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STUDIES ON EXPERIMENTAL AUTOIMMUNE THYMITIS IN GUINEA-PIGS

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SUMMARY

Normal and thymectomized outbred guinea-pigs were immunized with extracts of calf thymus, calf skeletal muscle or calf heart muscle emulsified in Freund's complete adjuvant. Immunization with either thymus or skeletal muscle produced a significant incidence of thymitis and a partial neuromuscular block in contrast to control animals either untreated or injected with an extract of calf lymph node or saline in Freund's complete adjuvant. The incidence of thymitis and partial neuromuscular block in animals that were injected with an extract of calf heart muscle in Freund's complete adjuvant was found not to be significant when compared to the control animals.

Neuromuscular transmission was studied using Copeland–Davis clip electrodes or a bipolar silver wire electrode threaded through the flexor digitorum muscle. The development of partial neuromuscular block in the test animals was found to be dependent on the presence of the thymus. All animals with a partial neuromuscular block had evidence of experimental thymitis.

These findings are in keeping with the hypothesis that a factor released by the thymus may be important in the development of the neuromuscular block characteristic of myasthenia gravis.

INTRODUCTION

The thymus gland in myasthenia gravis has one of three histological appearances (Castleman, 1966). About 10% of patients have thymomas. Of the remaining patients, the thymus is normal macroscopically and microscopically in 20% while in the other 80% germinal centres are conspicuously present in the thymic medulla. In about 35% of myasthenia patients without a thymoma and in approximately 95% of patients with associated thymoma, serum autoantibody can be demonstrated reacting with striations of skeletal muscle and heart muscle and with the cytoplasm of the thymus myoid cells (Strauss *et al.*, 1965). In attempts

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to produce similar histological appearances and antibodies with similar reactivity in experimental animals, Marshall & White (1961) reported that the formation of germinal centres in the thymic medulla could be produced by intrathymic injection of a typhoid–paratyphoid vaccine or diphtheria toxoid. Namba & Grob (1966) succeeded in raising skeletal muscle antibodies in rabbits by the repeated injections of a human skeletal muscle ribonucleoprotein in Freund's complete adjuvant (CFA). Goldstein & Whittingham (1966, 1967) and Goldstein & Hofman (1968) showed that guinea-pigs or rats injected with heterologous or homologous thymus or skeletal muscle in CFA developed a thymitis, serum antibodies to skeletal muscle and thymus myoid cells and also a partial neuromuscular block. In contrast to these positive findings, negative results in similar experiments have been reported by Strauss (1963) and by Parkes (1966).

This paper describes our own experience in the attempt to produce an experimental model of myasthenia gravis in guinea-pigs.

MATERIALS AND METHODS

Animals

Outbred guinea-pigs of both sexes, 4–6 months old were used.

Antigen preparation

Fresh thymus, skeletal muscle, heart muscle and lymph node from a young calf were homogenized in phosphate buffered saline, pH 7·2 (20% w/v). The homogenates were centrifuged for 15 min at 5000 rev/min and the supernatant emulsified in an equal volume of Freund's complete adjuvant (CFA).

TABLE 1. The number of animals injected with a given antigen subdivided according to the two methods used for electromyography and whether or not thymectomy had previously been done

No. of animals		Treatment
Thymus intact	Thymectomized	
Group A		
10		Calf thymus + CFA
10		Calf skeletal muscle + CFA
4		Calf lymph node + CFA
Group B		
9		Calf thymus + CFA
8		Calf skeletal muscle + CFA
9		Calf heart muscle + CFA
5		Calf lymph node + CFA
8		Saline + CFA
10		No treatment
	7	Calf thymus + CFA
	8	Calf skeletal muscle + CFA
	4	Calf lymph node + CFA

Immunization

Each animal was injected with 0·1 ml antigen in CFA in each hind footpad. The number of animals injected with a given antigen is as shown in Table 1. The animals were subdivided into two groups A and B according to the two different methods used for electromyography. In addition five thymectomized animals were immunized with 0·1 ml of a crude guinea-pig extract in CFA and given a boosting injection after 3 weeks in order to demonstrate that these animals could still develop an experimental autoimmune disease.

Skin tests

The development of delayed hypersensitivity to the antigen used for immunization was tested after 2 weeks by the intradermal injection of calf thymus, calf skeletal muscle and calf lymph node extracts prepared as for immunization except that their concentration was 50% w/v. Animals immunized with calf heart muscle in CFA were also skin tested with a 50% w/v calf heart muscle extract. In each instance 0·1 ml of saline and of the different antigen preparations were injected intracutaneously at different sites on the abdomen after the animals had been shaved. The tests were read 30 min, 1 hr and 24 hr after the injection, and the results were recorded positive if at the injection site an erythema with oedema developed with a diameter $\geqslant 1$ cm.

Detection of circulating antibodies

The indirect fluorescent antibody test (Weller & Coons, 1954) was used. Air dried unfixed sections of thymus, skeletal muscle, heart muscle and smooth muscle were cut at 4 μ using a SLEE cryostat at $-20°C$. Tissue specimens obtained from a young calf, a 2-week-old guinea-pig and from the experimental animals were frozen to $-70°C$ in an acetone–solid CO_2 mixture immediately after the animals were killed.

Two antisera were used for the demonstration of antibody. First a polyvalent rabbit anti-guinea-pig γ-globulin serum obtained from Hyland (Hyland Division, Travenol Laboratories, Los Angeles) and secondly a rabbit anti-guinea-pig IgG serum prepared in our laboratory by immunization of a rabbit with the IgG fraction of normal guinea-pig serum. The IgG fraction was prepared by using a combined salt precipitation and chromatographic procedure (Fahey & Terry, 1967) and the absence of other immunoglobulins was confirmed by immunoelectrophoresis.

The protein content of both antisera was adjusted to 10 mg protein/ml with normal unbuffered saline and the pH was then raised to approximately 9 with a carbonate–bicarbonate buffer.

Fluorescein isothiocyanate isomer I (FITC I) (British Drug Houses Ltd, Poole, England) was used in a concentration of 1 mg for each 100 mg protein. The conjugation was done in the cold at 4°C over a period of 18 hr. The uncoupled FITC I was removed by passage through a Sephadex G-50 column. The filtrate was precipitated with two-thirds of its volume of saturated ammonium sulphate and the precipitated fraction dialysed overnight against veronal buffer, pH 7·2. The final fluorescein–protein ratio was calculated on a Zeiss P.M.Q. II spectrophotometer at a wavelength of 495 and 280 mμ. The protein–fluorescein ratio was found to be 0·5 for the conjugated antiserum to polyvalent immunoglobulin and 0·56 for the conjugated antiserum to IgG.

All the animals were bled 14–17 days after immunization and the sera stored at $-20°C$. Undiluted serum and serum diluted 1:8 was applied to the frozen sections for 20 min. The

sections were then washed in veronal buffer (pH 7·2) for 30 min with continual gentle agitation. The fluorescein conjugated antisera were applied in a dilution 1:8 for 20 min and the sections were then given a final wash in veronal buffer, pH 7·2, for 1 hr with continual gentle agitation. The sections were mounted in 10% glycerol in saline and examined using a Gillet and Sibert microscope with a 100-W Iodine–Quartz lamp with primary filter 30/063 and ocular filter 10/285.

The indirect fluorescent antibody test was also used to detect the presence of antinuclear factor in the sera of the experimental animals, using frozen guinea-pig liver sections and the polyvalent anti-guinea-pig γ-globulin conjugated as described above.

Passive haemagglutination test

Fresh tanned sheep red cells were prepared according to the method described by Herbert (1967). Tanned sheep cells were sensitized with extracts of a guinea-pig thymus, skeletal muscle, heart muscle, smooth muscle, lymph node, thyroid and saline in a concentration of 0·1–0·25 mg tissue protein/ml. The sensitized sheep cells were incubated overnight at room temperature with doubling dilutions of the sera from the test and from the control animals. The test was read positive when haemagglutination was found in a titre $\geqslant 1:20$.

