole muscle the abnormality is recognized by a progressive reduction in the size of successive integrated action potentials in a train of stimuli, and, at the single neuromuscular junction *in vitro*, there is often a subthreshold response to nerve stimulation and a reduction in the amplitude of depolarizations produced by individual 'units' of transmitter escaping at rest (Hofmann and Stemmer, 1963; Elmqvist, Hofmann, Kugelberg, and Quastel, 1964). The reduction in the amount of depolarization produced by the transmitter is entirely adequate to account for all the features of neuromuscular block in the disease.

An autoimmune aetiology has been suggested for myasthenia gravis (Nastuk, Plescia, and Osserman, 1960; Simpson, 1960). Most patients have pathological changes in the thymus (Castleman and Norris, 1949) and 30% have circulating autoantibodies reactive with the striations of skeletal muscle and with myoid cells in the thymic medulla (Strauss, van der Geld, Kemp, Exum, and Goodman, 1965). Goldstein (1966, 1967) suggested that the histological changes should be interpreted as chronic inflammation of the thymus and that autoimmune thymitis is the fundamental lesion in myasthenia gravis. This was supported by the discovery of a deficit in neuromuscular conduction in guinea-pigs in which autoimmune thymitis was experimentally induced by immunization with thymus in Freund's

complete adjuvant (Goldstein and Whittingham, 1966, 1967). The electromyographic abnormalities consisted of a neostigmine reversible block to both single and tetanic nerve stimulation. The presence of an inflamed thymus was shown to be essential in the development of the experimental neuromuscular block, as thymectomized animals injected in exactly the same way with thymus and adjuvant showed no evidence of a deficit in neuromuscular conduction (Goldstein and Whittingham, 1966). The question remained whether the electromyographic changes present in the immunized thymus-intact animals were, in fact, indicative of the same abnormality as that described at single neuromuscular junctions in human myasthenics.

The present study carries the analysis of neuromuscular block in the experimental animals to the level of the single neuromuscular junction *in vitro* and *in vivo*. Our investigations show that animals immunized with homologous thymus in Freund's complete adjuvant develop changes at single end-plate regions qualitatively indistinguishable from those seen at the myasthenic neuromuscular junction.

METHODS

PREPARATION OF ANTIGEN Thymus and a portion of liver were dissected from weanling rats of the inbred Lewis strain. The tissues were separately homogenized in phosphate buffered saline pH 7.5 (20% wet wt./vol.) using a Teflon homogenizer. The homogenate was centrifuged at 7,000 rev/min for 10 minutes and the supernate was lyophilized. The lyophilizate was dissolved in distilled water to a final concentration of 10 mg/ml. Freund's complete adjuvant (FCA) was prepared by adding 8 mg/ml. ground dried *Mycobacterium tuberculosis* to a mixture of 85% Bayol F and 15% Arlacel. Equal volumes of aqueous tissue extract and FCA were emulsified for injection.

IMMUNIZATION OF RATS Female Lewis rats weighing 100-200 g were injected with either thymus in FCA or, as a control, liver in FCA. Each rat received 0.1 ml. into each hind footpad.

THYMIC HISTOLOGY Rats were killed with chloroform two weeks after immunization. The thymus was removed, fixed in 10% formalin, and sections were prepared in the usual manner and stained with haematoxylin and eosin.

ELECTROMYOGRAPHY Two weeks after immunization rats were anaesthetized with urethane, 750 mg/kg body weight intraperitoneally. The median nerve was stimulated beneath the axillary fold by needle electrodes 3 mm apart and the muscle action potentials were recorded with a bipolar coaxial needle electrode in the flexor digitorum. The nerve was stimulated with rectangular pulses of 0.15 msec duration from a Grass S-8 stimulator and a Grass SIU-4B stimulus isolation unit (Grass Instrument Company, Quincy, Massachusetts).

To ensure that the current output remained relatively constant during tetanic stimulation, a resistance of $2K \Omega$ was placed in series with the stimulating electrodes. The electromyographic response was recorded with a Tektronix 564 storage oscilloscope with a 2B67 time base and a 2A61 differential amplifier. Representative displays were photographed with a Tektronix C-12 Land

Polaroid camera (Tektronix, Inc., Portland, Oregon) (Fig. 1).

