Absence of cellular hypersensitivity to muscle and thymic antigens in myasthenia gravis¹

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SYNOPSIS Humoral antibodies to skeletal muscle and its components and to thymus have been demonstrated in the sera of patients with myasthenia gravis. A role for cellular hypersensitivity to similar antigens in the pathogenesis of the disease has been suggested by some reports of the presence of cellular immunity. A detailed immunological study using muscle and thymic antigens, including those prepared from the patients' own tissues, failed to confirm these findings. It is suggested that previous reports of cellular hypersensitivity represent the demonstration of an epiphenomenon.

There is good evidence that immunological mechanisms are involved in the pathogenesis of myasthenia gravis (Simpson, 1960, 1975). The strongest argument is the fact that the thymus gland is histologically abnormal in more than 80% of cases; in about 70% medullary germinal centres are found and in about 10% a thymoma is present (Castleman and Norris, 1949). The proportion of patients in whom myasthenia is associated with a thymoma has been reported as from 10–30% of cases but the incidence increases with age and the overall percentage is therefore approximately 10% (Castleman, 1955; Simpson, 1958). Medullary germinal centres similar to those found in myasthenia are also seen in other disorders presumed to have an auto-hypersensitivity basis—for example, thyrotoxicosis and Addison's disease (Sloan, 1943), thyroiditis (Gunn *et al.*, 1964), and systemic lupus erythematosus (Goldstein and Mackay, 1967). The centres have also, however, been seen in young normal subjects dying suddenly (Middleton, 1967) or undergoing cardiac surgery for congenital anomalies (Vetters and Barclay, 1973) and therefore their importance seems debatable (Vetters and Simpson, 1974).

There is a definite clinical association between myasthenia gravis and other presumed auto-

immune disorders—for example, rheumatoid arthritis (Simpson, 1960), systemic lupus erythematosus (Wolf and Barrows, 1966), Sjögren's disease (Brown *et al.*, 1968), pernicious anaemia (Simpson, 1960, 1964; Bletcher and Williams, 1967), pemphigus (Beutner *et al.*, 1968; Vetters *et al.*, 1973), autoimmune haemolytic anaemia (Cohen and Waxman, 1967; Halperrin *et al.*, 1966), Hashimoto's thyroiditis (Simpson, 1964), and thyrotoxicosis and hypothyroidism (Osserman *et al.*, 1967; Simpson, 1968). Serological abnormalities including rheumatoid factor, anti-nuclear antibodies, thyroid and haemolytic auto-antibodies have been frequently demonstrated in myasthenia (van der Geld *et al.*, 1963; Adner *et al.*, 1964; Simpson, 1964). Abnormalities in IgA metabolism have also been found in these patients (Behan *et al.*, in preparation). Histologically, skeletal muscles from myasthenic patients show round cell infiltrates (lymphorrhages), non-specific findings which may suggest an immune response.

Humoral antibodies to skeletal muscle or its components have been demonstrated in the serum of myasthenic patients by a variety of techniques, including immunofluorescence (Strauss *et al.*, 1965; Nastuk *et al.*, 1966; Vetters, 1967), tanned red cell agglutination (Djanian *et al.*, 1964), and precipitation in gels (Shulman *et al.*, 1966). Strauss *et al.* (1965) originally demonstrated that the serum of 30% of myasthenics, and of almost all patients with myas-

¹ This work was supported by the Muscular Dystrophy Group of Great Britain.

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(Accepted 9 May 1975.)

thenia gravis and a thymoma, contained antibody to muscle demonstrable on immunofluorescent staining. They used 900 control cases consisting of normal subjects and patients with a great variety of neurological disorders and showed that all but one were negative. Other workers have reported positive immunofluorescence in normal undiluted serum but Strauss used serum at a dilution of 1 in 60 and considered the reaction that he found was specific and characteristic of the disease.

The role of these antibodies in disease pathogenesis, however, is doubtful since antibodies are not present in the majority of patients with myasthenia and, even when present, there tends to be no correlation between the antibody titre and the course and severity of the disease (Oosterhuis *et al.*, 1967). Mothers with myasthenia have been found to show high antibody titres with no symptomatology in the neonate and the reverse has been described (Oosterhuis *et al.*, 1966). Again, patients with a thymoma often have high titres of anti-muscle antibody and yet show no evidence of myasthenia even on detailed electrophysiological testing (McFarlin *et al.*, 1966). Theoretically, it is also difficult to account for a transmission defect produced by anti-muscle antibodies involving myofibrils rather than by a process which affects the motor end-plates.