Absorption procedures

The specificity of the serum antibodies in the experimental animals immunized with thymus, skeletal muscle or heart muscle in CFA for thymus lymphocytes and for thymus myoid cells and skeletal muscle and heart muscle striations was studied by absorption experiments. Calf and guinea-pig tissue extracts of thymus, skeletal muscle, heart muscle, smooth muscle, thyroid, lymph node and liver were prepared as for immunization. Two parts of tissue extract and one part of undiluted serum were incubated in a water-bath at 37°C and shaken for 1 hr. The absorption of the antibody was then studied using the indirect immunofluorescence technique with the polyvalent rabbit anti-guinea-pig γ-globulin serum conjugated with FITC I.

Other serological procedures

To determine whether the serum antibodies were complement fixing all the sera were inactivated by heating at 56°C for 30 min. Cryostat sections of guinea-pig thymus, skeletal muscle, heart muscle and smooth muscle were tested in duplicate. In each instance one of the sections was treated with inactivated serum followed by the polyvalent rabbit antiserum against guinea-pig γ-globulin conjugated with FITC I (see above). The other section was treated with guinea-pig serum and one drop of fresh complement and then with rabbit anti-serum against guinea-pig complement conjugated with FITC I (F–P ratio 0·417).

Cryostat sections of thymus, skeletal muscle and heart muscle tissue from all the animals were tested by the direct immunofluorescence method for the *in vivo* binding of γ-globulin using a polyvalent rabbit serum against guinea-pig γ-globulin conjugated with FITC I.

To test if the serum autoantibodies of animals immunized with skeletal muscle in CFA were directed against a muscle ribonucleoprotein, frozen guinea-pig muscle sections were incubated for 1 hr at 45°C with a bovine pancreas ribonuclease (British Drug Houses Ltd, Dorset, England) in a concentration of 5 mg/ml in phosphate buffered saline, pH 7·2, and afterwards used in the indirect immunofluorescence test as described above.

Histology

Specimens of guinea-pig thymus, spleen and cervical lymph nodes were fixed in Carnoy solution overnight. One tissue block of skeletal muscle, of heart muscle and of smooth muscle (stomach wall) was fixed in 10% formol saline. All tissue sections were stained with haematoxylin and eosin (H & E) and the thymus sections were stained also with methyl green pyronin, periodic acid–Schiff (PAS) and silver impregnation.

Electromyography

Fourteen to 17 days after immunization each guinea-pig was anaesthetized with Nembutal®, 35 mg/kg body weight.

Two techniques were used to record the compound muscle action potential.

In the animals of Group A, a fine insulated silver wire (0·05 mm diameter) was used for recording. A loop of this wire was attached to the amplifier imput and soldered connections checked by noting the absence of 50 cycle interference. The loop was then twisted and drawn through the exposed flexor digitorum of the animal's right front leg using a small suture needle. The end of the twisted double wire was cut, and the ends of each wire were bent to make small hooks. The resulting micro-bipolar electrode system was then drawn back into the muscle body (Fig. 1). During recording the exposed muscle was maintained at

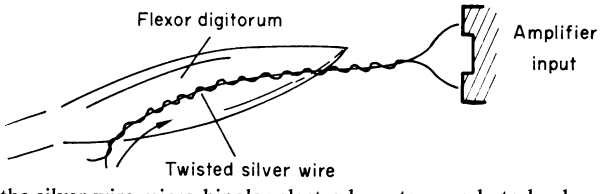

FIG. 1. Shows the silver wire micro-bipolar electrode system ready to be drawn back into the muscle body. The insulation is removed from a very small area at the end of each wire.

body temperature by covering with paraffin heated to 39°C. In animals of Group B, Copeland–Davies clip electrodes (Copeland & Davies, 1964) supplied by Electro-Physiological Instruments Ltd, Edinburgh, were used. The clips penetrated the unexposed muscle through the skin. Each clip measured 1 cm across. The two clip electrodes were placed as close together as possible. By this method recordings were made on both front limbs after they had been shaved.

In both Groups A and B the median nerve was exposed in the axilla and stimulated using silver wire electrodes (diameter 0·5 mm and shaped as small hooks) 2 mm apart. The nerve stimulation was supramaximal with a pulse duration of 100 msec. In each instance a single compound muscle action potential was observed at a sweep speed of 2 msec/cm. For tetanus a stimulation frequency of 50/sec was used and the first ten muscle action potentials observed at a sweep speed of 20 msec/cm. The first twenty-five muscle action potentials were also observed at a sweep speed of 50 msec/cm. The effect of Tensilon® on a single action potential as well as on a tetanus was tested 5–10 min after the injection of 50 μg Tensilon® intramuscularly into the right hind leg. A double pulse unit (2 GIRO, Electro-Physiological Instruments, E.P.I. Edinburgh) was used for stimulation and a recording system was designed by Electro-Physiological Instruments Ltd, Edinburgh, E.P.I. Its pre-amplifier had a gain of 1000 and was used at a time constant of 25 msec. It was incorporated in a Tele-equipment oscilloscope Model (D52). For photographic recording a Tektronix oscilloscope 502 was used with a Shackman polaroid camera (Model No. P.L.I.).

Chi-square analysis was performed to measure the significance of the incidence of thymitis in the experimental and in the control animals. Statistical evaluation of the thymus weight and the electromyographic response in the different animal groups was made using the Student *t*-test.

RESULTS

Thymic histology

Thymitis was considered to be present if there was an accumulation of small and medium