Single stimuli of increasing voltage were delivered until a maximal electromyographic response was obtained, and the voltage was then doubled for supra-maximal stimulation. The stimulator was then programmed to deliver 10 supramaximal stimuli at a rate of 50/sec. The electromyographic response of each animal was tested at four separate sites in each forearm, repeating each tetanus several times for each position of the recording electrodes. When the results at a given site were not reproducible it was assumed that there had been a shift in position of the electrode within the muscle and the data were discarded.

SPONTANEOUS SUBTHRESHOLD ACTIVITY AT THE NEUROMUSCULAR JUNCTION *In vivo* studies were performed on the small intertransversarius muscles of the rat tail prepared in the manner described by Steg (1964). The animal was lightly anaesthetized with pentobarbital sodium, 50-70 mg/kg body weight, and a longitudinal skin incision was made in the tail. The skin was pinned aside to expose the underlying fascia and long tendons. These were removed under the dissecting microscope, exposing the muscle bundles. The tail was kept submerged in warm mineral oil. The micro-electrodes were inserted into the end plate regions under visual control.

In vitro studies were performed on forearm muscles removed from rats lightly anaesthetized with ether. The small forearm muscles were dissected free from each other and placed in an oxygenated physiological solution of the composition described by Liley (1956). Under a dissecting microscope the individual muscles were cleaned of fat and connective tissue, and each was pinned by its tendons of origin and insertion to a silicone rubber holder. To ensure proper oxygenation the fusiform muscles were pinned to the holders with their tendons spread as far laterally as possible, thus converting the specimen to a flat translucent sheet. The preparations were also stretched longitudinally by about 10-15% of their resting length and were kept slightly raised off their holders by angulation of the tendon pins. The temperature was set from 33-35°C and varied less than 0.5°C during a given experiment. The muscles were washed in oxygenated solution for at least one hour before testing.

The direct-coupled input time constant of the first stage cathode follower (Bioelectric Instruments, Inc.) was adjusted to a minimum value (50-150 msec), and micro-electrodes of relatively low impedance were used (4-8 M Ω). To ensure that the recording characteristics of the electrode had not changed as a result of plugging while being driven through successive fibres, a small calibration current pulse was sent through the preparation and the electrode to ground (Bioelectric Instruments, Inc.). With any visible change in the recorded characteristics of the test pulse the micro-electrode was replaced.

By following to their ends the small nerve terminals that could be seen under the microscope in both *in vitro* and *in vivo* preparations, it was possible to insert micro-electrodes near end plate regions. Muscle fibres were impaled in a search for a group having membrane

FIG. 1. Electromyographic response of rat forearm muscle to supramaximal median nerve stimulation at a rate of 50/sec. There is a decline in successive muscle action potentials in the thymus-immunized animal by contrast with the sustained response of the control animal.

potentials of 60 mV or more and spontaneous depolarizations or miniature end-plate potentials (m.e.p.p.s) arising very near the electrode. When a fibre was entered and m.e.p.p.s were found a preliminary series of single oscilloscope sweeps was observed and photographed at a fast sweep rate (Fig. 2). In this way the average depolarization rates of the m.e.p.p.s could be assessed and only fibres having m.e.p.p.s with rise times of less than 1.5 msec were considered to be focally impaired at the end-plate. All others were rejected. If the majority of m.e.p.p.s at a junction were focal a record was made at a slow film speed for 10-20 sec, with the oscilloscope time base generator off. These records were made with DC coupled input so that changes in the level of the resting membrane potential could also be followed. Results were not included in the analysis if the resting membrane potential of a fibre fell below 60 mV while m.e.p.p.s were being recorded.

The amplitude of m.e.p.p.s was determined by direct measurement from the centre of the baseline 'noise' to the peak of each signal. The electronic 'noise' level varied considerably from fibre to fibre and with different micro-electrodes and it is probable that in some cases signals of very low amplitude were obscured, but errors on this basis would have affected both control and test groups. For each fibre a histogram of m.e.p.p.s amplitudes was drawn up and the peak of the histogram, or the m.e.p.p. amplitude occurring most frequently, was taken as the mean for that fibre. In most instances the peak of this histogram was above the noise level. The mean m.e.p.p. amplitude for each animal was an arithmetical mean of the values thus obtained for each fibre. Similarly, for each animal an arithmetical mean was obtained for the m.e.p.p. frequency and resting membrane potentials at individual junctions.

