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Mitogen and muscle extract induced *in vitro* proliferative responses in myasthenia gravis, dermatomyositis, and polymyositis[1]

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SYNOPSIS We have compared the lymphocyte *in vitro* proliferative response induced by muscle extract, phytohaemagglutinin, and pokeweed mitogen in peripheral blood lymphocytes of patients with myasthenia gravis, polymyositis, dermatomyositis, and normal subjects. Similar mean responses to antigen and mitogen were seen in cells of all groups of patients. The proliferative responses induced by muscle did not differ in thymic cells from myasthenics as compared with those from cardiac surgery controls. Our findings do not support the hypothesis that cell-mediated immunity to muscle or major impairment of lymphocyte responses to mitogens occurs in these diseases.

Myasthenia gravis, polymyositis, and dermatomyositis represent disorders of the neuromuscular system in which a possible autoimmune aetiology has been suggested. These disorders have been investigated using several *in vitro* parameters that are considered to correlate with cell-mediated hypersensitivity (Bloom, 1971). These include *in vitro* lymphocyte proliferative response, buffy coat migration inhibition, and direct cell-mediated lymphocytolysis of muscle cultures. Using such techniques, studies have been published suggesting that cell-mediated hypersensitivity to muscle is a concomitant and perhaps causative mechanism of both myasthenia gravis (Alpert *et al.*, 1972; Goust *et al.*, 1974) and polymyositis (Currie *et al.*, 1971; Johnson *et al.*, 1972; Dawkins and Mastaglia, 1973; Esiri *et al.*, 1973; Goust *et al.*, 1974; Haas and Arnason, 1974). Evidence has likewise been presented, using lymphocyte mediated cytotoxicity and inhibition of thymic cell migration, suggesting that there are cells within the myasthenic thymus sensitized to muscle (Armstrong *et al.*, 1973). We sought to investigate the *in vitro* proliferative response to muscle extract in the peripheral blood of patients with myasthenia gravis as well as in patients with polymyositis and dermatomyositis and to compare these with the responses in blood lymphocytes of controls. The *in vitro* proliferative response of peripheral blood lymphocytes from such subjects were all assessed after stimulation by the non-specific mitogens, phytohaemagglutinin, and pokeweed mitogen. In addition, the proliferative responses induced by skeletal muscle were compared in thymic cells obtained from myasthenic patients and cardiac surgery patients.

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METHODS

PATIENTS Patients with myasthenia gravis, defined by variable voluntary skeletal muscle weakness responsive to anticholinesterase administration (Simpson, 1969; Rowland and Layzer, 1973), were divided into two groups: one group consisted of 21 patients who had not undergone therapeutic thymectomy and the other group consisted of 12 patients who had undergone thymectomy three months to four years before study. This latter group

included patients with and without lymphoepithelioid thymoma. Twelve patients with polymyositis or dermatomyositis had unequivocal clinical disease accompanied by elevated serum enzymes and, in most cases, diagnostically compatible findings in a muscle biopsy (Pearson, 1969; Rowland and Layzer, 1973). The normal controls consisted of 11 subjects (ages 15–59 years) with no known neurological or immunological diseases.

For the study of the *in vitro* proliferative response of thymic cells induced by muscle extract, tissue was obtained at therapeutic thymectomy or from small thymic biopsies obtained at open heart surgery for non-rheumatic heart disease. The glands removed at thymectomy in myasthenic patients showed thymic hyperplasia in five cases and lymphoepithelioid thymomas in three others. No patients or control subjects had been on corticosteroids or cytotoxic drugs.