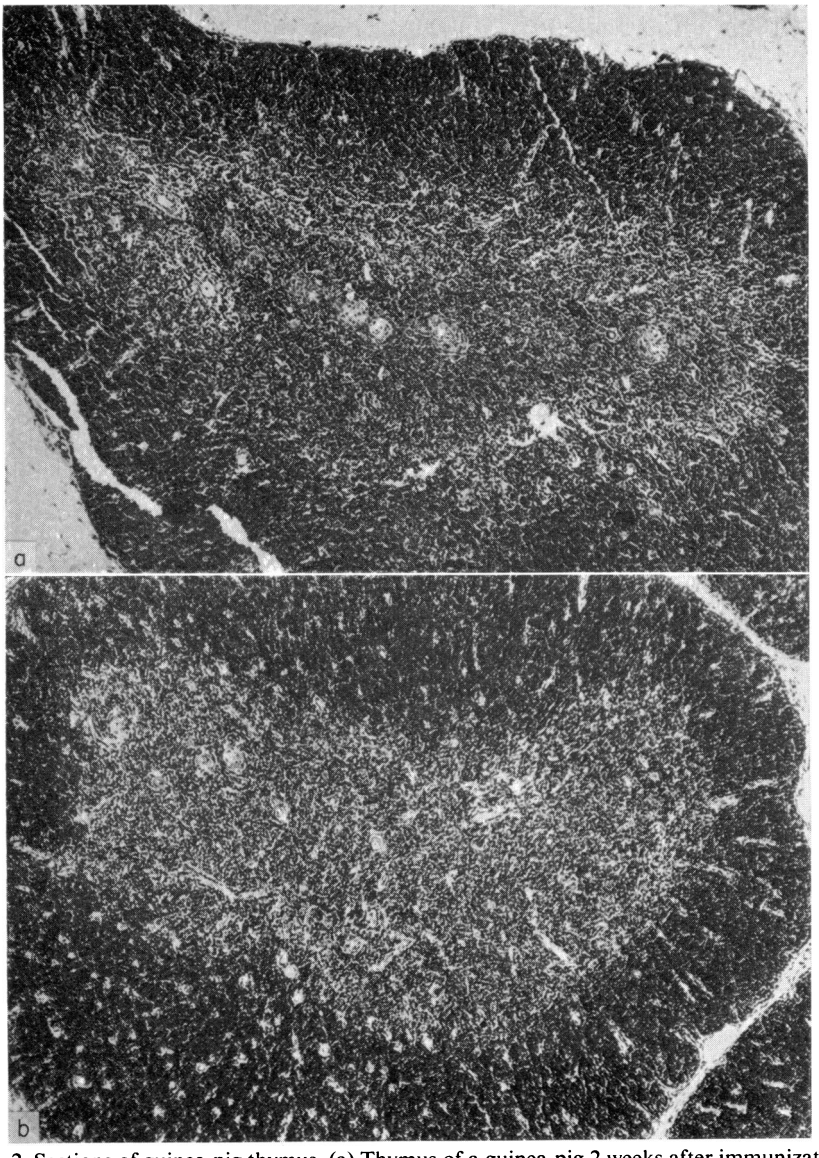

FIG. 2. Sections of guinea-pig thymus. (a) Thymus of a guinea-pig 2 weeks after immunization with calf heart muscle in CFA. Note the accumulation of lymphocytes around the Hassall's corpuscles in the thymic medulla. H & E, ×70. (b) Normal thymus from a control animal showing an even dispersion of lymphocytes in the medulla. H & E, ×70.

sized lymphocytes in the thymic medulla. The accumulation of lymphocytes tended to occur around the Hassall's corpuscles (Fig. 2a). This picture was quite distinct from the histology of the normal thymus gland in the guinea-pigs in which there is a relatively even dispersion of small lymphocytes throughout the medulla of the thymus (Fig. 2b). Staining with silver impregnation showed that in some of the thymus glands there was a breakdown of the fine medullary reticulum fibre structure with the formation of a reticulum fibre barrier at the cortico-medullary border. This change was not dependent on the presence of thymitis, since it was also observed in guinea-pig thymus that was otherwise normal. There appeared to be no differences in the plasma cells, Hassall's corpuscles or cortex in the presence or absence of thymitis.

TABLE 2. Incidence of positive delayed hypersensitivity and incidence of experimental thymitis in experimental and control guinea-pigs 14 days after immunization

Thymus status	No. of animals	Antigen injected	Positive delayed hypersensitivity* (test tissue)					Incidence of thymitis	Thymus weight (mean+SD)
			Thymus	Skeletal muscle	Heart muscle	Lymph Node	Saline		
Intact	19	Thymus	18	13	NT†	16	0	11	0·49±0·25
	18	Skeletal muscle	16	13	NT	11	0	9	0·56±0·24
	9	Heart muscle	9	8	9	8	0	2	0·51±0·16
	9	Lymph node	7	6	NT	8	0	1	0·55±0·16
	8	Saline	0	0	NT	0	0	1	0·47±0·11
	10	–	0	0	NT	0	0	0	0·5 ±0·22
Thymectomized	8	Thymus	6	4	NT	6	0	–	–
	7	Skeletal muscle	7	6	NT	7	0	–	–
	4	Lymph node	4	1	NT	4	0	–	–

* No. of animals with positive skin reactions after 24 hr.
† NT, Not tested.

The incidence of thymitis (Table 2) recorded in animals injected with calf thymus in CFA (eleven out of nineteen) and in those immunized with calf skeletal muscle in CFA (nine out of eighteen) is significantly higher than the incidence in control animals (two out of twenty-two) with P values of $<0{\cdot}001$ and $<0{\cdot}01$, respectively. The occurrence of thymitis in two out of nine animals immunized with heart muscle in CFA was not significant when compared with that in the control animals ($P>0{\cdot}5$). Likewise there was no statistical difference in the incidence of thymitis in animals injected with lymph node in CFA (one out of nine) or in animals injected with saline in CFA (one out of eight) compared to untreated guinea-pigs (none out of ten).

Thymus weight

The weight of the thymus in the different animal groups is given in Table 2. No significant differences were observed between the groups ($P>0{\cdot}2$).

Histology of other tissues

No abnormalities were noted in sections of spleen, lymph node and smooth muscle either in the experimental or in the control animals. In one guinea-pig immunized with thymus an inflammatory reaction was found in a muscle section taken from the proximal part of the

right hind leg. In one animal immunized with skeletal muscle an inflammatory focus in the myocardium was recorded. A small perivascular focus of inflammation was also observed in the myocardium in two of the guinea-pigs injected with heart muscle in CFA. No abnormalities in skeletal muscle or myocardium were noted in the remaining test or control animals. All guinea-pigs immunized with a crude thyroid extract in CFA after thymectomy developed histological evidence of mild to severe thyroiditis.

Delayed hypersensitivity

The number of animals giving a positive skin reaction for delayed hypersensitivity after 24 hr is shown in Table 2. No tissue specific reaction was obtained. In about 30% of the immunized animals the skin reaction showed a central necrosis after 24 hr. No immediate type of reaction was seen in any of the animals at 30 min and 1 hr after the injection. None of the animals injected with saline and none of the untreated guinea-pigs gave a positive reaction to any of the test tissues. No difference was recorded in the development of delayed hypersensitivity in guinea-pigs when the thymus was intact or after thymectomy (Table 2).

Circulating antibodies

The antibodies detected in the serum 14 days after immunization with the various tissues is given in Table 3. Results obtained in the indirect immunofluorescence test using heterologous (calf) homologous (2-week-old guinea-pigs) and autologous tissue sections were similar.

TABLE 3. Serological studies in experimental and control guinea-pigs 13 days after immunization

Thymus status	No. of animals	Antigen injected	Indirect immunofluorescence test*				ANF†
			Thymus		Skeletal muscle (striations)	Heart muscle (striations)	
			Cytoplasm of lymphocytes	Myoid cells			
Intact	19	Thymus	17	0	0	0	5
	18	Skeletal muscle	0	17	17	17	6
	9	Heart muscle	0	3	5	5	5
	9	Lymph node	(8)	0	0	0	3
	8	Saline	0	0	0	0	0
	10	–	0	0	0	0	0
Thymectomized	8	Thymus	7	0	0	0	(2)
	7	Skeletal muscle	0	7	7	7	(1)
	4	Lymph node	(4)	0	0	0	0

Numbers in parentheses indicate that the reactions are only weakly positive.

* Using autologous tissue sections and serum dilutions 1:8.

† Using homologous guinea-pig liver sections and undiluted serum in the indirect fluorescence test.

In about 10% of the control animals the undiluted sera gave weak but definite fluorescence reactions with the striations of skeletal muscle and heart muscle and the cytoplasm of lymphocytes and myoid cells of the thymus. When the test sera were diluted 1:8 no positive staining was found with the sera of the control animals. No significant difference ($P > 0{\cdot}5$)

was recorded in the incidence of circulating antibodies in thymectomized and intact animals.

