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DELAYED HYPERSENSITIVITY TO MUSCLE AND THYMUS IN MYASTHENIA GRAVIS AND POLYMYOSITIS

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SUMMARY

Delayed hypersensitivity reactions appear to be important in the two main muscular human diseases involving autoimmune anomalies. Using the leucocyte migration test (LMT), a significant abnormality was detected in twelve out of fourteen polymyositis (PM) patients, and in forty out of forty-six myasthenics, in the presence of muscle antigens. No abnormal reactions in the presence of monkey thymus in the LMT were observed amongst the PM patients, as opposed to twenty-one abnormal reactions out of forty-two myasthenics. Secretion of migration inhibition factor (MIF) in the presence of muscular antigens is in accordance with what is known about hypersensitivity reactions during the course of polymyositis. In myasthenia gravis (MG), delayed hypersensitivity to muscle antigens was found to be frequent, and this also applies to thymic antigens, which are considered important in this disease. The role of the T lymphocytes in the neuromuscular junction still remains hypothetical.

INTRODUCTION

Two groups of muscular illnesses occur with a certain frequency among the autoimmune diseases: myasthenia gravis (MG) and polymyositis (PM) (or dermatomyositis (DM)). Recent studies have shown the presence of lymphocytes, cytotoxic to cultured muscle cells, during PM and DM (Currie, 1970, 1971; Currie, Saunders & Knowles, 1971; Kakulas, 1966). In MG, the lymphoblast transformation test had yielded controversial results (Housley & Oppenheim, 1967). It would be interesting to study the cell-mediated immunity during the course of the two diseases, whose causes, evolution and immunological profiles are very different, using an '*in vitro*' cellular immunity test, i.e. the leucocyte migration test (LMT), thus expanding on the initial studies (Alpert *et al.*, 1970). This technique may be considered a good test for delayed hypersensitivity (Søborg & Bendixen, 1967; Rosenberg & David, 1970).

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MATERIALS AND METHODS

Patients and diseases

Myasthenia gravis (MG). Forty-six patients were tested, some of them on several occasions during the course of the disease; at the time of testing, nineteen were in class III or IV, as defined by Alpert *et al.* (1971) twenty-six in classes I, IIA or IIB, and one patient, who did not show any of the usual electromyographical signs, but whose fatigue symptoms were alleviated by mytelase treatment, was also classified under this diagnosis.

Polymyositis and dermatomyositis (PM and DM). Fourteen patients were tested, some on several occasions during immunosuppressive treatment. Their diagnosis was based on clinical data, an elevated level of muscular enzymes (aldolase, creatine kinase and lactatedehydrogenase), and the histological examination of muscle and skin biopsies. One patient had PM associated with a visceral neoplasia.

Other neuro-muscular disorders. Thirty-three patients were tested, and divided into two groups. The first group of fourteen patients had striated muscle abnormalities. Six of these had various thyroid disorders such as thyroiditis or hypothyroidism (five cases) and exothalmic ophthalmoplegia (one case); the others consisted of two cases of Steinert's disease, one case of muscular dystrophy of the Walton–Nattras type, three cases of sclerodermias and two cases of Lambert–Eaton syndrome. The second group of nineteen patients had neurological diseases without muscular involvement—polyradiculoneuritis (eight cases), multiple sclerosis (eight cases) and central nervous system tumours (three cases).

Normal subjects. Twenty volunteers in good health were studied in the same way.

The leucocyte migration test (LMT)

The LM test was performed according to the technique of Bendixen & Søborg (1969), with modifications as described elsewhere (Goust, 1971; Kott, Genkins & Rule, 1973; Moulias *et al.*, 1970).

Twenty millilitres of blood were obtained by venepuncture under sterile conditions, and incubated for 1 hr at 37°C, with 2500 i.u. of calcium heparin. The supernatant and buffy coat were transferred to sterile plastic tubes, diluted 1:1 with 199 medium, centrifuged for 10 min at 150 *g*, and then washed three times in 199 medium, centrifuging for 8 min at 150 *g*. The white blood cells were then counted and diluted in 199 medium, supplemented with 10% sterile, filtered foal serum, in order to obtain a final cell concentration of 1×10^4 white blood cells/ml.

