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ENDOCRINE FUNCTION OF THE THYMUS AFFECTING NEUROMUSCULAR TRANSMISSION

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SUMMARY

Neuromuscular transmission was studied with micro-electrodes in thymus-deficient Lewis rats thymectomized within 3 days of birth and in Lewis rats bearing increased thymic tissue in the form of multiple isogeneic thymic grafts. The amplitude of miniature end-plate potentials (MEPPS) was increased in thymus-deficient animals and decreased in animals with increased thymic tissue.

These data suggest that a substance we have previously termed thymin is being secreted by the normal thymus in physiologically active amounts which inhibit transmission at the neuromyal synapse. The proposed substance thus appears to act like a hormone. It is suggested that in myasthenia gravis autoimmune thymitis causes the release of toxic amounts of thymin which produce the characteristic myasthenic neuromuscular block.

INTRODUCTION

The association of pathological changes in thymus with impairment of neuromuscular transmission is well documented in the human disease myasthenia gravis (Castleman & Norris, 1949). The suggestion that myasthenia gravis is an autoimmune disease (Simpson, 1960; Nastuk, Plescia & Osserman, 1960) is supported by serological and clinical studies (Strauss *et al.*, 1960; White & Marshall, 1962; Feltkamp *et al.*, 1963) but the evidence is against an autoimmune reaction being directly implicated in the genesis of the neuromuscular block (Strauss *et al.*, 1966; McFarlin, Engel & Strauss, 1966).

From histopathological studies of the thymus in myasthenia gravis it was suggested that an autoimmune reaction is directed towards the thymus and that autoimmune thymitis is the thymic lesion basic to myasthenia gravis (Goldstein, 1966, 1967). This concept was supported by the findings in an animal model of myasthenia gravis, experimental autoimmune thymitis (Goldstein & Whittingham, 1966, 1967). Guinea-pigs immunized with thymus in Freund's complete adjuvant developed autoantibodies, delayed hypersensitivity

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and histological thymitis, and an associated neuromuscular block was demonstrated by electromyography. The neuromuscular block was considered to be caused by a humoral substance released from the inflamed thymus since animals thymectomized before immunization developed the same immune responses but had no evidence of neuromuscular block.

Subsequently, a substance causing myasthenic neuromuscular block was demonstrated directly in extracts of normal bovine thymus (Goldstein, 1968) and this substance was termed 'thymin'. Since thymin is present in normal thymus and is released in excess with inflammation of the thymus the question arose as to whether thymin is being normally secreted by the thymus in physiological amounts which affect neuromuscular transmission. We therefore studied neuromuscular transmission in thymectomized rats and also in rats bearing increased amounts of thymic tissue in the form of multiple thymus grafts. Spontaneous sub-threshold activity at the neuromyal synapse was studied because the amplitude of miniature end-plate potentials (MEPPS) provides a sensitive indicator of neuromuscular transmission, being depressed both in myasthenia gravis (Hofmann & Stemmer, 1963; Elmqvist *et al.*, 1964) and in experimental autoimmune thymitis (Goldstein & Hofmann, 1968).

MATERIALS AND METHODS

Inbred Lewis rats (Microbiological Associates, Inc.) were used for all experiments. Alternate littermates were thymectomized or sham-operated within 3 days of birth by the technique of Miller (1960). For the grafting experiments, thymus and spleen were obtained from rats in the 1st week of life. Four-week-old female littermates each received ten grafts of thymus or, as a control, spleen; these were implanted subcutaneously beneath the dorsolateral skin (Metcalf, 1966).

The neonatally operated rats were weaned and separated by sexes at 4 weeks and females were studied at 5–8 weeks and males at 10–12 weeks. The presence of residual thymus was looked for in all cases and a thymic weight was obtained in most cases (Table 1). The grafted animals were studied at 10–12 weeks and the weight of the thymus and of graft tissue was obtained in most cases (Table 2). Lewis rats are isogeneic and the grafts were well taken and appeared normal. There was normal differentiation of thymus into cortex and medulla and no evidence of thymitis. Thymus-grafted animals had a mean total weight of thymic tissue 2·5 times greater than spleen-grafted controls.