RESULTS

THYMIC HISTOLOGY There were accumulations of

FIG. 2. Focal m.e.p.p.s *in vivo* and *in vitro* showing rise times of less than 1.5 msec; each frame represents from 10 to 25 sweeps superimposed. The micro-electrode in each instance was considered to be in the region of the fibre end-plate.

lymphocytes in the thymic medulla of nine thymus-immunized animals, the changes being similar to those described in experimental autoimmune thyroiditis in guinea pigs (Goldstein and Whittingham, 1967). These changes were not observed in control animals immunized with liver.

ELECTROMYOGRAPHY The amplitude of the initial muscle response to supramaximal nerve stimulation varied considerably at different sites in the same muscle. The mean amplitude at 72 sites in nine thymus-immunized animals was 6.1 ± 3.6 mV (Mean $\pm$ S.D.), this being significantly decreased ($P < 0.01$) by comparison with the mean amplitude of 7.9 ± 4.2 mV at 80 sites in 10 control animals. There is thus evidence in the thymus-injected animals of a partial block to the passage of a single impulse at the neuromuscular junction.

The fractional decline in muscle response during 50/sec nerve stimulation was obtained by the ratio of the amplitude of the 10th to the 1st muscle action potential at sites where the responses were reproducible, and control and thymus-immunized preparations could then be compared. In control animals the lowest recorded decline was $69 \pm 4\%$ by the 10th impulse and, since the probability of a decline to 60% or less by chance was very low ($P > 0.01$), it was considered that any preparation declining to 60% or less of the initial response during stimulation had an abnormality of neuromuscular transmission. On this basis, none of the control animals showed a defect, whereas of the nine thymus-immunized animals, seven were abnormal ($P < 0.001$) (Fig. 3).

SPONTANEOUS SUBTHRESHOLD ACTIVITY AT THE NEUROMUSCULAR JUNCTION A comparison of the amplitudes of miniature end-plate potentials (m.e.p.p.) in control and thymus-immunized animals revealed a significant decrease in the latter group, both *in vivo* and *in vitro*. These results are illustrated in Figs. 4-6 and summarized in Table I.

The findings *in vivo* were compared for 125 junctions in nine thymus-immunized rats and 85 junctions in eight control rats. The amplitude of the m.e.p.p.s was 0.43 ± 0.04 mV (Mean $\pm$ S.D.) in thymus-immunized animals, and this was significantly decreased ($P < 0.001$) by comparison with the amplitude of 0.62 ± 0.08 mV in control animals. The resting membrane potential was similar in both groups, averaging 76 ± 5 mV in thymus-immunized animals and 78 ± 4 mV in the control animals. Similarly, the frequency of m.e.p.p.s was similar in both groups being 1.2 ± 0.6 /sec in the thymus-immunized animals and 1.1 ± 0.3 /sec in the control animals.

FIG. 3. Scattergram to show the electromyographic response to supramaximal nerve stimulation at a rate of 50/sec. From the control animals a decline in the tenth response to less than 60% of the first was unlikely ($P < 0.01$). Seven of nine thymus-immunized animals had a significant decline in muscle action potentials ($P < 0.001$).

The *in vivo* reduction of m.e.p.p. amplitude in thymus-immunized animals was also present *in vitro*. Data were obtained from 87 junctions of nine immunized rats and 77 junctions in six controls. In the immunized group the amplitude of m.e.p.p.s was 0.50 ± 0.09 mV, this being significantly lower ($P < 0.02$) than the amplitude in control animals, 0.62 ± 0.10 mV. There were no significant differences ($P > 0.10$) between the resting membrane potentials in the thymus-immunized animals (80 ± 6 mV) and the control group (83 ± 2 mV). Similarly the frequency of m.e.p.p.s was not significantly different ($P > 0.10$) in thymus-immunized animals (9.9 ± 5.5 /sec) and control animals (9.2 ± 4.0 /sec). The

higher frequency of spontaneous activity *in vitro* can be attributed to mild depolarization of the nerve terminals that may have occurred during preparation (Parsons, Hofmann, and Feigen, 1965) and to the slight stretch of the muscles necessary in mounting to insure adequate oxygenation (Hutter and Trautwein, 1956).