The original suggestion of Simpson (1960) of a cell-mediated immunity in myasthenia gravis has, therefore, been reconsidered. Several reports have suggested that such mechanisms are involved but the results have been conflicting. It was decided to investigate cellular hypersensitivity to a variety of muscle and thymic antigens in myasthenia using a standardized and reproducible *in vitro* technique (Soborg and Bendixen, 1967; Rosenberg and David, 1970). These antigens were prepared similarly (1) to those used in other reports which had claimed positive cell mediated immune responses (Alpert *et al.*, 1972; Armstrong *et al.*, 1973; Kott *et al.*, 1973; Goust *et al.*, 1974), or those which had been claimed to induce an experimental thymitis and a partial neuromuscular block in guinea-pigs (Kalden *et al.*, 1973), and (2) to those antigens which react specifically with the sera of patients with myasthenia (Aarli, 1972). In addition, control non-muscle antigens were used to which the patients

demonstrated delayed hypersensitivity by skin testing. Finally, a group of patients were tested immediately before and within a week after thymectomy with antigens prepared from their own thymus and skeletal muscle so as to obviate the possible effect of histocompatibility antigens.

METHODS

SUBJECTS *Patients A* Fifteen patients with myasthenia gravis of varying stages of severity, none of whom had undergone thymectomy.

Patients B Nine patients with myasthenia gravis who were tested before and after thymectomy.

Patients C Thirty control subjects consisting of 10 normal subjects and 20 patients with a variety of neurological disorders affecting the central and peripheral nervous system: sciatica (five), peripheral neuropathy (two), meningioma (two), glioma (three), cerebrovascular haemorrhage (two), cerebrovascular thrombosis (four), subarachnoid haemorrhage (two).

ANTIGENS 1. *Streptokinase/streptodornase* (Vari-dase, Lederle Laboratories) (SKSD) This was used at a concentration of 300 units/ml tissue culture fluid.

2. *Muscle antigens* Three categories of muscle antigens were prepared from skeletal muscle obtained at thymectomy, other operations, or necropsy and dissected as free as possible of connective tissue, fat, and blood. One of the five antigens listed below was then prepared.

a. *Preparation of muscle homogenate* Fresh muscle was placed in a sterile dish, diced with a scalpel blade, and then homogenized in phosphate buffered saline pH 7.2, as a 20% w/v homogenate, in a Sorval Omnimix Blender. It was then centrifuged for 15 minutes at maximum speed and aliquots of the supernatant were stored at -20°C and used within three weeks of preparation. Before use, the antigens were frozen and thawed once, then diluted with RPMI 1640 tissue culture fluid to obtain the desired dilution. Dose response curves were used initially to determine cytotoxicity of the antigen. The concentrations finally used were 1/100 and 1/50 dilutions of the original 20% w/v homogenate.

b. *Preparation of soluble and microsomal fractions of muscle* The method of Kalden *et al.* (1973) was followed. Fresh skeletal muscle tissue was homogenized (20% w/v) in phosphate buffered saline

pH 7.2. The homogenate was centrifuged for 10 minutes at 2 500 *g* and the supernatant then used to prepare the fractions needed. First the supernatant was centrifuged for 30 minutes at 10 000 *g* at 4°C. The sediment was discarded and the supernatant further centrifuged in a Beckman vacuum ultracentrifuge at 105 000 *g* for one hour. The final sediment obtained (so-called microsomal fraction) and the supernatant (so-called soluble fraction) were lyophilized. The lyophilized fractions were prepared for use by dissolving in RPMI 1640 tissue culture fluid and making up to concentrations of 500 µg/ml (microsomal fraction) and 500 µg/ml and 250 µg/ml (soluble fraction). These concentrations were determined to be non-cytotoxic by previous dose-response measurements.

c. Preparation of citric acid extract of muscle Skeletal muscle was thawed, minced, and washed in cold phosphate buffered saline repeatedly until the supernatant was free of all discoloration. A citric acid extract was then prepared as described by Espinosa and Kaplan (1968) and used by Aarli (1972). The procedure included repeated extractions with 0.85% NaCl before treatment with 0.05 M citric acid. The final extract obtained was lyophilized. It was easily soluble in RPMI 1640 and concentration response curves were used in order to obtain a concentration of the extract which did not produce non-specific inhibition in the controls. In most cases the concentration used was 50 µg/ml.