Spontaneous sub-threshold activity at the neuromuscular junction was studied *in vivo* in the small intertransversarius muscles of the rat tail prepared in the manner described by Steg (1964). The preparation of the anaesthetized animals, the insertion of the microelectrodes, and the methods of recording were as described previously (Goldstein & Hofmann, 1968; Roberts & Thesleff, 1965).

Records were used for calculations if the fibre was easily impaled and maintained a resting membrane potential greater than 60 mV. To ensure that all data were obtained from end-plate regions only focal MEPPS with rise times of less than 1·5 m-sec were selected (Fig. 1), an average of twelve such records being obtained for each animal. From these records the mean MEPP amplitude, MEPP frequency and resting membrane potential was calculated for each animal and the mean and standard deviation calculated for each group (Tables 1 and 2). Student's *t*-test was used to assess the statistical significance of differences between groups.

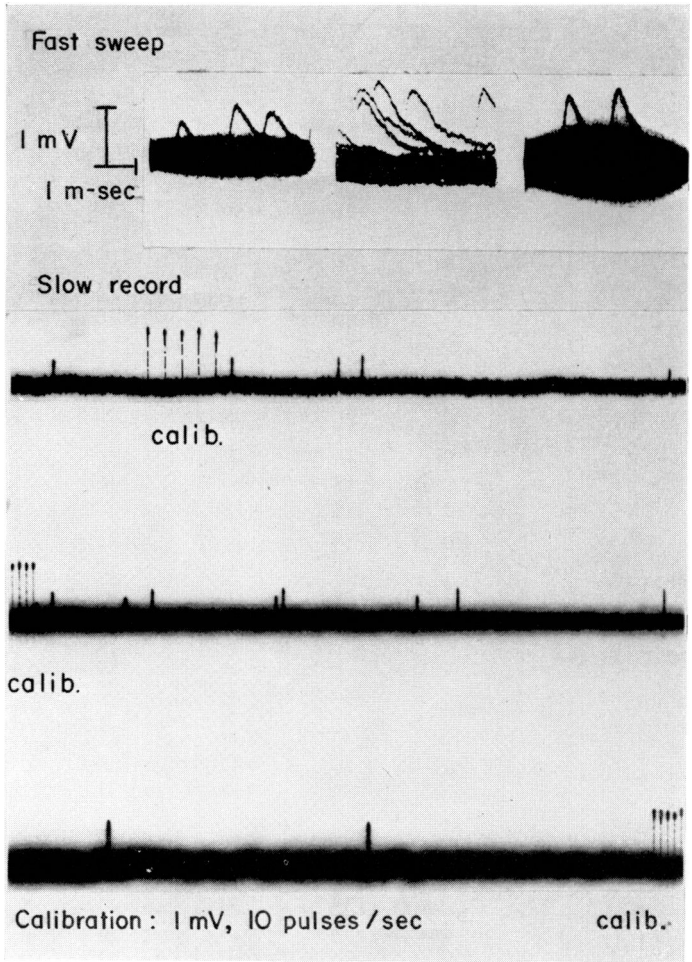

FIG. 1. Representative records of MEPPS recorded *in vivo*. The fast sweeps show that the insertions are focal with MEPP rise times of less than 1·5 m-sec. The slow records show MEPPS clearly visible above electrical 'noise'; calibration pulses (calib.) are superimposed.

TABLE 1. Electrophysiological data from *in vivo* micro-electrode study of Lewis rats sham-operated or thymectomized within 3 days of birth

