Lymphocyte function in myasthenia gravis

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SUMMARY Mitogen-induced blastoid transformation of peripheral blood lymphocytes from patients with myasthenia gravis was studied using a microplate culture technique and evaluated with ^3H -thymidine incorporation. It was found that both phytohaemagglutinin and pokeweed mitogen responses decreased significantly in patients with myasthenia gravis. In myasthenic crisis, indices of stimulation by phytohaemagglutination became very low. The autologous plasma neither inhibited nor facilitated mitogenic responses of lymphocytes. The decreased mitogen responsiveness of lymphocytes suggests that part of the T lymphocyte function is subnormal in myasthenia.

An autoimmune pathogenesis of myasthenia gravis has frequently been postulated on the basis of clinical (Simpson, 1960) and laboratory investigations (Nastuk *et al.*, 1960; Strauss *et al.*, 1960; Almon *et al.*, 1974; Lindstrom *et al.*, 1976a,b). Since there are abnormalities in the myasthenic thymus, either thymic hyperplasia or thymoma (Castleman, 1966), and the thymus is known to be a central organ of the immune system (Miller, 1961; Komuro and Boyse, 1973), lymphocytes from patients with myasthenia gravis may function abnormally, causing the autoimmune processes.

The number of peripheral blood lymphocytes decreases during remission after thymectomy (Joske *et al.*, 1958). There were no significant differences in peripheral blood lymphocyte subpopulations of myasthenia gravis patients and control subjects (Abdou *et al.*, 1974; Shirai *et al.*, 1976). Delayed hypersensitivity induced by 1-chloro-2, 4-dinitrobenzene has been reported to be either reduced (Adner *et al.*, 1966) or normal (Kornfeld *et al.*, 1965) in patients with myasthenia gravis. Lymphocytes of myasthenic patients caused a more severe systemic graft-versus-host reaction than normal human lymphocytes (Namba *et al.*, 1969). Studies of lymphocyte function using in vitro culture techniques have reported normal mitogenic responsiveness to phytohaemagglutinin (PHA) in both thymectomised and non-thymectomised

patients (Housley and Oppenheim, 1967) and to PHA and pokeweed mitogen (PWM) (Lisak and Zweiman, 1975).

As for cellular immunity in myasthenia gravis, thymocytes and peripheral blood lymphocytes have been reported sensitised to muscle antigens (Alpert *et al.*, 1972), acetylcholine receptor protein (Abramsky *et al.*, 1975), or thymus tissue antigens (Goust *et al.*, 1974; Kawanami *et al.*, 1976).

In this study we examined lymphocyte functions by measuring mitogen responsiveness to PHA for T cells (Clot *et al.*, 1975) and PWM in a lower concentration for B cells (Mellstedt, 1975) with respect to change in myasthenic crisis, change after thymectomy, and the effect of serum factor.

Methods

Defibrinated venous blood was obtained with a heparinised syringe. An equal volume of 0.9% sodium chloride solution was mixed with the blood. The lymphocyte suspension was cleared from the red cells and polymorphous leucocytes by centrifugation through a Ficoll-Conray gradient. The lymphocyte layer remaining at the interface was removed into an equal volume of Eagle's MEM, washed three times, and finally resuspended one million lymphocytes per ml in RPMI 1640 containing penicillin (500 units/ml), streptomycin (50 $\mu\text{g}/\text{ml}$) and 20% heat-inactivated fetal calf serum (Gibco).

Cultures were done using microplates (Falcon plastics, No. 3040) (Knoke *et al.*, 1974). Each cul-

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ture was done in triplicate; 0.2 ml of the cell suspension (200 000 cells) were dispensed into each well, and further additions were made in 0.01 ml increments. The plates were covered with adhesive-backed mylar film (Falcon No. 3044) and incubated in a humidified atmosphere of 5% CO₂–95% air at 37°C. Deoxyribonucleic acid synthesis was measured by the incorporation of ³H-thymidine into acid insoluble material. After 72 hours, all cultures were labelled with 0.01 ml (0.5 microCi; 2.6 Ci/mmol) of ³H-thymidine in phosphate buffered saline. After four hours pulse at 37°C, the cells were harvested and trichloroacetic acid insoluble radioactivity was evaluated by a liquid scintillation counter (Beckman LS 250). The ratio of uptake of ³H-thymidine by the stimulated culture to the uptake of simultaneously incubated unstimulated cultures was calculated as the stimulation index. The significance of the difference was measured using Student's *t* test.