3. Thymus antigens Human thymus obtained at operation for thymectomy was dissected free of connective tissue, fat, and blood vessels. It was washed repeatedly in cold phosphate buffered saline and then frozen to -20°C unless used at once to prepare the antigens.

a. Preparation of thymus homogenate Fresh thymus tissue was minced, then homogenized in 20% w/v in RPMI 1640 using a Sorval Omnimix blender. It was filtered once through cotton gauze and then frozen and thawed once before being stored at -20°C until use. It was used at a final concentration of 1/20 of the original 20% w/v homogenate.

b. Preparation of thymus soluble antigen The method of Goust *et al.* (1974) was used. Fresh thymus was minced and suspended in 10% w/v in 0.5 M saline and then homogenized in a Sorval Omnimix blender at maximum speed in bursts of 20 seconds. The homogenate was then spun at 1 000 *g* for 15 minutes and the lipid-free supernatant lyophilized. The above operations were all carried out at 4°C. Finally,

protein concentration was adjusted to give a final concentration of 100 µg/ml.

SKIN TESTING Patients and control subjects were skin tested by an intradermal injection into the volar surface of the arm of 0.1 ml of a normal saline solution containing 5 units and, if unreactive to this, a saline solution containing 10 units of streptokinase/streptodornase. The results were examined at 24 and 48 hours and were considered to be positive when the raised indurated area was greater than 1.0 cm in diameter.

In vitro TECHNIQUE OF MACROPHAGE INHIBITION The leucocyte migration test was performed according to the technique of Soborg and Bendixen (1967) with modifications as indicated. Essentially 50 ml venous blood was taken under sterile conditions into a syringe containing 1 000 units of preservative-free heparin and 10 ml dextran. The blood was then allowed to sediment at 37°C for 20 minutes. All but the bottom 0.5 cm of leucocyte-rich plasma was removed and the suspension then centrifuged at 250 *g* for 10 minutes. Nine volumes of 0.83% ammonium chloride were added and the cells resuspended and left in this solution for four minutes exactly. (The ammonium chloride lysed the red cells present.) The cells were then centrifuged at 200 *g* and washed twice in RPMI 1640 tissue culture fluid and then once in RPMI 1640 to which 10% fetal calf serum had been added and 1.0% penicillin and streptomycin. After the final wash the cell pellet was suspended in the residual fluid and a trial capillary filled and sealed with Critoseal. This capillary was centrifuged at 200 *g* for four minutes and the concentration of the cell suspension remaining was adjusted so that after dilution with antigen (1/5 dilution) a length of 2-3 mm of the capillary would be filled with packed cells. Usually between 2.2 and 2.8 ml of cell suspension of the required concentration was obtained, meaning that a total of between 22 and 28 chambers (each containing two capillaries) could be set up. The cell suspension was then divided into at least nine aliquots of 0.2 ml. Each aliquot was incubated with 0.05 ml of one of the various antigens (and one aliquot had 0.05 ml of RPMI 1640 alone added) for two hours at 37°C. Thus a minimum of two chambers was set up for each antigen and the controls. If the number of cells permitted, the aliquots of each cell suspension and the antigen were increased accordingly. At the end of the two hour incubation period haematocrit tubes were filled with the cell suspensions sealed at one end with Critoseal and centrifuged at 200 *g* for four minutes. The tubes were cut at the cell-fluid interphase and the cell bearing ends of two capillaries placed in each small culture chamber

(planchette: Univers, Meckaniska, Sweden) secured at one end with silicone grease (Dow Corning, vacuum silicone grease). The chambers were filled with tissue culture medium RPMI 1640, sealed with a greased cover slip, and incubated for 18 hours at 37°C. The migration surfaces were then examined under a Leitz Diavert inverted microscope with drawing attachment and the area of migration drawn on white paper. This area was measured with a planimeter. The migration index was then calculated thus:

migration index

$$= \frac{\text{surface area in presence of antigen}}{\text{surface area in absence of antigen}} \times 100$$

Each result is the average of at least four capillary measurements: unless the measurements agreed within 15% the result was discarded. An inhibition of migration of more than 30% was considered to be a significant result.