Out of twenty-seven guinea-pigs injected with thymus twenty-five gave a positive fluorescence reaction with the cytoplasm of the thymus lymphocytes (Fig. 3). No reaction was recorded with skeletal muscle or with heart muscle sections. A similar but weak reaction was found in seven out of nine guinea-pigs injected with lymph node. Twenty-four out of

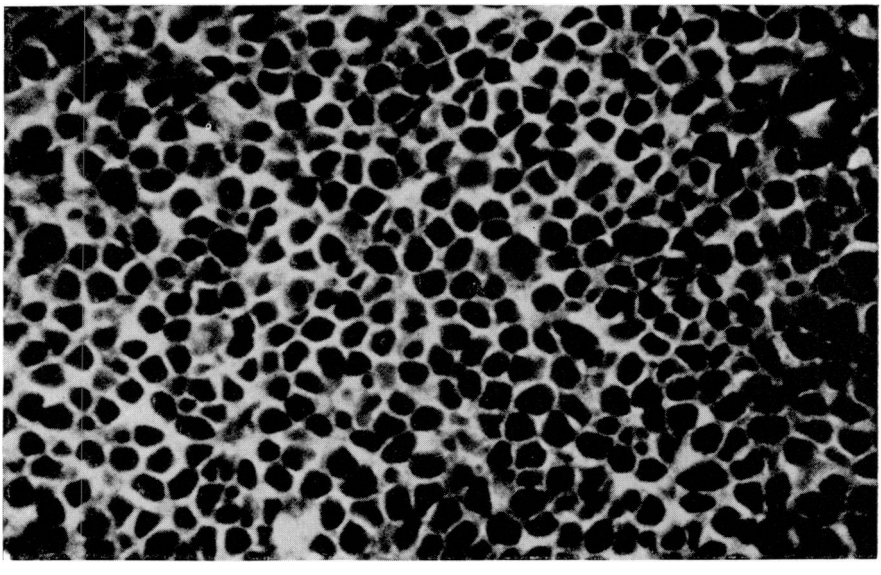

FIG. 3. Indirect immunofluorescence test using a cryostat section of guinea-pig thymus and the serum of a guinea-pig 2 weeks after immunization with calf thymus in CFA. There is a positive reaction with the cytoplasm of the thymus lymphocytes. ×320.

twenty-five animals immunized with skeletal muscle and five out of nine animals with heart muscle showed positive fluorescence reactions with the cytoplasm of the thymus myoid cells (Fig. 4a), and with the striations of skeletal muscle (Fig. 4b) and heart muscle (Fig. 4c). A few sera also showed positive fluorescence reactions with Hassall's corpuscles and weak staining of the connective tissue septa in the thymus (Fig. 4a). None of the sera reacted with smooth muscle. Identical fluorescence reactions were obtained using conjugates prepared from a rabbit anti-guinea-pig polyvalent γ-globulin serum and from a rabbit anti-guinea-pig IgG serum.

Table 4 shows the distribution of the antibody titres in the test and control animals. In general the results compare favourably with those obtained by the indirect immunofluorescence test. In animals immunized with thymus in CFA the tanned red cell titre ranged up to 1:1280, and in animals sensitized with skeletal muscle in CFA the titres ranged up to 1:2560 (Fig. 5). The results demonstrate the tissue specificity of the serum antibodies raised in the experimental animals injected with different tissue homogenates, since guinea-pigs immunized with thymus in CFA reacted only with thymus or lymph node sensitized red cells and not with erythrocytes sensitized with skeletal muscle, heart muscle, smooth muscle or thyroid extracts nor with the saline control. A similar but weaker reaction pattern was found in guinea-pigs immunized with lymph node in CFA. Animals injected with skeletal muscle or heart muscle in CFA reacted only with these tissues, with the exception of three

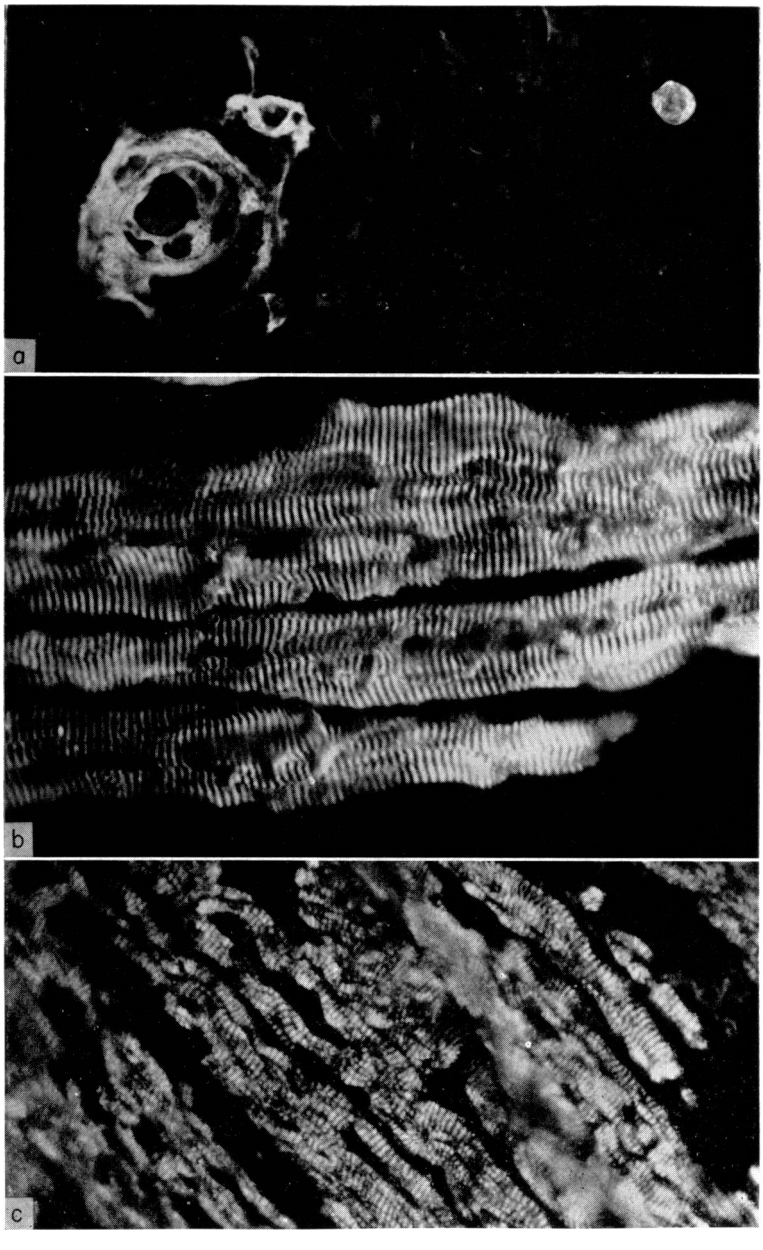

FIG. 4. Indirect immunofluorescence tests using sections of guinea-pig thymus, skeletal muscle and heart muscle and the serum from a guinea-pig 2 weeks after immunization with calf skeletal muscle in CFA. (a) Positive reaction with the cytoplasm of a thymus myoid cell and with a Hassall's corpuscle. There is also weak reaction with the connective tissue septa. ×280. (b) A positive reaction with the cross striations of skeletal muscle. ×630. (c) A positive reaction with the cross striations of heart muscle. ×630.

TABLE 4. Serum antibody titre of experimental and control guinea-pigs obtained by the passive haemagglutination test after 14 days immunization

Thymus status	No. of animals	Antigen injected	Test tissue												
			Thymus			Skeletal muscle			Heart muscle			Smooth muscle	Lymph node		Thyroid	Saline
			1:20	1:40	⩾1:80	1:20	1:40	⩾1:80	1:20	1:40	⩾1:80	⩾1:20	1:20	⩾1:40	⩾1:20	⩾1:20
Intact	19	Thymus	5*	4	6	—	—	—	—	—	—	—	7	6	—	—
	18	Skeletal muscle	3	—	—	3	3	8	4	7	3	—	1	—	1	—
	9	Heart muscle	—	—	—	4	3	—	4	3	—	—	—	—	—	—
	9	Lymph node	4	—	—	—	—	—	—	—	—	—	4	—	—	—
	8	Saline	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	10	—	—	—	—	—	—	—	—	—	—	—	1	—	1	—
Thymectomized	8	Thymus	3	2	—	—	—	—	—	—	—	—	—	—	—	—
	7	Skeletal muscle	1	—	—	2	1	3	2	1	3	1	—	—	—	—
	4	Lymph node	1	1	—	—	—	—	—	—	—	—	—	1	—	—

* No. of animals showing antibody titre at the serum dilutions indicated.

guinea-pigs which in addition showed a weak reaction with thymus sensitized cells. This latter finding is explained by the presence of a common antigen in skeletal muscle, heart muscle and thymus.