Aliquots of the cell suspensions were placed in graduated capillary tubes in order to give 1×10^5 cells/tube. One end of the tube was then sealed in a flame, and the tubes were centrifuged for 10 minutes at 200 *g*. The tubes were then cut at the packed cell supernatant interface and the cell-bearing ends were placed in sterile, siliconized glass culture dishes. Foal serum supplemented 199 medium was then added, with or without test antigen, and the cultures were covered with coverslips and incubated at 37°C for 18 hr. The migration surfaces were then magnified 200 times (Durst 609 magnifier), outlined, and the surfaces area measured. The migration index (MI) is calculated as follows: MI = [Mx (surface in the presence of antigen)]/[Mo (surface in the absence of antigen)]. Each scored result is the average of at least two measurements. Unless the measurements agreed within 15%, the result was rejected as non-significant.

Antigens

Monkey muscle (MM) and thymus (MT) antigens were extracted from samples removed immediately after the sacrifice of the animal (*Macca mulatta*) at the Pasteur Institut, and immediately frozen at −20°C. After thawing, the fragments were de-lipidized, minced and suspended 10% w/w in 0·5 M saline, and homogenized with an 'Ultra-Turrax 3' in bursts of 20 sec. After centrifugation for 15 min at 9000 rev/min, the lipid-free supernatant was lyophylized. All the above operations were performed at +4°C. After a Biuret protein estimation the concentration of each of the antigens was adjusted to 100 and 10 μg/ml of protein in culture medium.

A human muscle extract, obtained from an operating theatre explant, was prepared in the same way, and used at protein concentrations of 500 and 200 μg/ml.

RESULTS

Normal subjects

In healthy volunteers, no reaction to muscular and thymic antigens were ever observed. The MI to these different antigens is found to lie between 0·94 and 0·98, with extreme values

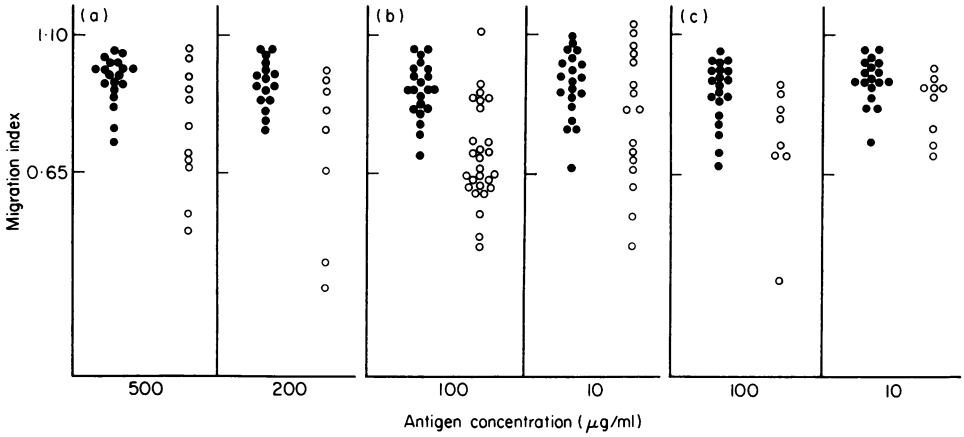

FIG. 1 (a) Migration index of normal subjects (●) and of patients suffering from diseases other than MG and PM (○) against human striated muscle. (b) Migration index of normal subjects (●) and of patients suffering from neuromuscular disorders other than MG of PM-DM (○) in the presence of simian striated muscle. (c) Migration index of normal subjects (●) and of patients suffering from neuromuscular disorders other than PM-DM and MG (○), against simian thymus.

of 0·69 and 1·08. Thus, any MI value below 0·65 and above 1·10 may be considered abnormal (Fig. 1), and was scored positive.