The effect of autologous plasma was examined in culture using medium RPMI 1640 containing 20% autoplasm in place of fetal calf serum.

Phytohaemagglutinin (PHA-P, Difco) was taken up in 5 ml of saline solution and diluted 1:100 in culture medium, (150 µg/ml). Aliquots of 0.01 ml were added to each well containing 200 000 lymphocytes. Pokeweed mitogen (PWM, Gibco) was also diluted 1:100 and added to each well of culture plates to a final concentration of 5 µg/ml.

The lymphocytes from 18 non-thymectomised patients with myasthenia gravis, including three thymomas (14 women aged 22 to 51 years and four men aged 20 to 73 years), 13 thymectomised patients with myasthenia gravis including four thymomas (eight women and five men aged 22 to 53 years) and 13 normal control subjects (eight women and five men aged 22 to 47 years) were examined for mitogenic responsiveness of lymphocytes. All patients had generalised myasthenia and were classified as Osserman's adult type 2. At the time of our study, the patients were being treated with acetylcholine esterase inhibitors.

The effect of autoplasm on PHA and PWM responses was examined in lymphocytes from 10 and six myasthenic patients, respectively. Six normal control subjects were tested.

Intrathymic lymphocytes were removed from the thymus of two patients with myasthenia gravis and one with thymoma. Mitogen responsiveness was examined as well as lymphocyte subpopulations. Intrathymic lymphocyte suspensions were prepared by cutting the thymus into small fragments and gently pressing them between two glass slides in Eagle's MEM. Connective tissue and tissue clumps were then eliminated by passage through

a nylon screen. Lymphocyte fractions were obtained by Conray-Ficoll gradient centrifugation at 400 *g* for 30 minutes. After washing, the cells were resuspended to one million/ml of RPMI 1640 containing 20% fetal calf serum.

The subpopulations of lymphocytes were detected by the rosette forming method described by Yata *et al.* (1973), with slight modifications. In our study, the spontaneous rosette forming capacity of lymphocytes with sheep red blood cells (SRBC) was used for a T cell marker. The presence of receptor for complement on lymphocytes was used to search for B cells. For the anti-SRBC sera, either whole anti-SRBC rabbit serum or its IgM fraction was used. These were designated erythrocyte-haemolysin-complements rosette forming cells, EA(H)C-RFC, and erythrocyte-IgM-complements rosette forming cells, EA(M)C-RFC (Itoyama *et al.*, to be published).

Results

PHA RESPONSE

The mitogenic responses of lymphocytes from patients with myasthenia gravis and normal control subjects are shown in Fig. 1. The mean PHA responses of 13 normal controls, 18 non-thymectomised patients, and 13 thymectomised patients were 123.9±16.3, (mean±SE), 59.7±7.7, and 62.5±12.0, respectively, (Table 1). The mean stimulation indices of both the thymectomised and non-thymectomised groups of patients with myasthenia gravis were lower than those of normal control subjects (*P*<0.01).

We studied three patients in myasthenic crisis (Table 2). The lymphocytes were obtained before steroid administration. The PHA-SI decreased remarkably during myasthenic crisis and returned to its pre-crisis level during recovery from the crisis.

The stimulation indices for PHA were lower in intrathymic lymphocytes from myasthenic thymus or thymoma than in peripheral blood lymphocytes. The study on the subpopulations of the lymphocytes revealed that the percentage of erythrocyte (SRBC)-rosette forming cells was not lower than in peripheral blood lymphocytes (Table 3).

PWM RESPONSE

The results of lymphocyte transformation by PWM are shown in Fig. 2. The mean value of the PWM-SI of lymphocytes from 10 normal control subjects, 17 non-thymectomised, and 13 thymectomised myasthenia gravis patients were 43.3±5.1 (mean±SE), 24.1±3.9, and 22.5±3.6, respectively. Whether or not the thymus had been

Fig. 1 Lymphocyte transformation with phytohaemagglutinin (PHA) diluted to 1 : 100. SI (stimulation index): the ratio of uptake of ^3H -thymidine by the stimulated culture to the uptake of a simultaneously incubated unstimulated culture was calculated. Stimulation index (SI)=mean cpm of triplicate culture with mitogen/mean cpm of triplicate culture without mitogen. MG=myasthenia gravis; thymex=thymectomised. Dotted lines indicate mean values.