The serum antibodies in guinea-pigs immunized with thymus or lymph nodes could be selectively absorbed with thymus or lymph node extracts (calf or guinea-pig) prepared as for immunization. Similar extracts of calf and guinea-pig skeletal muscle, heart muscle, smooth muscle, thyroid and liver were ineffective in this respect. Antibodies in the serum of

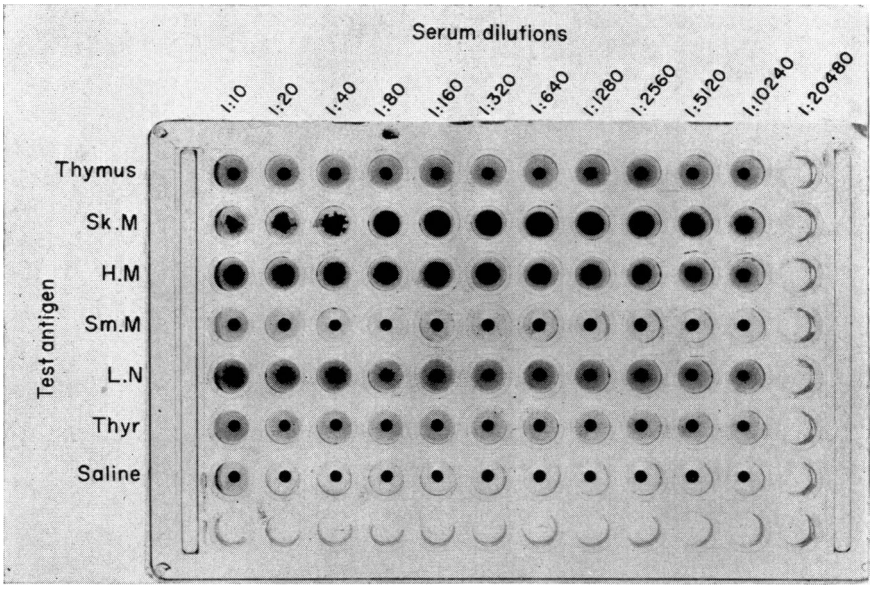

FIG. 5. Passive haemagglutination tests demonstrating the reaction pattern of serum antibodies from a guinea-pig 2 weeks after immunization with calf skeletal muscle in CFA. Agglutination occurred with sheep red cells sensitized with skeletal muscle (Sk.M) or with heart muscle (H.M) but not with cells sensitized with thymus, smooth muscle (Sm.M), lymph node (L.N) or thyroid (Thyr) extracts.

guinea-pigs immunized with skeletal muscle were readily absorbed by an extract of skeletal muscle but only weakly by an extract of heart muscle. Antibodies in the sera of animals immunized with heart muscle were readily absorbed by either skeletal muscle or heart muscle.

The detection of the antibodies by the indirect immunofluorescence test was not dependent on complement. Inactivation of the sera did not affect the results. Consistently negative results were obtained when positive sera by the indirect immunofluorescence method were inactivated, applied to the appropriate tissue sections, the sections then treated with fresh guinea-pig complement and counterstained with a rabbit anti-guinea-pig complement conjugate.

Definite positive tests for antinuclear factor were found in seven out of nine guinea-pigs immunized with thymus in CFA, six out of eighteen animals immunized with heart muscle and skeletal muscle in CFA and three out of nine guinea-pigs immunized with lymph node in CFA. Weak reactions for ANF were observed in three out of five guinea-pigs thymectomized before immunization with thymus or skeletal muscle (Table 3). No correlation was found between the incidence of thymitis and the presence of ANF in the serum.

Direct immunofluorescence tests for guinea-pig γ-globulin in thymus, skeletal muscle and heart muscle sections gave negative results in both the experimental and in the control animals.

Pretreatment of skeletal muscle, heart muscle and thymus sections with a ribonuclease prevented positive immunofluorescence reactions with skeletal muscle and heart muscle striations as well as with thymus myoid cell cytoplasm, but it did not prevent positive staining of the Hassall's corpuscles and connective tissue septa in the thymus.

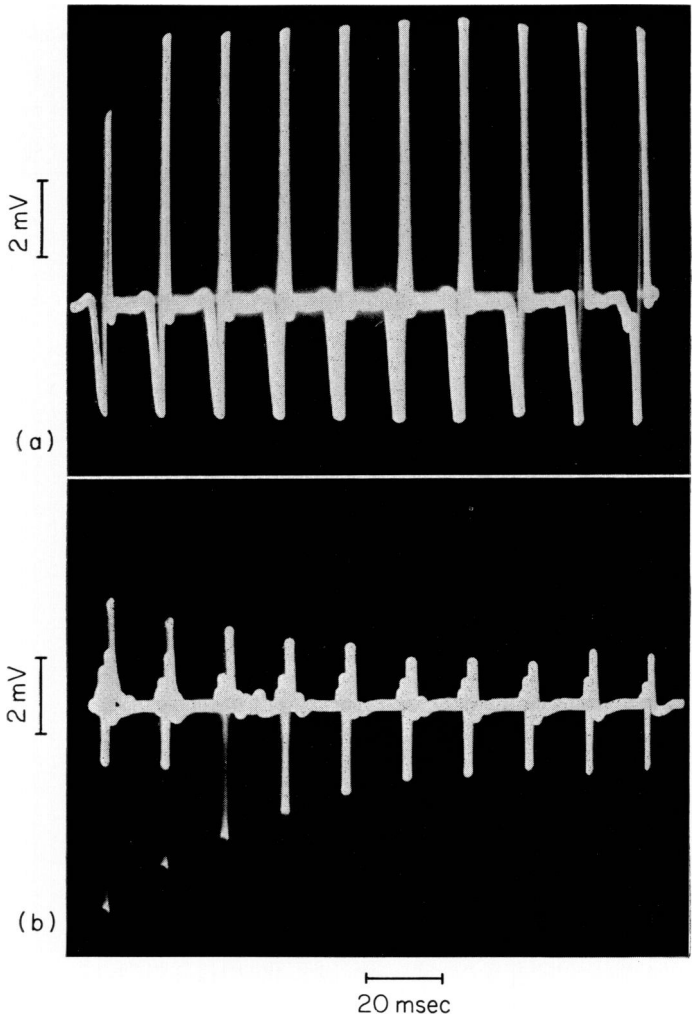

FIG. 6. Record of the muscle action potentials in the flexor digitorum following supramaximal nerve stimulation at a rate of 50/sec using Copeland–Davies clip electrodes. (a) Normal response in which the height of the successive muscle responses is maintained. The height of the first action potential as shown is foreshortened by the photographic tube. (b) Decreasing response in an animal immunized with calf heart muscle in CFA and in which thymitis was present. The tenth action potential is 36·6% of the first.