Other neuromuscular disorders

Of the various neuromuscular diseases (Fig. 1), none of the patients who had brain tumours or who had had vascular accidents gave positive results. On the other hand, of the eight polyradiculoneuritis patients, three showed a depressed MI in the presence of muscular extracts, and in two of these a neuromuscular biopsy revealed an infiltration of lympho-monocytes at the muscular level. The existence of a similar reaction in three out of five

patients with multiple sclerosis is even more surprising. The two patients with Steinert's disease and the patient with the Walton–Nattras type muscular dystrophy also evidenced a significant depression of the MI.

In the thyroid diseases, three patients did not react abnormally to muscle antigens, but one patient with exophthalmia showed a depression of the MI.

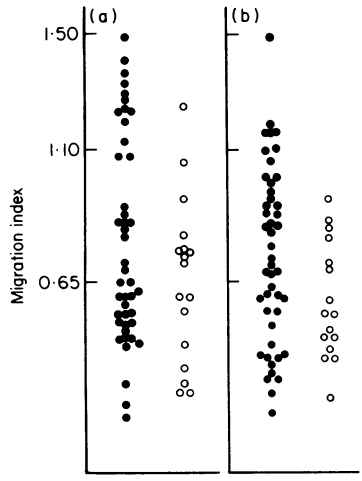

FIG. 2. Migration index in the presence of monkey striated muscle for patients suffering from PM (○) and MG (●); inhibition of migration is significant under 0·65; augmentation of migration over 1·10 is sometimes observed. Two concentrations of antigen were used: (a) 100 μg/ml; (b) 10 μg/ml.

Polymyositis and dermatomyositis

In PM (Fig. 2), positive responses to muscle antigens were observed in twelve out of fourteen patients tested. Two patients were tested on several occasions during a course of treatment with corticoid alone, or, in the second case, corticoids coupled with methotrexate. In the latter patient, a progressive improvement of the muscle inflammatory symptoms was observed, followed by the satisfactory recovery of one motile function, which was concomitant with an increase of the MI, and then a reversion to normal of the MI value, which remained stable even after the cessation of the immunosuppressive therapy. In the first patient, the hitherto positive results became negative, with an accompanying improvement in the muscular force. Positive responses to the muscle extracts were observed despite the daily administration of 40–70 mg of Δ-cortisone.

In the presence of thymic antigen, none of the eight patients tested gave a positive response (Fig. 3).

Myasthenia gravis

In the case of myasthenic patients, an abnormal response to one of the three antigens occurred in forty-five out of forty-six patients tested. The responses to monkey striated muscle (Fig. 2) were abnormal in forty patients. An inhibition of leucocyte migration was frequently observed (thirty-five cases). Surprisingly, augmentation of migration was exhibited in ten cases at one or both antigenic concentration.

In the presence of monkey thymus extract (Fig. 3), twenty-one abnormal responses were

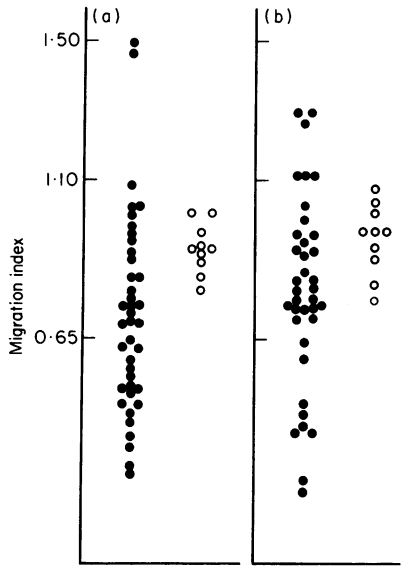

FIG. 3. Migration index in the presence of monkey thymus for patients suffering from MG (●) and PM-DM (○). Two antigenic concentrations were used: (a) 100 μg/ml; (b) 10 μg/ml.