Table 1 *In vitro* lymphocyte transformation by mitogens

		PHA-SI		PWM-SI
Normal	n=13	123.9 ± 16.3	n=10	43.5 ± 5.1
MG	n=18	59.7 ± 7.7*	n=17	24.1 ± 3.9*
MG-Thymex	n=13	62.5 ± 12.0*	n=13	22.5 ± 3.6*

*P < 0.01, mean ± SE.

PHA-SI=stimulation indices with PHA; PWM-SI=stimulation indices with pokeweed mitogen; MG=myasthenia gravis; MG-Thymex=thymectomised patients with MG; n=numbers of patients or normal control subjects examined.

removed, the means of the PWM stimulation indices for the group of patients with myasthenia gravis were significantly lower than those of the control subjects ($P < 0.01$).

EFFECT OF PLASMA

Study of the effect of plasma on lymphocyte trans-

Table 2 *Lymphocyte transformation during and after myasthenic crisis*

Patient	Age (yr)	Sex	Time of study	PHA-SI
NB	22	F	In crisis	2.63
			25 days after	122.7
FO	26	M	In crisis	9.03
			6 days after	70.8
			15 days after	151.4
HD	22	F	In crisis	5.5
			11 months after	83.3

PHA-SI=stimulation indices with PHA; crisis=at the stage of myasthenic crisis.

Table 3 *PHA responses and subpopulations of lymphocytes obtained from myasthenic thymus or thymoma*

Patient	Age (yr)	Sex	Origin	PHA-SI	E-RFC	EA(H)C-RFC
NY	18	F	Thymoma	12.0*	89.7 (%)	12.3 (%)
			Thymus	8.0*	90.3	10.0
NI	52	F	Thymus	18.7†	95.7	4.0
MK	25	M	Thymus	25.0*	73.0	22.0

PHA-SI=stimulation index with PHA. The concentration of PHA was described as the ratio of dilution from the 5ml vial of PHA (Difco). * = 1:40, † = 1:100. E-RFC, erythrocyte rosette forming cells; EA(H)C-RFC, erythrocyte haemolysin complements rosette forming cells.

formation revealed that there was no definite inhibition or facilitation of mitogenic responses in both PHA and PWM stimulation (Fig. 3).

Discussion

The *in vitro* mitogen responsiveness of lymphocytes from patients with myasthenia gravis has been reported as normal. Housley and Oppenheim (1967) compared the capacity of lymphocytes from thymectomised and non-thymectomised myasthenics to react *in vitro* to PHA and found no differences between them. They used Eagle's MEM with autologous plasma as medium and cultured for five days. Abdou *et al.* (1974) reported that the peripheral blood lymphocytes of patients with myasthenia gravis and control subjects proliferated without significant differences in response to PHA in cultures carried out for three to five days in Medium 199 with autologous plasma or fetal calf serum. Lisak and Zweiman (1975) found no impairment of peripheral blood lymphocyte responses to mitogens, PHA and PWM, in myasthenia gravis. They cultured for five days and the degree of proliferative response was assayed by the incorporation of ^3H -thymidine added 18 hours before termination of the culture. Hammarström *et al.* (1975) found no significant differences in the

Fig. 2 Lymphocyte transformation with pokeweed mitogen, 5 $\mu\text{g/ml}$ in final concentration. SI= stimulation indices, MG=myasthenia gravis, thymex=thymectomised.

responsiveness of thoracic duct lymphocytes from patients with myasthenia gravis compared to normal peripheral blood lymphocytes. Although their cultures were performed using Eagle's MEM with 10% CO_2 , the lymphocytes had been stored in liquid nitrogen with dimethylsulphoxide added to the medium until culture.

These reports differed from our study in the duration of cultures, culture media, concentration of mitogens, and the method of lymphocyte preparation. Autologous plasma was occasionally used in place of calf serum. It is well known that the mitogenic responses of lymphocytes reach their maximum level on the second to the third day, and the viability of lymphocytes decreases remarkably during longer culture periods (Knight *et al.*, 1968). Autologous plasma modifies the lymphocyte responses to mitogens as shown in Fig. 3, and many humoral factors (Newberry *et al.*, 1973; Han, 1974; Hsu, 1976; Mendelsohn *et al.*, 1977) are known to have an effect on lymphocyte functions.

Results of the present study revealed that lymphocytes from patients with myasthenia gravis had a lower response to mitogens, both PHA and

Fig. 3 Plasma effect on lymphocyte transformation. The mitogenic stimulation indices of lymphocytes from patients with myasthenia gravis and normal control subjects are illustrated. Circles indicate patients and squares show normal controls. Open circles or squares indicate stimulation indices of lymphocyte culture using fetal calf serum. Closed circles or squares indicate the PHA-SI of cultures in media including autoplasmal instead of fetal calf serum.