Neuromuscular transmission

The behaviour of the first ten muscle action potentials during tetanic supramaximal nerve stimulation was used to study neuromuscular transmission (Harvey & Masland, 1941). The ratio of the amplitude of the tenth to the first action potential was noted. In the control animals the lower confidence limit for this ratio was 0·81. The chance that the ratio would be

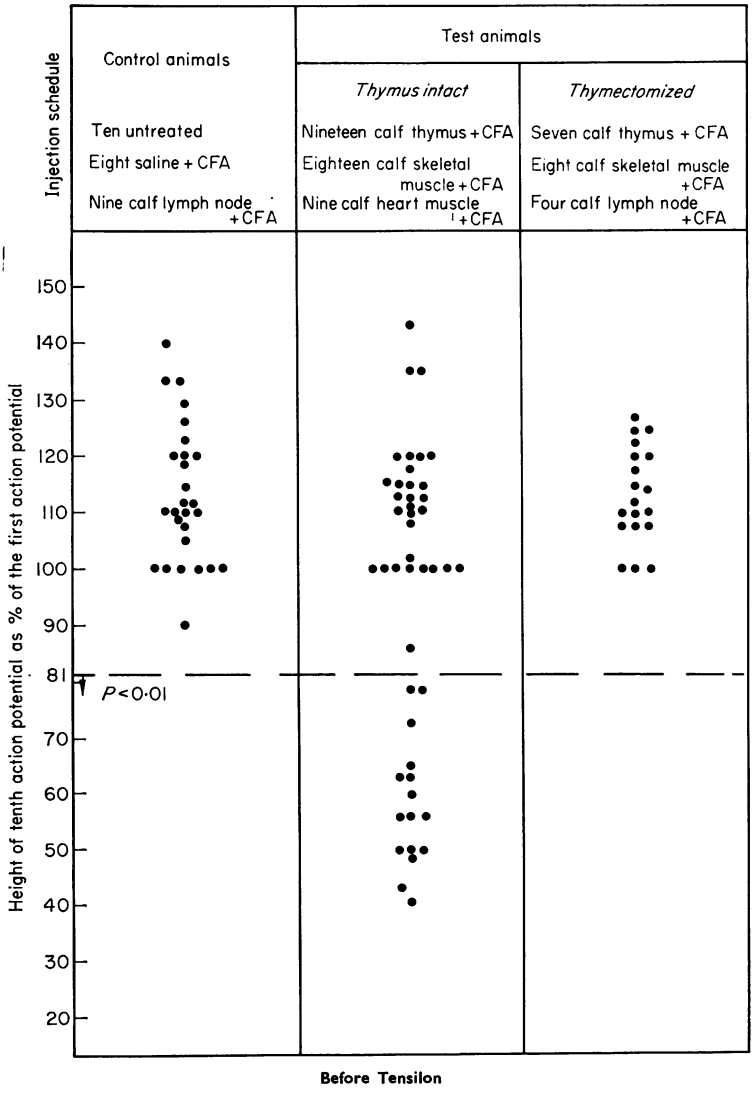

FIG. 7. The electromyographic response to supramaximal nerve stimulation at a rate of 50/sec in animals of Groups A and B combined before the injection of Tensilon. In animals of Group B, the individual results shown are the mean of the recordings in each front limb. From the findings in control animals, a decline in the height of the tenth action potential to less than 81% of the first was unlikely ($P<0·01$). Seven of the thymus-immunized, seven of the skeletal muscle-immunized and two of the heart muscle-immunized animals had a significant decline in the muscle action potentials compared to the tetanus pattern observed in the control animals.

smaller than this is unlikely ($P<0{\cdot}01$). Therefore, guinea-pigs showing a ratio of less than 0·81 were regarded as having abnormal neuromuscular transmission (Fig. 6a and b).

In the animals of Group A, three out of ten guinea-pigs immunized with thymus in CFA and four out of ten animals immunized with skeletal muscle in CFA showed a decline in the successive muscle responses over the first ten stimuli to $63\pm23{\cdot}4\%$ (mean$\pm$SD) and to

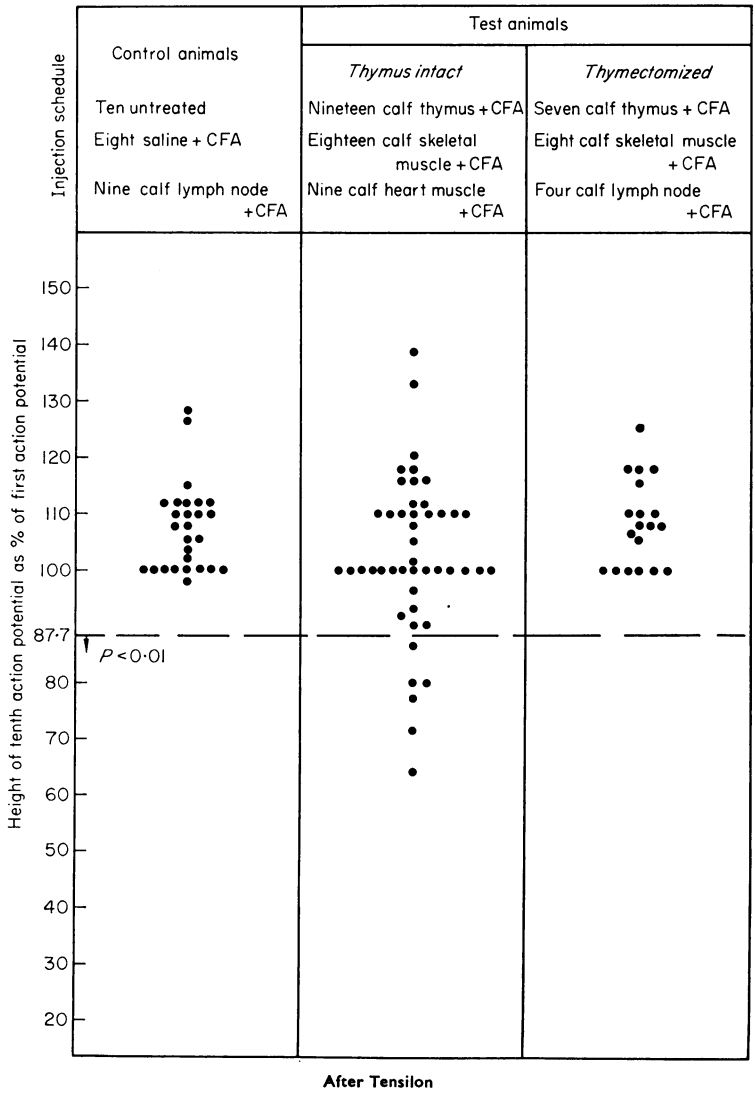

FIG. 8. The same as Fig. 7 but 10 min after 50 ng Tensilon intramuscularly. The lower confidence limit in the control animals was 87%. Note, in comparison to Fig. 7, the improvement in the tetanus muscle reaction in all animals with a partial neuromuscular block. In six guinea-pigs, however, the tetanus pattern was still subnormal.

$59{\cdot}3\pm6{\cdot}0\%$ of the initial action potentials, respectively. In contrast, in the control animals of this group an increase in the action potentials to $118{\cdot}3\pm36{\cdot}9\%$ was observed (Fig. 7).

In those animals with a partial neuromuscular block a significant improvement in their successive muscle action potentials was observed 5–10 min after the intramuscular injection of 50 μg Tensilon ($P<0{\cdot}05$ for thymus-immunized, and $P<0{\cdot}02$ for skeletal muscle-immunized animals, respectively). The tetanus pattern, however, remained subnormal. No marked affect was found in the tetanus pattern of control animals after Tensilon (Fig. 8).