	Thymus weight (mg)	No. of fibres	Mean MEPP amplitude (mV)	Mean MEPP frequency (per sec)	Mean resting membrane potential (mV)
FEMALE: TESTED AT 5–8 WEEKS					
Sham-operated					
	410	16	0·65	1·0	85
	NR	13	0·60	0·9	83
	405	14	0·58	0·7	81
	400	8	0·57	0·7	82
	NR	12	0·56	0·9	84
	325	10	0·56	0·9	83
	210*	9	0·56	0·8	79
Mean ± SD	350	12	0·58 ± 0·03	0·8 ± 0·1	83 ± 2
Thymectomized					
	0	12	0·73	0·5	83
	0	13	0·70	0·7	82
	0	11	0·67	0·6	82
	0	13	0·67	0·8	80
	0	13	0·64	1·0	85
	0	14	0·58	1·9	77
Mean ± SD	0	13	0·67 ± 0·05	0·9 ± 0·5	82 ± 3
Significance of differences between groups†			$P < 0·01$	NS	NS
MALE: TESTED AT 10–12 WEEKS					
Sham-operated					
	425	16	0·49	1·2	76
	230*	12	0·44	1·2	82
	365	12	0·42	1·2	80
	305	19	0·41	0·7	77
Mean ± SD	331	15	0·44 ± 0·04	1·1 ± 0·2	79 ± 3
Thymectomized					
	0	14	0·54	0·8	81
	0	11	0·54	0·9	87
	0	13	0·52	0·9	83
	0	15	0·52	1·2	81
	0	12	0·50	1·3	79
Mean ± SD	0	13	0·52 ± 0·02	1·0 ± 0·2	82 ± 3
Significance of differences between groups†			$P < 0·01$	NS	NS

NR, Not recorded; NS, not significant, $P > 0·10$.

* Partial thymectomy.

† Student's *t*-test.

TABLE 2. Electrophysiological data from *in vivo* micro-electrode study of female Lewis rats bearing multiple grafts of thymus or spleen

	Thymus weight (mg)	Graft weight (mg)	Total weight of thymic tissue (mg)	No. of fibres	Mean MEPP amplitude (mV)	Mean MEPP frequency (per sec)	Mean resting membrane potential (mV)
Spleen-grafted controls							
	390	130	390	9	0·74	0·8	84
	460	100	460	11	0·71	1·2	88
	375	850	375	7	0·70	1·1	90
	335	228	335	9	0·69	0·6	85
	NR	100	NR	10	0·59	0·4	87
	350	310	350	11	0·56	1·0	75
	NR	NR	NR	11	0·77	0·6	87
Mean ± SD	382	286	382	10	0·68 ± 0·08	0·8 ± 0·3	85 ± 5
Thymus-grafted							
	435	1050	1485	10	0·58	1·3	79
	360	750	1110	13	0·42	1·0	80
	325	550	875	9	0·50	0·8	76
	310	570	880	10	0·47	0·8	84
	375	600	975	12	0·58	0·5	79
	470	360	830	11	0·56	0·9	83
	375	480	855	7	0·44	0·9	72
	345	310	655	13	0·40	0·8	82
Mean ± SD	374	584	958	11	0·49 ± 0·07	0·9 ± 0·2	79 ± 4
Significance of differences between groups*					$P < 0·001$	NS	$P < 0·02$

NR, Not recorded; NS, not significant, $P > 0·01$.

* Student's *t*-test.

RESULTS

Thymectomy experiment

MEPP amplitudes of all the male animals, tested at 10–12 weeks, were consistently lower than those of females, tested at 5–8 weeks (Fig. 2). Within each group, however, comparing animals fully matched except for the presence or absence of the thymus there was a significantly higher MEPP amplitude in thymectomized animals ($P < 0·01$) (Fig. 2). In thymectomized females the MEPP amplitude was 0·67 ± 0·05 mV (mean ± SD) compared with 0·58 ± 0·03 mV in sham-operated animals while for males the values were 0·52 ± 0·02 mV for thymectomized and 0·44 ± 0·04 mV for sham-operated animals. Resting membrane potentials and MEPP frequencies were similar in all these groups (Table 1).

Thymus-grafting experiment

Animals with thymus grafts, bearing a mean weight of thymus 2·5 times greater than spleen-grafted controls, had a significantly lower ($P < 0·001$) MEPP amplitude of 0·49 ±

0·07 mV by comparison with 0·68±0·08 mV in matched spleen-grafted controls (Fig. 3). The MEPP frequencies were similar in both groups but there was a slightly lower ($P<0·02$) resting membrane potential in thymus-grafted (79±4 mV) as opposed to spleen-grafted (85±5 mV) animals (Table 2).