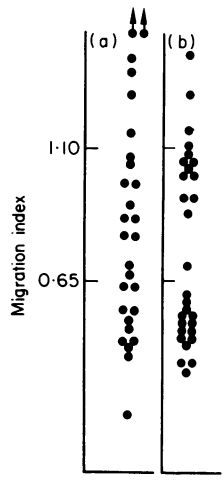

FIG. 4. Migration index in the presence of human striated muscle in MG at two different antigenic concentrations: (a) 500 μg/ml; (b) 200 μg/ml.

observed out of forty-two significant results (50%) at an antigen concentration of 100 μg/ml. In ten of these cases, only one concentration of antigen yielded a positive response. In two cases in the same test, an inhibition of migration at 100 μg/ml became an enhancement at 10 μg/ml; the reverse was observed in one patient.

Thirty-nine test results were significant when human muscle extract was used (Fig. 4); of these twenty-six (66%) were abnormal. Here also, one can observe the alternation of inhibition and enhancement of leucocyte migration according to the concentration of

antigen used. In MG, with this technique, an augmentation of the MI seems as significant as a depression.

Forty-five out of forty-six (97·8%) myasthenics gave abnormal results in the presence of one of the three antigens (Fig. 5). Eleven patients responded to both muscle antigens; twenty patients exhibited abnormal responses to both monkey thymus and muscle extracts. Nine patients reacted only with monkey muscle, four responded only to human muscle and one responded only to monkey thymus. No correlation appears to exist between the clinical and pathological data, the presence or absence of a thymoma, thymectomy performed either recently or up to 5 years before, and a recent recrudescence or a stable clinical status do not appear to influence the results of the test.

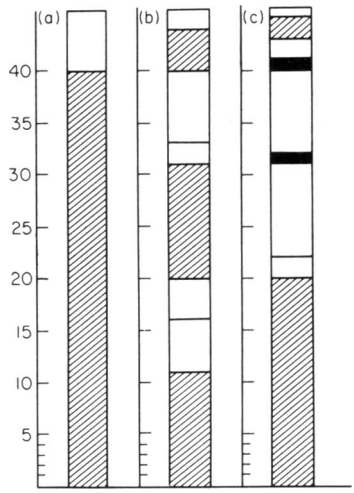

FIG. 5. Comparison of results obtained for each patient in forty-six cases of MG for each antigen tested. (a) Monkey striated muscle. (b) Human striated muscle. (c) Simian thymus. The hatched areas represent the positive results for the antigen tested, the open areas represent negative results for the antigen tested, and the solid areas represent untested antigen.

DISCUSSION

An inhibition of leucocyte migration may signify the release of MIF by the activated lymphocytes, but the enhancement observed in several cases is difficult to explain. Søborg & Halberg (1968) have already described this phenomenon in patients suffering from thyroditis. Falk *et al.* (1972) have also noticed this effect during the rejection phase of kidney graft transplantations. These authors consider that an increase of the MI following a depression signifies that the graft rejection is being controlled, and is probably due to the appearance of enhancing antibodies. A phenomenon of this nature might be responsible for the results obtained with two cases of PM described above. Here, a very important increase of the MI precedes a normalization of muscle-lysis test and LM test results, concomitant with a clinical amelioration.

Recently, using the Søborg and Bendixen technique, Stevenson (1973) has observed an enhancement of migration in the presence of hydrocortisone at concentrations in the region

of physiological doses. He attributes this finding to the secretion of an enhancing factor by mononucleated cells which enhances the migration of polymorphs, this factor being different from MIF. The presence of such an enhancing factor could well be postulated to explain some of our results.