AUTOLOGOUS ANTIGENS Group B patients were tested for possible cell-mediated immunity to their own muscle and thymus. Their peripheral blood was withdrawn before thymectomy and set up as described in the presence of crude homogenates of muscle and thymus obtained at operation. The experiment was repeated one week later using the same antigens.

RESULTS

Delayed hypersensitivity, as demonstrated by inhibition of macrophage migration greater than 30%, was observed in all patients tested with the SKSD antigen. Excellent correlation was observed between this finding *in vitro* and the demonstration of cutaneous reactivity to SKSD with thickened, indurated lesions greater than 1.0 cm *in vivo*.

No inhibition of any significant degree (greater than 30%) could be found in normal subjects or in the three groups of patients, to any of the antigens prepared from thymus and muscle (Figs 1, 2, and 3).

It can be seen that thymectomy had no influence on the results of testing with SKSD antigens, as measured *in vitro*. Similarly, thymectomy did not influence the negative results to muscle and thymic antigens in the patients tested with autologous antigens before and after operation.

DISCUSSION

We were able to demonstrate that the *in vitro* technique of macrophage migration inhibition

FIG. 1 Migration Index of peripheral blood leucocytes on contact with muscle and thymic antigens, in 10 normal controls and 20 patients with neurological disorders other than myasthenia gravis. Δ = normal controls. $\bullet$ = patients with neurological disorders.

FIG. 2 Migration Index of peripheral blood leucocytes on contact with muscle and thymic antigens, in 15 non-thymectomized patients with myasthenia gravis.

FIG. 3 Migration Index of peripheral blood leucocytes on contact with muscle and thymic antigens, in nine patients with myasthenia gravis, before and after thymectomy. ● = before thymectomy. △ = after thymectomy.

detected cell-mediated immunity to streptokinase/streptodornase (SKSD) but we were unable to show similar sensitivity to any of the skeletal muscle or thymic antigens employed in our experimental subjects. These results, therefore, conflict with those of other workers who, using the same technique, claim to have found hypersensitivity to muscle and thymic antigens in patients with myasthenia.

Alpert *et al.* (1972) obtained positive inhibitory responses to crude skeletal muscle and myosin-containing fractions in 14 of 21 myasthenics. They considered inhibition of migration of more than 30% to be significant: 12 of their patients showed this degree of inhibition but inhibition to the same antigens of up to 10% was also present in their control subjects. No positive control antigens were used in the system—for example, PPD, candida, or SKSD but with non-specific proteins, such as meconium extract, they obtained greater than 20% migration inhibition in some patients. A strong positive correlation was noted between the degree of cell-mediated immunity and the titre of humoral muscle antibodies.

Armstrong *et al.* (1973), using thymic lymphocytes, found cellular hypersensitivity to crude muscle and crude thymus antigens in myasthenic patients but also reported similar sensitivity to muscle in nearly 50% of their control subjects. In addition, they obtained a positive response in a patient with a thymoma who did not have myasthenia gravis. Kott *et al.* (1973) showed positive *in vitro* responses to crude muscle and myosin-containing fractions as antigen in 63% of their patients with myasthenia gravis. They noted, however, poor correlation between cell-mediated immunity and the presence of humoral antibodies but they were able to demonstrate a correlation between the stage of the disease and cell-mediated or humoral immunity. The authors stated that tuberculin was used as a positive control antigen but these particular results were not presented.

Goust *et al.* (1974) found cell-mediated immunity to muscle and thymic antigens in 45 of their 46 patients. They used monkey muscle and thymus and human muscle as antigens. They demonstrated that some of their patients responded only to human muscle and others only to monkey muscle and one to monkey thymus

only. They noted that 'no correlation appears to exist between the clinical and pathological data' and presence of a thymoma, thymectomy, or a changing clinical state did not influence the results of their test. No control antigens of any sort were used and some of their control subjects had inhibition of 20–35% to the muscle and thymus used as antigen. Positive responses to the muscle extracts were also found in 12 of 14 patients with polymyositis but without myasthenia gravis.

A criticism that can be made of all these reports is that positive control antigens were not used. In addition, dose response curves were not employed to measure cellular hypersensitivity when present. In our experiments, increasing the dose of antigen had no effect, whereas if sensitivity were present one would expect some increase of inhibition with an increase in antigen concentration. Furthermore, Goust *et al.* (1974) noted that enhancement of macrophage migration was present in 10 cases at one or both of the antigen concentrations used and they stated that 'in myasthenia gravis, with this technique, an augmentation of the migration index seems as significant as a depression'. Augmentation is found in the macrophage migration inhibition test when no sensitivity exists, so that this statement is open to criticism.