LYMPHOCYTE PROLIFERATION Peripheral blood of all subjects was studied using a modification of the method of Zweiman *et al.* (1969). In brief, heparinized blood was sedimented with Dextran and a lymphocyte-enriched supernatant obtained. These cells were washed two times and then suspended in a final concentration of 5×10^5 lymphocytes/ml in a culture medium containing penicillin, streptomycin, and glutamine. Agammaglobulinaemic fetal calf serum was added to a final concentration of 10%. Cells were cultured in 5% CO_2/air at 37°C for five days. The degree of proliferative response was assayed by the incorporation of tritiated thymidine added 18 hours before termination of the cultures. This proliferation was expressed as:

$$\text{Stimulation Index (SI)} = \frac{\text{mean cpm of replicate cultures with mitogen or antigen}}{\text{mean cpm of replicate cultures without mitogen or antigen}}$$

Cultures from all patients were incubated with phytohaemagglutinin (PHA-P; Difco Laboratories) and pokeweed mitogen in at least two concentrations (PWM; Difco Laboratories). Lymphocyte cultures from all patients were also incubated with varying doses of a saline soluble muscle extract prepared by the method of Currie *et al.* (1971). A stimulation index (SI) of $\geqslant 3$ in the presence of any concentration of muscle extract was considered a positive response.

Thymic cells were prepared under sterile conditions by mincing the thymic tissue and then passing them through a no. 80 steel mesh screen (Abdou *et al.*, 1974). The cells were cultured at a concentration of 1×10^6 cells/ml with varying concentrations of muscle for five days. The degree of stimulation (SI) was determined in a manner identical to that described for the peripheral blood lymphocytes.

RESULTS

The peripheral blood lymphocyte proliferative responses of all subjects are shown in the Table. There were no significant differences among the

TABLE

LYMPHOCYTE *IN VITRO* PROLIFERATIVE RESPONSES TO MITOGENS AND MUSCLE IN NEUROMUSCULAR DISEASE

	*PHA**			*PWM**			*Muscle†*	*Control‡*
	1:10	*1:100*	*Peak*	*1:10*	*1:100*	*Peak*		
Mg§ No Tx	74¶ ±19	84 ±23	88 ±21	32 ±7	37 ±7	37 ±7	2.7 ±1.2	1334 ±256
Post-Tx	73 ±20	69 ±17	81 ±19	38 ±11	24 ±6	39 ±10	1.2 ±1.0	1043 ±254
PM–DM§	77 ±24	90 ±27	127 ±28	47 ±4	48 ±6	49 ±9	1.1 ±0.1	901 ±309
Normal§	67 ±18	102 ±27	93 ±23	35 ±8	36 ±8	37 ±9	1.1 ±0.1	1209 ±287

* PHA- phytohaemagglutinin-P; group mean responses to PHA; 1:10 and 1:100: 0.1 ml of these concentrations added to 0.9 ml of cell suspension; peak: mean of greatest response regardless of concentration of individual subjects. PWM: pokeweed mitogen.
† Muscle: saline muscle extract; peak response represented.
‡ Group mean counts per minute (cpm) of unstimulated (control) cultures.
§ MG No TX: Myasthenia gravis; no thymectomy; MG post-Tx: Myasthenia gravis post-thymectomy; PM–DM; polymyositis and dermatomyositis; normals: normal adult subjects.
¶ Stimulation index (SI) = $\frac{\text{mean cpm with mitogen or antigen}}{\text{mean cpm without mitogen or antigen}}$

groups in the mean degrees of the proliferative responses to PHA at any concentration studied. There was no difference in the response to PWM or to muscle extract. Spontaneous uptake of the isotope was similar in unstimulated cultures (cells cultured in the absence of mitogen or antigen) in lymphocytes obtained from the three groups. Muscle extract induced proliferative responses of $\geqslant 3$ were seen in lymphocytes of only two subjects. One was a patient with myasthenia gravis and thymoma; the SI was 14. Because of the patient's age (72 years) and the relatively well-controlled status of his myasthenia gravis, he did not undergo therapeutic thymectomy. The other was a woman with generalized myasthenia undergoing thymectomy and with thymic hyperplasia-thymitis at surgery; the SI was 22.