In the animals of Group B, two out of nine guinea-pigs immunized with heart muscle in CFA showed a decline to $60{\cdot}6\pm10{\cdot}0\%$ (mean$\pm$SD) in the right and to $71{\cdot}4\pm2{\cdot}2\%$ in the

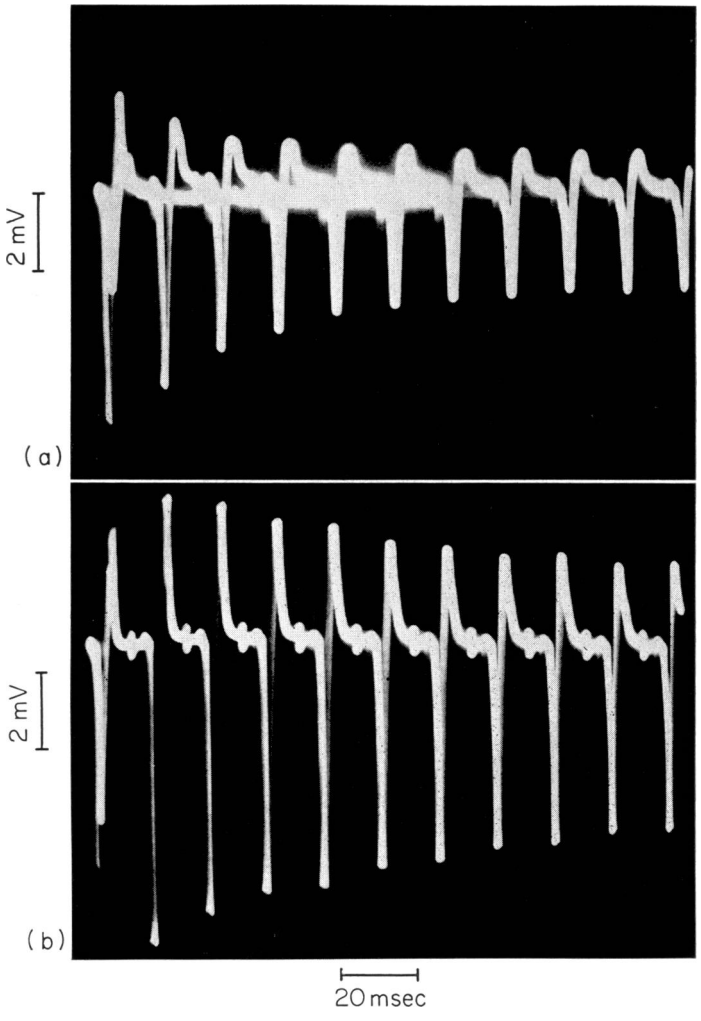

FIG. 9. Muscle action potentials in the flexor digitorum following supramaximal nerve stimulation at a rate of 50/sec. The recordings were made before and after the injection of Tensilon in a guinea-pig with thymitis 2 weeks after immunization with calf thymus in CFA. (a) Before the injection of Tensilon the height of the tenth action potential is 32% of the first. (b) Ten minutes after the injection of Tensilon the decremental response is markedly improved, the tenth muscle action potential being 65% of the first. The height of the first action potential as shown is foreshortened by the photographic tube.

left front limb. Three out of eight animals immunized with skeletal muscle in CFA showed a decline in their muscle action potentials over the first ten stimuli to $64 \pm 28{\cdot}1\%$ in the right and to $51 \pm 11{\cdot}7\%$ in the left front limb. In four out of nine guinea-pigs immunized with thymus in CFA, a decline was found to $62{\cdot}8 \pm 29{\cdot}6\%$ in the right and to $66{\cdot}9 \pm 10{\cdot}7\%$ in the left front limb. In one of the animals immunized with skeletal muscle, a marked

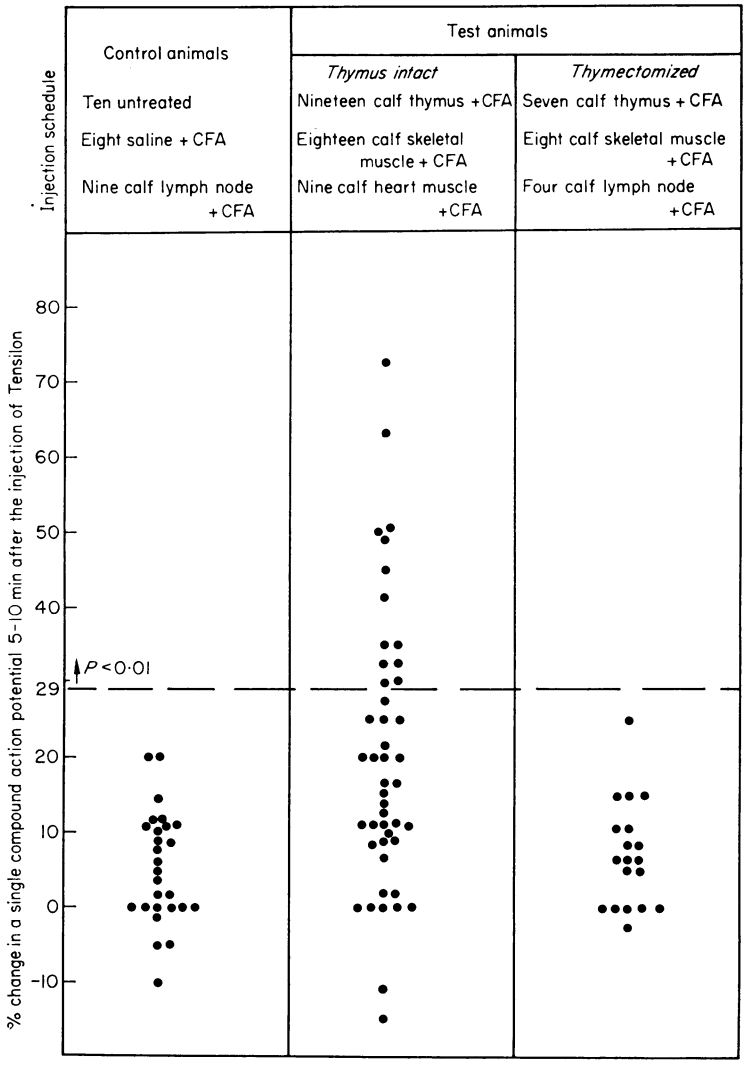

FIG. 10. The change in a single compound muscle action potential after the injection of Tensilon in animals of Groups A and B. In guinea-pigs of Group B, the results for the individual animals are shown as the mean of the recordings in the right and left front limbs. From the results obtained in the control animals a rise in a single compound action potential after Tensilon of more than 29% was unlikely ($P<0{\cdot}01$). The action potentials of six guinea-pigs immunized with thymus, six immunized with skeletal muscle and one immunized with heart muscle in CFA showed a significant increase after the injection of Tensilon.

difference in the muscle tetanus pattern was found between the front limbs with a decline in the successive muscle responses to 78·9% in the right and to 40% in the left front limb. In the control guinea-pigs of this group an increase in the successive muscle responses to 111·3±23·5% in the right and to 110·4±25·6% in the left front leg was recorded. A similar increase to 113·1±17·5% in the right and to 110·4±20·1% in the left front limb was found in those animals which were thymectomized and 1 week later immunized with either thymus or skeletal muscle in CFA. Again after the injection of 50 μg Tensilon® a significant improvement in the muscle tetanus pattern was observed in animals showing a partial neuromuscular block ($P<0·05$, $P<0·02$ and $P<0·01$ for animals immunized with heart muscle, skeletal muscle and thymus, respectively), but the tetanus pattern did not revert to a completely normal picture in all animals (Fig. 9a and b). In the control animals of this group no effect was seen (Fig. 8) after the injection of Tensilon.

In those animals of Group A in which a significant decline in the successive muscle responses was observed, the height of a single muscle action potential following a single nerve stimulus was 3·0±0·57 mV (mean±SD). This was not significantly different from the mean height of a single action potential in the control animals of this group, 3·6±0·48 mV ($P>0·1$). The mean height of a single action potential in guinea-pigs of Group B which had shown a partial neuromuscular block during tetanic nerve stimulation was 7·17±2·84 mV in the right and 7·14±3·85 mV in the left front limb. This was significantly different ($P<0·01$) from the single muscle responses of 9·59±2·4 mV in the right and 10·85±6·24 mV in the left front leg of the control animals and of the guinea-pigs thymectomized prior to immunization. The height of a single action potential in animals thymectomized prior to immunization (with 12·44±6·02 mV right and 11·73±4·4 mV six out of eight) was not significantly different ($P>0·1$) from that recorded in the control animals of this group.