DISCUSSION

Micro-electrodes in the motor end-plates record spontaneous subthreshold depolarizations termed miniature end-plate potentials (MEPPS); these are believed to represent the depolari-

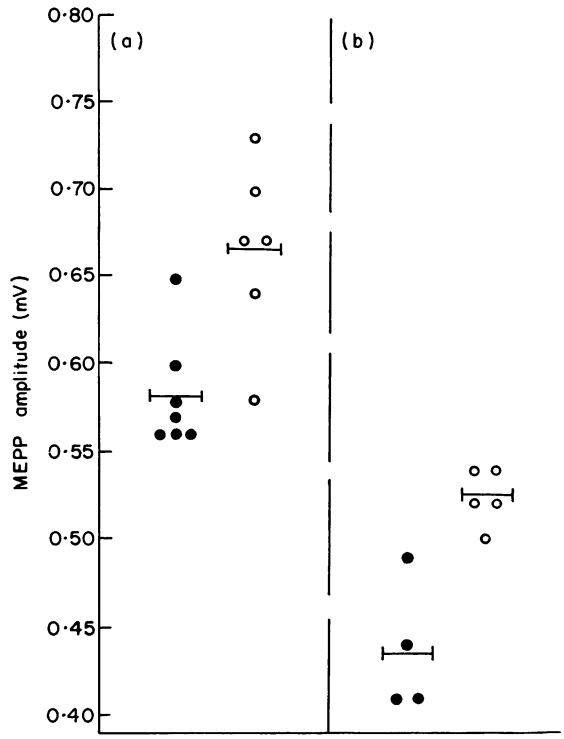

FIG. 2. Mean MEPP amplitudes in sham-operated (●) and thymectomized (○) Lewis rats. There was a consistent difference between males and females (see text) but within each group thymectomized animals had a significantly higher ($P<0·01$) MEPP amplitude (horizontal bars equal mean of each group). (a) Females tested at 5–8 weeks. (b) Males tested at 10–12 weeks.

zation caused by the smallest amount (quantum) of transmitter that can escape from a single release site (Fatt & Katz, 1952). In myasthenia gravis there is a decrease in amplitude of MEPPS and the reduction in amount of depolarization produced by the transmitter is entirely adequate to account for all the features of neuromuscular block in the disease (Hofmann & Stemmer, 1963; Elmqvist *et al.*, 1964). In experimental autoimmune thymitis a decrease in MEPP amplitude can be similarly demonstrated (Goldstein & Hofmann, 1968). We have suggested that the neuromuscular block of myasthenia gravis is caused by the release, with autoimmune thymitis, of the neuromuscular blocking substance thymin (Goldstein, 1966, 1968; Goldstein & Whittingham, 1966, 1967); the amplitude of MEPPS would, therefore, seem a sensitive measure of the action of thymin.

To determine if thymin were being secreted by the normal thymus in physiologically active amounts we studied spontaneous sub-threshold activity in thymus-deficient rats and rats bearing increased thymic tissue. To avoid artifacts owing to the preparation of the tissue *in vitro*, all records were obtained *in vivo* from the motor end-plate region of the segmental tail muscles of the rat.

In the thymectomy experiment we found that the MEPP amplitudes of all the male animals, tested at 10–12 weeks, were consistently lower than those of the females, tested at 5–8 weeks (Fig. 2). We are uncertain as to the reason for this difference. Katz & Thesleff (1957) have