In polymyositis, Kakulas (1966), and Currie *et al.* (1971; Currie, 1970, 1971; Mastaglia & Currie, 1971) have demonstrated the existence of a direct lymphocyte cytotoxic effect against muscle cells in tissue culture. Currie *et al.* consider that this cytotoxic effect is correlated with lymphoblast transformation, following culture in the presence of muscle antigen of xenogeneic origin. The positive results obtained with the LMT in the presence of xenogeneic antigens in twelve of the fourteen patients studied here, when more than half of them were undergoing corticoid therapy at the time of the test, corroborate the hypothesis that delayed hypersensitivity plays an important role in this disease. Other than direct lymphocyte cytotoxicity, the secretion of lymphokine would also account for the release of MIF observed here by the LMT, and the recently demonstrated release of lymphotoxic factor (LTF) (Johnson, Fink & Ziff, 1972), which could also be responsible for the cytolytic process observed in the muscle at some distance from the lymphocyte invasion focus. Humoral immunity seems to be unimportant; the search for antimuscle antibodies usually yields negative results (Caspary, Gubbay & Stern, 1964; Penn, Shotland & Rowland, 1971; Stern, Rose & Jacobs, 1967). Whitaker & King-Engel (1972) have nevertheless shown IgG and C_3 deposits in the intima of muscular vessels in cases of infantile dermatomyositis, sometimes associated with occlusions of these vessels, which make one think that these lesions may be caused by circulating immune complexes. The inflammatory reaction in such cases is very weak. Although the antigen has not been identified, it is not impossible that the virus-like particles detected in the muscle and skin of several of these patients (Chou, 1968) (Sato *et al.*, 1971) could also been found in the immune complexes.

Delayed hypersensitivity reaction in the adult patients studied here seem confined to muscular antigens: not one positive reaction was observed in the presence of thymic extracts. Therefore the antigen involved in PM and DM gave no cross-reaction with thymus and was different from the one involved in MG.

Kalden *et al.* (1969) found, in fact, an autoimmune thymitis after immunization with thymic or muscular extracts emulsified in Freund's complete adjuvant, as well as a 'partial neuromuscular block'. In human MG, a delayed hypersensitivity reaction is always displayed to the thymus. Fifty per cent of the patients tested showed abnormal leucocyte migrations in the presence of thymic antigen. In the other diseased patients used as controls, the only patients who showed a depression of the MI was suffering from thyroiditis. The relationship between thyroid disorders and MG have long been established (Drachman, 1962; Sahay, Blendis & Greene, 1965) and therefore this patient's reaction is not surprising. In MG, the role of the thymus is becoming increasingly apparent; thymic abnormalities exist in 80% of MG patients (Oosterhuis, Bethllem & Feltkamp, 1968). According to Goldstein (Goldstein & Hoffmann, 1971; Goldstein & Whittingham, 1967; Goldstein, 1968) an autoimmune thymitis could be responsible for the neuromuscular abnormalities due to secretion of an excess of a hormonal polypeptide, thymine (Goldstein & Manganaro, 1971). Ossermann *et al.* (Alpert *et al.*, 1971; Papatestas, Osserman & Kark, 1971; Papatestas *et al.*, 1971) have observed that even in the absence of a thymoma, recovery after thymectomy is a very lengthy process, and is proportional to the number of intra-thymic germinative foci. The delay of several years before a complete recovery is effected is nevertheless an obstacle in accepting

the exclusive role of thymine in the neuromuscular block; the excision of this secretory organ should make the disease regress rapidly if the thymic hormone alone is the cause of the neuromuscular block.

The role of the thymus in the maintenance of long-lived lymphocytes is also important in other respects. In thymectomized myasthenic patients, the interval between surgery is as long for clinical improvement as for a diminution of cellular immunity to antigens (Kornfeld *et al.*, 1965).

The diminution of T lymphocytes after thymectomy could therefore be considered indirectly responsible for the improvement. The therapeutical efficacity of antilymphocyte serum and immunosuppressive drugs (Rowland, 1971) could be explained on the same grounds.

The relationship between thymic anomalies and lymphocyte delayed hypersensitivity reactions to thymic and muscular antigens described by Alpert *et al.* (1970), Rule & Kornfeld (1971), Kott *et al.* (1973) and ourselves is not certain. In particular, this reaction to muscle antigens is not peculiar to myasthenia, as it can be found in polymyositis and other muscular afflictions. On the other hand, we have found that in some patients this reaction persists for up to 5 years after thymectomy.

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