DISCUSSION

The present studies reveal that rats injected with homologous thymus and adjuvant develop in their electromyographic responses and spontaneous subthreshold end-plate activity electrophysiological changes qualitatively very similar to those reported in myasthenia gravis in man. These abnormalities were present two weeks after immunization and were not extensive enough in the animals we have used to cause obvious clinical weakness.

In the present experiments Lewis rats were immunized with thymus obtained from inbred Lewis rats. Since these tissues were histocompatible they contained no antigens 'foreign' to the animals being immunized. Thus the action of the Freund's complete adjuvant was to initiate an immune reaction to thymic antigens also present in the animals' own thymus—that is, to initiate a damaging autoimmune reaction to thymus and inflammation of the thymus.

In the electromyographic studies the amplitude of response to a single supramaximal nerve shock varied considerably from area to area within a given muscle, as would be expected from the known characteristics of bipolar needle recording electrodes in a population of contracting units. However, the decrease in mean amplitude of the integrated electromyographic potential in rats with experimental autoimmune thymitis, as in humans with myasthenia gravis, indicates a partial block to the passage of single impulses from nerve to muscle, and, as in the clinical disease, the block is reversed with a known cholinesterase inhibitor, neostigmine (Goldstein and Whittingham, 1966).

The neuromuscular block of rats with experimental autoimmune thymitis became more pronounced during repetitive nerve stimulation at 50 impulses/sec as evidenced by a decline in successive muscle action potentials. The same finding is also characteristic of human myasthenia gravis, as has been known for many years.

The changes in spontaneous activity at the neuromuscular junctions of rats with experimental autoimmune thymitis also resembled the findings in human myasthenia gravis. The amplitudes of m.e.p.p.s were decreased in *in vitro* preparations of forearm muscles from immunized rats just as they are in *in vitro* preparations of intercostal muscle

FIG. 4.

FIG. 5.

FIG. 6.

FIG. 4. Representative records of m.e.p.s in vivo showing m.e.p.s clearly visible above the electrical 'noise'. M.e.p.p. amplitude was decreased in thymus-immunized animals.

FIG. 5. Records of m.e.p.s in vitro showing decreased amplitude in thymus-immunized animals. M.e.p.p. frequencies were similar in control and thymus-immunized animals. Each trace is a representative record from a different animal.

FIG. 6. Scattergram to show mean m.e.p.p. amplitudes in 32 rats, obtained from focal records at 374 neuromuscular junctions. There was a significant decrease of m.e.p.p. amplitude in thymus-immunized animals both in vivo (0.43 ± 0.04 mV from 0.62 ± 0.08 mV in controls, $P < 0.001$) and in vitro (0.05 ± 0.09 mV from 0.62 ± 0.10 mV in controls, $P < 0.02$).

TABLE 1

SUMMARY OF THE MICROPHYSIOLOGICAL DATA AT 374 NEUROMUSCULAR JUNCTIONS OF 18 RATS WITH EXPERIMENTAL AUTOIMMUNE THYMITIS AND 14 CONTROL RATS IMMUNIZED WITH LIVER IN FCA¹

<i>In vivo</i>					<i>In vitro</i>				
	<i>M.e.p.p. amplitude</i>	<i>Resting membrane potential</i>	<i>M.e.p.p. frequency</i>	<i>Junctions studied</i>		<i>M.e.p.p. amplitude</i>	<i>Resting membrane potential</i>	<i>M.e.p.p. frequency</i>	<i>Junctions studied</i>
	(mV)	(mV)	(no./sec)	(no.)		(mV)	(mV)	(no./sec.)	(no.)
Controls	0.72	78	1.3	3		0.74	87	4.8	4
	0.69	79	1.8	11		0.71	82	8.7	13
	0.65	78	0.9	13		0.68	80	13.9	14
	0.65	77	1.1	17		0.64	82	11.4	11
	0.61	79	0.9	15		0.51	82	12.0	22
	0.58	84	0.9	12		0.50	82	4.0	13
	0.57	69	0.8	10					
	0.48	79	1.2	4					
Mean $\pm$ S.D	0.62 $\pm$ 0.08	78 $\pm$ 4	1.1 $\pm$ 0.3	11 $\pm$ 5		0.62 $\pm$ 0.10	83 $\pm$ 2	9.2 $\pm$ 4.0	13 $\pm$ 6
Thymus-immunized	0.50	83	1.1	12		0.67	77	19.4	15
	0.48	80	1.2	19		0.54	84	4.2	9
	0.45	72	0.9	13		0.54	81	8.5	9
	0.43	75	1.0	13		0.54	74	7.4	4
	0.42	72	0.9	12		0.47	85	10.9	10
	0.41	83	1.1	14		0.44	84	6.2	11
	0.40	73	0.6	15		0.43	71	9.9	12
	0.39	72	3.3	12		0.43	75	18.3	13
	0.39	75	0.9	15		0.42	91	4.4	4
Mean $\pm$ S.D	0.43 $\pm$ 0.04	76 $\pm$ 5	1.2 $\pm$ 0.6	14 $\pm$ 2		0.50 $\pm$ 0.09	80 $\pm$ 6	9.9 $\pm$ 5.5	10 $\pm$ 4
Student's t-test	T	6.620	0.954	0.4000		2.652	1.364	0.173	
	f	15	15	15		13	13	13	
	P	<0.001	>0.10	>0.10		0.02	>0.10	>0.10	