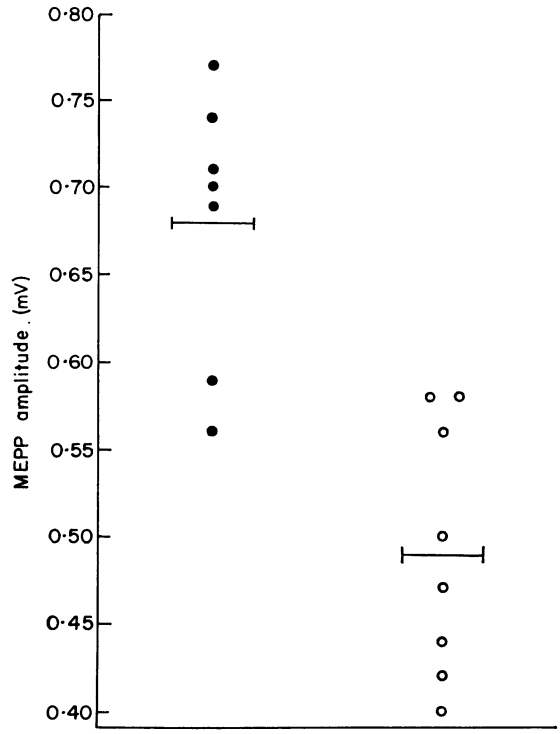

FIG. 3. Mean MEPP amplitudes in thymus-grafted animals (○) and spleen-grafted controls (●). Thymus-grafted animals had a significantly lower ($P < 0{\cdot}001$) MEPP amplitude (horizontal bars equal mean of each group). Female Lewis rats, grafted at 4 weeks and tested at 10–12 weeks.

shown that the MEPP amplitude is related not only to the amount of transmitter substance in a quantum and the post-synaptic efficiency of the transmitter substance but also to the electrical resistance of the muscle fibre. Larger fibres have a lower electrical resistance and it was found that there was a ten-fold range of mean MEPP amplitude in different fibres, the smallest amplitudes being recorded in the largest fibres. It may be, therefore, that the older male rats had increased fibre size by comparison with the younger females, thus accounting for the consistently smaller amplitude of MEPPS in these animals.

Within each group, however, comparing motor end-plates in the segmental tail muscles of animals fully matched except for the presence or absence of the thymus there was a significantly higher MEPP amplitude in thymectomized animals. Since thymin is considered to

depress the amplitude of MEPPS (*vide supra*) this finding suggests that thymin *is* being secreted by the normal thymus, removal of which removes this inhibitory influence on neuromuscular transmission. This conclusion was supported by the findings in the rats with multiple isogeneic thymic grafts. The decrease in MEPP amplitude in these animals having a greater amount of normal thymic tissue again suggests that thymin is being secreted by the normal thymus, with more thymus producing more thymin and resulting in a detectable depression of MEPP amplitude. These experiments therefore suggest that a substance we have called thymin is being secreted by the normal thymus in physiologically active amounts which inhibit transmission at the neuromyal synapse; thymin thus appears to act like a hormone.

The thymus is essential to the development of immunological competence (Miller, 1961), and influences the maturation of lymphoid stem cells to antigen-sensitive cells capable of initiating an immune response (Miller & Osoba, 1967). A humoral basis for this immunological function has been suggested since thymus grafts in cell tight 'Millipore' chambers restore the immunological competence of neonatally thymectomized mice (Levey, Trainin & Law, 1963; Osoba & Miller, 1963). Goldstein, Slater & White (1966) have described a substance thymosin, extracted from bovine thymus, which causes lymphopoiesis *in vivo*, and Trainin & Linker-Israeli (1967) have reported the action of a thymic extract which restores immunological competence in neonatally thymectomized mice. The relationship of thymin to thymic humoral substances affecting the maturation of lymphocytes and immunological competence remains unknown. It may be that the thymus is an endocrine organ secreting several hormones.

Myasthenia gravis has provided the clue to the presence of an endocrine function of the thymus affecting neuromuscular transmission and it is suggested that in this disease autoimmune thymitis causes the release of toxic amounts of thymin which produce the characteristic myasthenic neuromuscular block.

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REFERENCES

Castleman, B. & Norris, E.H. (1949) The pathology of the thymus in myasthenia gravis: A study of 35 cases. *Medicine (Baltimore)*, **28**, 27.

Elmqvist, D., Hofmann, W.W., Kugelberg, J. & Quastel, D.M.J. (1964) An electrophysiological investigation of neuromuscular transmission in myasthenia gravis. *J. Physiol. (Lond.)*, **174**, 417.

Fatt, P. & Katz, B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J. Physiol. (Lond.)*, **117**, 109.

Feltkamp, T.E.W., Van Der Geld, H.W.R., Kruyff, K. & Oosterhuis, H.J.G.H. (1963) Antinuclear factor in myasthenia gravis. *Lancet*, **i**, 667.

Goldstein, G. (1966) Thymitis and myasthenia gravis. *Lancet*, **ii**, 1164.