From the study in the control animals, it could be said that a rise in a single muscle action potential after the intramuscular injection of 50 μg Tensilon by more than 29% would be unlikely ($P<0·01$). However, out of the forty-six animals in Groups A and B, thirteen guinea-pigs showed an increase in a single muscle action potential after the injection of Tensilon beyond 29% with a range between 29 and 73%. All these animals had evidence of a partial neuromuscular block during tetanic supramaximal nerve stimulation. In one guinea-pig immunized with thymus in CFA, one immunized with skeletal muscle in CFA and in one immunized with heart muscle in CFA with a significant decline in the successive muscle responses during tetanic supramaximal nerve stimulation, an increase in a single action potential of only 20–25% was recorded after the injection of Tensilon (Fig. 10).

DISCUSSION

In contrast to the negative findings published by Strauss (1963) and by Parkes (1966), who were unable to produce a model of myasthenia gravis in experimental animals, the present paper describes the production of thymic abnormalities and of partial neuromuscular block in guinea-pigs by immunization with either calf thymus or calf skeletal muscle extract emulsified in CFA. Our findings are similar to those reported by Goldstein & Whittingham (1966, 1967).

The thymic abnormalities consisted of an accumulation of small and medium sized lymphocytes around the Hassall's corpuscles in the thymic medulla. This is referred to as a thymitis. Germinal centres were not seen. A distortion of the fine reticulum fibre structure in the

thymic medulla, with the formation of a reticulum fibre barrier at the cortico-medullary border, did not always correspond with the presence of thymitis as described by Goldstein & Whittingham (1967). Our own unpublished experiments as well as recent results obtained by Goldstein & Hofmann (1968) and Goldstein, Strauss & Pickeral (1969) show that the incidence of thymitis may be increased by immunization of rats or guinea-pigs with homologous thymus or skeletal muscle tissue in CFA.

Experimental thymitis was also produced in two out of nine animals immunized with calf heart muscle in CFA which was, however, statistically not significantly different from the control animals. Both these animals showed evidence of partial neuromuscular block. The potency of heart muscle to initiate an experimental thymitis is probably due to a common antigen shared by skeletal muscle, heart muscle and thymus myoid cells (Van der Geld & Strauss, 1966; Beutner *et al.*, 1962). The low incidence of thymitis using heart muscle as antigen may be explained by a smaller content of thymus specific antigens in heart muscle tissue. In a careful study by Goldstein *et al.* (1969) on the capacity of thymus and skeletal muscle tissue to induce experimental thymitis, thymic tissue was found to be much more antigenic than skeletal muscle tissue, probably because of a smaller content of thymus specific antigen in the muscle tissue.

Lymphorrhages in skeletal muscle and/or myocarditis have been described in about 23% of myasthenia gravis patients (Fenichel, 1966; Mendolow & Genkins, 1954). Comparable histological changes were only observed in one out of twenty-seven guinea-pigs immunized with thymus in CFA which showed a lymphocytic infiltration in a muscle section from the upper hind limb. In one out of twenty-five guinea-pigs injected with skeletal muscle in CFA a local lymphocytic infiltration with necrosis of the muscle tissue was seen in the myocardium. Both these animals showed the features of experimental thymitis and a partial neuromuscular block. In two out of nine animals immunized with heart muscle in CFA a local lymphocytic infiltration in the myocardium was observed. The low incidence of myocardial lesions in our experiments compared to that described by Davies *et al.* (1964) is probably due to the different methods used for antigen preparation and to the different immunization schedules. Furthermore, a higher incidence of myositis in skeletal muscle may have been achieved if tissue had been examined from a larger selection of sites. None of the control animals showed lymphorrhages in skeletal muscle or myocarditis.

We were not able to correlate the development of thymitis with any pattern of delayed hypersensitivity or circulating antibody response. Guinea-pigs immunized with thymus or lymph node in CFA developed serum autoantibodies reacting with the thymus lymphoid cells but not with the myoid cells or with skeletal muscle or heart muscle probably because of insufficient myoid antigen in the calf thymus extract used for immunization (Van der Geld & Strauss, 1966). Guinea-pigs immunized with skeletal muscle or heart muscle in CFA developed antibodies against the striations of skeletal muscle, cross-reacting with heart muscle striations and thymus myoid cells. In this respect, the antibodies raised in the experimental animals had the same reactivity as serum antibodies found in patients with myasthenia gravis. However, in patients with myasthenia, there are two types of antibody reacting with muscle antigen; one of them is complement fixing and reacts with skeletal muscle only and the other is not complement fixing and reacts with both skeletal muscle and heart muscle (Van der Geld & Strauss, 1966; Beutner *et al.*, 1962). Complement fixing antibodies reactive with muscle antigens could not be produced in the guinea-pig.

A rapid decline in successive muscle responses to supramaximal nerve stimulation and

reversion to a normal tetanus pattern in response to an anticholine-esterase drug is characteristic of myasthenia gravis (Harvey & Masland, 1941; Desmedt, 1966). We have confirmed results reported by Goldstein & Whittingham (1966) that a similar pattern of a neuromuscular block can be produced in experimental animals by immunological techniques. More recently Goldstein & Hofmann (1968) have been able to demonstrate in rats with experimental thymitis a decrease in the miniature muscle end-plate potentials which is another characteristic feature of myasthenia gravis in man (Hofmann & Stemmer, 1963; Thesleff, 1966).

In contrast to bipolar needle electrodes which are frequently used for electromyographic recordings, we employed either Copeland–Davies clip electrodes or a fine twisted silver wire drawn through the muscle as recording systems. Both these techniques were found to be superior to bipolar needle electrodes for electromyography in guinea-pigs in terms of stability in the recording of a constant number of muscle fibre action potentials during the test procedure. Using Copeland–Davies clip electrodes a larger number of contracting units were recorded than by the fine silver wire method. Both these methods, however, have the same limitation as the bipolar needle electrodes in so far that they do not record the same constant number of contracting units in different animals. This fact may explain why the single action potential following supramaximal nerve stimulation in animals with experimental thymitis was not always significantly lower than the action potentials recorded in the control animals. Thus, when these types of electrodes are used, the recording of the muscle tetanus pattern during supramaximal nerve stimulation is the better parameter for detecting partial neuromuscular block.

The fact that in some of the animals Tensilon did not significantly improve the height of single compound action potentials and that in some animals with a partial neuromuscular block Tensilon did not restore the tetanic muscle reaction to normal may have been due to insufficient dosage of Tensilon, particularly as this was given intramuscularly. On the other hand, in patients with myasthenia gravis Tensilon does not invariably restore neuromuscular transmission to normal (Osserman & Genkins, 1966).

The occurrence of a marked difference in the muscle tetanus pattern in the front limbs of one guinea-pig immunized with skeletal muscle in CFA suggests that the degree of neuromuscular block produced in experimental animals may not be the same throughout the whole of the skeletal musculature.

The presence of thymitis in all animals with a partial neuromuscular block and the observation that a neuromuscular block could not be induced in thymectomized animals by immunization procedures indicate that the thymus gland is important in the production of experimental myasthenia gravis. Furthermore, in patients with myasthenia gravis of short duration thymectomy may be beneficial. Thymectomy in animals with thymitis and partial neuromuscular block has been shown to restore neuromuscular transmission to normal within 3–4 days in contrast to hemi-thymectomy or sham-thymectomy in such animals when it takes 7–10 days for the neuromuscular transmission to come back to normal (Kalden, Williamson & Irvine, 1969 in preparation). These observations support the hypothesis that an autoimmune mechanism may be important in at least some cases of myasthenia gravis. Strauss *et al.* (1966) and Goldstein (1966) have suggested that the thymus may be a source of a factor which has normally a regulative influence on neuromuscular transmission but which blocks neuromuscular transmission when secreted in excess, as may happen in the presence of thymitis. However, attempts to extract from the thymus a substance with such

properties have so far produced only equivocal results (Wilson, Obrist & Wilson, 1953; Rider, 1955; Parkes & McKinna, 1967; Goldstein, 1968).

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