A non-specific inhibition of macrophage migration may occur due to differing histocompatibility antigens. Falk *et al.* (1970) showed that non-sensitized lymphocytes were stimulated to produce macrophage inhibiting factor on contact with different histocompatibility antigens. We used autologous antigens in some of our experiments in order to eliminate this effect but the phenomenon may help to explain, in part, the inhibition noted by other workers.

Our findings do agree with those of experimenters who used different *in vitro* methods to try to detect cell-mediated immunity to muscle and thymic antigens in patients with myasthenia gravis. Houseley and Oppenheim (1967) and Lisak and Zweiman (1975), employing the technique of measuring lymphocyte proliferative responses, were unable to show any sensitivity in patients with myasthenia. Similarly, Abramsky *et al.* (1975), using a lymphocyte transformation method, found no response to crude muscle antigen in the myasthenic patients they investigated.

The inhibition which has been observed is most likely an epiphenomenon. Indeed, Alpert *et al.* (1972) point out in the interpretation of their results that the findings need not reflect a primary immunological effect but rather 'may simply reflect a cellular response to tissue alteration resulting from another cause'. This was also the opinion of Lisak and Zweiman (1975) who stated 'it would be somewhat surprising if such reactivity (cell-mediated immunity) was pathogenic in the face of the disparate clinical features of the two disorders' (the other condition they referred to was polymyositis in which cell-mediated immunity has been shown by Currie *et al.* 1971).

The specificity and significance of cell-mediated immune responses to muscle in myasthenia gravis is questioned because of the demonstration of similar sensitivity in patients with muscular dystrophy (Caspary *et al.*, 1971), polymyalgia rheumatica (Esiri *et al.*, 1973), and Guillain-Barré syndrome (Caspary *et al.*, 1971). Furthermore, Kott *et al.* (1971) have reported sensitivity to central nervous system antigens in patients with myasthenia gravis and Caspary *et al.* (1971) have found similar reactivity to these same antigens in patients with muscular dystrophy.

The possibility that muscle and thymic antigens might be involved in the pathogenesis of myasthenia gravis, however, is also suggested by the work of Goldstein and Whittingham (1966) and Kalden and Irvine (1969) who claim that myasthenia gravis can be produced in guinea-pigs by immunizing them with these antigens. Several other investigators, however, have been unable to confirm their findings (Kaufmann *et al.*, 1969; Namba and Grob, 1969; Veters *et al.*, 1969; Behan, 1974). None of the animals immunized by the former workers developed clinical disease and subtle electrophysiological changes were used as the criteria for diagnosis of myasthenia. These changes are non-specific and open to differing interpretations (Veters *et al.*, 1969; Brooks, 1971). The antigens used by Kalden *et al.* (1973) failed to elicit any positive responses in our system.

The occurrence of myasthenia gravis in association with diseases in which there is anergy—for example, chronic lymphatic leukaemia (Cohen and Waxman, 1967), lymphosarcoma (Simpson, 1960), Hodgkin's disease, systemic

lupus erythematosus (Wolf and Barrows, 1966), and sarcoidosis (Simpson, 1960; 1964)—mitigates against cell-mediated hypersensitivity being involved in the pathogenesis of the disease. Fudenberg (1971) has postulated that there is a depression of T-cell function rather than a hyper-allergic state in the autoimmune disorders. In fact, myasthenic patients show impaired immune responses to dinitrochlorobenzene (DNCB) sensitization (Adner *et al.*, 1964).

An association between certain histocompatibility antigens and myasthenia has also been demonstrated recently (Pirkanen *et al.*, 1972; Behan *et al.*, 1973). HLA-8 has been found to occur with a very high frequency in myasthenia gravis, gluten enteropathy, dermatitis herpetiformis, and autoimmune thyroiditis (Dausset *et al.*, 1974). It is claimed that HLA-8 is associated with disorders of impaired immunity (Da Costa *et al.*, 1974).

Our work, therefore, provides no evidence to support the theory that cell-mediated immunity to muscle or thymic antigens is involved in the pathogenesis of myasthenia gravis. The most likely explanation for the previous reports of such cellular hypersensitivity is that these findings represent an epiphenomenon and not true cell-mediated immunity.

The encouragement and advice of Professor J. R. Anderson are gratefully acknowledged.

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