Goldstein, G. (1967) Thymic germinal centres in myasthenia gravis: a correlative study. *Clin. exp. Immunol.* **2**, 103.

Goldstein, G. (1968) The thymus and neuromuscular function. A substance in thymus which causes myositis and myasthenic neuromuscular block in guinea pigs. *Lancet*, **ii**, 119.

Goldstein, G. & Hofmann, W.W. (1968) Electrophysiological changes similar to those of myasthenia gravis in rats with experimental autoimmune thymitis. *J. Neurol. Neurosurg. Psychiat.* **31**, 453.

Goldstein, A.L., Slater, F.D. & White, A. (1966) Preparation, assay and partial purification of thymic lymphopoietic factor (thymosin). *Proc. nat. Acad. Sci. (Wash.)*, **56**, 1010.

GOLDSTEIN, G. & WHITTINGHAM, S. (1966) Experimental autoimmune thymitis. An animal model of human myasthenia gravis. *Lancet*, **ii**, 315.

GOLDSTEIN, G. & WHITTINGHAM, S. (1967) Histological and serological features of experimental autoimmune thymitis in guinea-pigs. *Clin. exp. Immunol.* **2**, 257.

HOFMANN, W.W. & STEMMER, E.A. (1963) Subthreshold activity at normal and myasthenic end-plates. *Neurology* (*Minneap.*), **13**, 227.

KATZ, B. & THESLEFF, S. (1957) On the factors which determine the amplitude of the miniature end-plate potential. *J. Physiol.* (*Lond.*), **137**, 267.

LEVEY, R.H., TRAININ, N. & LAW, L.W. (1963) Evidence for function of thymic tissue in diffusion chambers implanted in neonatally thymectomized mice. Preliminary report. *J. nat. Cancer Inst.* **31**, 199.

MCFARLIN, D.E., ENGEL, W.K. & STRAUSS, A.J.L. (1966) Does myasthenia serum bind to the neuromuscular junction. *Ann N.Y. Acad. Sci.* **135**, 656.

METCALF, D. (1966) *The thymus: Its Role in Immune Responses, Leukaemia Development and Carcinogenesis.* Springer Verlag, New York.

MILLER, J.F.A.P. (1960) Studies on mouse leukaemia. The role of the thymus in leukaemogenesis by cell-free leukaemic filtrates. *Brit. J. Cancer*, **14**, 93.

MILLER, J.F.A.P. (1961) Immunological function of the thymus. *Lancet*, **ii**, 748.

MILLER, J.F.A.P. & OSOBA, D. (1967) Current concepts of the immunological function of the thymus. *Physiol. Rev.* **47**, 437.

NASTUK, W.L., PLESCIA, O.J. & OSSERMAN, K.E. (1960) Changes in serum complement activity in patients with myasthenia gravis. *Proc. Soc. exp. Biol.* (*N.Y.*), **105**, 117.

OSOBA, D. & MILLER, J.F.A.P. (1963) Evidence for a humoral thymus factor responsible for the maturation of immunological faculty. *Nature* (*Lond.*), **199**, 653.

ROBERTS, D.V. & THESLEFF, S. (1965) Neuromuscular transmission in vivo and the actions of decamethonium: a micro-electrode study. *Acta anaesth. scand.* **9**, 165.

SIMPSON, J.A. (1960) Myasthenia gravis: a new hypothesis. *Scot. med. J.* **5**, 419.

STEG, G. (1964) Efferent muscle innervation and rigidity. *Acta physiol. scand.* Suppl. 225, **61**, 1.

STRAUSS, A.J.L., SEEGAL, B.C., HSU, K.C., BURKOLDER, P.M., NASTUK, W.L. & OSSERMAN, K.E. (1960) Immunofluorescence demonstration of a muscle binding complement-fixing serum globulin fraction in myasthenia gravis. *Proc. Soc. exp. Biol.* (*N.Y.*), **105**, 184.

TRAININ, N. & LINKER-ISRAELI, M. (1967) Restoration of immunologic reactivity of thymectomized mice by calf thymus extracts. *Cancer Res.* **27**, 309.

WHITE, R.G. & MARSHALL, A.H.E. (1962) The autoimmune response in myasthenia gravis. *Lancet*, **ii**, 120.