Several patients were studied pre- and post-thymectomy; no consistent pattern was noted in the responses of their lymphocytes to either mitogens or muscle extract. In the muscle extract-thymic cell cultures, the mean SI of cells from myasthenic patients (2.2 ± 0.6) was not statistically different from that of the control group (1.2 ± 0.6). In one subject, where muscle extract induced an SI of 22 in blood lymphocytes, the SI in thymic cells cultured with muscle was 5.1.

DISCUSSION

In this study no evidence could be found for an obvious lymphocyte immunological defect in myasthenia, polymyositis, or dermatomyositis as manifested by deficient mitogen induced responses (Daguillard, 1972). It is conceivable that a more subtle defect in generalized immune responsiveness might be demonstrated in any of the studied disease states utilizing other mitogens and varying durations of lymphocyte culture. However, in another study in which peripheral blood lymphocytes were isolated by Ficoll-Hypaque gradient centrifugation, there was no difference in the *in vitro* PHA or PWM induced proliferative response after three or five days of culture (Abdou *et al.*, 1974).

In this study, we found no consistent evidence of muscle extract stimulated *in vitro* proliferation of blood lymphocytes from myasthenics. It is true that a stimulation index of $\geqslant 3$ was seen in cells of two of 21 non-thymectomized myasthenics, whereas such stimulation was not seen in the lymphocytes of any of the normal subjects. However, the mean response to muscle of cells of the myasthenic group as a whole was not significantly different from that of normal subjects. It is of note that thymus cells of one of the two myasthenics with muscle stimulated blood lymphocytes were also stimulated by the muscle extract.

A search for *in vitro* evidence of cell-mediated reactivity to tissue antigens has been made in several studies of myasthenics by a variety of techniques. Ambramsky *et al.* (1975) reported increased proliferation of lymphocytes from myasthenics cultured with acetylcholine receptors from electric eels. The antigen-induced inhibition of *in vitro* migration of the buffy coat has been utilized in a number of laboratories (Søborg and Bendixin, 1967) as a presumptive measure of preexistent cell-mediated immunity to tissue antigens. Using this technique, significant inhibition of migration of leucocytes from patients with myasthenia or polymyositis induced by several fractions of muscle extract has been reported (Alpert *et al.*, 1972; Goust *et al.*, 1974). Armstrong *et al.* (1973) have demonstrated a similar inhibition by muscle extract of the migration of thymic cells obtained from myasthenics at thymectomy.

In comparative studies, we found no *in vitro* stimulation by muscle extract of blood lymphocytes from any of the polymyositis or dermatomyositis patients. Such stimulation has been reported previously (Currie *et al.*, 1971), but the responses in one of the reports were quite modest (mean SI—2.2). No stimulation index was described in the other study (Esiri *et al.*, 1973). Other presumptive parameters of cell-mediated immunity have been utilized in the study of polymyositis and dermatomyositis. Currie *et al.* (1971) found that lymphocytes of such patients exerted toxic effects on cultured fetal muscle. Johnson *et al.* (1972) reported toxic effects in fetal muscle cultured in the presence of lymphocytes from patients with polymyositis and autologous muscle. Dawkins and Mastaglia (1973) reported similar findings in cultures containing extracts of normal, rather than autologous, muscle. More recently, Haas and Arnason (1974) found increased creatine phosphokinase release from fetal muscle cultures in

the presence of lymphocytes from patients with polymyositis.

In an approach using an electrophoretic macrophage migration inhibition test, it has been claimed (Caspary *et al.*, 1971) that immune reactivity is present in lymphocytes of patients not only with polymyositis but also in patients with muscular dystrophy.

Although the methodology has varied in the reports just described, there is a suggestion of similar immunological reactivity against muscle in myasthenia and polymyositis. It would be somewhat surprising if such reactivity were pathogenetic in face of the disparate clinical features of the two disorders. It is possible that any immunological activity against muscle was a secondary event and/or an epiphenomenon.

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