¹Thymus-immunized animals had decreased m.e.p.p. amplitudes *in vivo* and *in vitro*, but their resting membrane potentials and m.e.p.p. frequencies were similar to those of control animals.

biopsies from humans with myasthenia gravis (Hofmann and Stemmer, 1963; Elmquist *et al.*, 1964; Thesleff, 1966). The forearm muscles were specifically selected for microphysiological comparison because these same muscles had shown the electromyographic abnormality. As in human material, the muscle fibres from the immunized rats showed no abnormality of the resting membrane potential that might account for a reduction in the depolarization produced by a single 'quantum of transmitter'. Neither was there any difference between test and control animals in the frequency of m.e.p.p.s. Thus in experimental autoimmune thymitis, as in myasthenia gravis, there appears to be either a reduction in the amount of acetylcholine in the individual quanta randomly released from the nerve terminal or there is reduced availability of the post-junctional membrane receptors whose combination with transmitter evokes end-plate depolarizations.

These similarities to the clinical disorder suggest that the condition induced in the rats with the present technique is indeed similar to myasthenia gravis and that this preparation may be of value in further comparative studies.

In previous experiments it has been shown that

the neuromuscular block of animals with experimental autoimmune thymitis requires the actual presence of the inflamed thymus and that it is not directly related to the immune response of the animal, for animals thymectomized before immunization developed the same immune responses but showed none of the electrophysiological abnormalities (Goldstein and Whittingham, 1966, 1967). It seems likely, therefore, that, as a result of an autoimmune reaction within it, the inflamed thymus liberates some substance into the circulation and that this substance causes the neuromuscular block at the periphery.

The present experiments do not establish the manner in which the proposed humoral substance causes neuromuscular block; in experimental animals it appears unlikely, however, that it is simply a curare-like competitive inhibitor, for the block was not reversed by washing for several hours in physiological solutions *in vitro*. If the proposed substance has an action primarily on the motor nerve terminals, this also is not washed off *in vitro*, and there remains the possibility that it acts by interfering intracellularly with the binding of acetylcholine molecules as 'quanta'. In any case, whether this agent released from the inflamed thymus

of our experimental animals acts by affecting pre- or post-synaptic structures at the neuromuscular junction, the defect that it produces is sufficiently like that of myasthenia gravis to renew interest in the relationship between the thymus and the mechanisms of human neuromuscular transmission.

SUMMARY

Experimental autoimmune thymitis was produced in inbred Lewis rats by immunization with Lewis rat thymus in Freund's complete adjuvant. The electromyographic records from rats with experimental autoimmune thymitis showed decreased amplitude of muscle action potentials with single supramaximal nerve stimuli and a progressive decline of muscle responses to nerve stimulation at 50/sec.

Spontaneous subthreshold activity at the neuromuscular junction was studied with intracellular micro-electrodes. Rats with experimental autoimmune thymitis had changes similar to those found in biopsied specimens from patients with myasthenia gravis in that there was decreased amplitude of miniature end plate potentials (m.e.p.p.s), while m.e.p.p. frequency and the resting membrane potential of the muscle fibres remained normal.

Inflammation in the thymus is considered to cause the release of a circulating substance whose site of action may be either in the motor nerve ending or the post junctional membrane receptors. This substance causing the experimental neuromuscular block was considered *not* to be a simple curare-like competitive inhibitor, as its effect was not diminished *in vitro*, even after prolonged washing of specimens.

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