PWM. Only in two reports were similar mitogenic responses in lymphocytes from patients with myasthenia gravis described (Arimori *et al.*, 1974; Simpson *et al.*, 1976). This lower responsiveness may be due to the difference in lymphocyte subpopulation. Our study showed (Itayama *et al.*, to be published) that the percentage of E rosette forming cells both in thymomatous and non-thymomatous patients did not differ from normal controls. Only B cells detected by EA(H)C-RFC were significantly increased in myasthenia gravis. The EA(H)C-RFC are supposed to include B cells and T cells bearing Fc receptors. Increase of T cells bearing Fc receptors was also reported in Japanese myasthenics (Yata *et al.*, 1977). Mitogen responsiveness of the T lymphocyte bearing Fc receptor has been noted as normal to PHA and concanavalin A (Stout and Herzenberg, 1975). However, Moretta *et al.* (1976) reported that the T lymphocyte bearing Fc receptor of IgG showed

a lower response to PHA. The low mitogen responsiveness of lymphocytes from myasthenic patients may be explained partly by the change of subpopulation of T lymphocytes, with increase of the T lymphocyte bearing Fc receptors. These lymphocytes can be helper or suppressor T cells and may cause production of autoantibodies or cellular sensitisation.

Depression of T lymphocyte function has been found in patients with malignancy and other autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis. Decrease of T lymphocytes and the presence of natural thymocytotoxic autoantibodies have been noted with exacerbation of systemic lupus erythematosus. Recently, loss of the suppressor function of T lymphocytes has been reported in patients with active systemic lupus erythematosus (Morimoto, 1978), although it remains to be determined whether these changes are primary or not.

To investigate the cause of the suppressed response to mitogens in lymphocytes from patients with myasthenia gravis, we examined the effect of autologous plasma. No definite inhibition of mitogenic response was found with either PHA or PWM. This means that humoral factors, such as those seen in liver diseases or carcinoma, cannot be a reason for the lower mitogenic response.

As far as we know, there has been no report on lymphocyte function during myasthenic crisis. In our study, the responsiveness to PHA became extremely low during myasthenic crisis. Humoral factors, including steroid hormones and cellular immunological events at crisis, may be the key to the problem.

There was no significant correlation between the level of immunoglobulin, IgG, IgM, IgA, and the mitogen responsiveness of lymphocytes from these myasthenic patients. The titre of antibody for acetylcholine receptor protein was not measured. Myasthenic serum has been reported to have increased antibodies to acetylcholine receptor protein and the antibodies are thought to be one of the causes of myasthenia gravis (Lindstrom *et al.*, 1976a; Monnier and Fulpius, 1977). It is known that lymphocytes have cholinergic receptors on their surface membranes (Hadden *et al.*, 1973; Strom *et al.*, 1972). Secondly, one of the reasons for the lower mitogenic response in myasthenic lymphocytes may be a modification induced by an immunological reaction between acetylcholine receptor on the lymphocytes and the antibody to the acetylcholine receptor of the muscle membrane.

According to the thymopoietin theory (Goldstein and Schlesinger, 1975), myasthenic serum

includes thymopoietin, which has the capacity to impair neuromuscular transmission in vivo and to induce expression of T cell antigens in vitro (Basch and Goldstein, 1975), that is TL antigen in mice. In mice, TL positive lymphocytes are present in the cortex of the thymus as premature thymocytes (Konda *et al.*, 1973). In the human, intrathymic lymphocytes have a lower PHA response than peripheral blood lymphocytes (Table 3; Armstrong *et al.*, 1973; Abdou *et al.*, 1974). In mixed leucocyte reactions, thymic cells from patients with myasthenia gravis and thymic hyperplasia were reported capable of stimulating autologous peripheral blood lymphocytes (Abdou *et al.*, 1974). In myasthenic crisis, the concentration of thymopoietin in serum is supposed to increase remarkably. It may cause changes in receptors on T lymphocytes and thus decrease PHA response in vitro to a point below normal. As a third possibility, it is conceivable that in human myasthenia gravis the receptors on T lymphocytes are modified by thymopoietin, resulting in reduced response to mitogens. More simply, in myasthenic crisis, intrathymic lymphocytes may be released from the thymus into peripheral blood, causing a reduced response to mitogens. Both anti-acetylcholine receptor antibodies and thymopoietin may cause the reduced response of lymphocytes to mitogens in myasthenia gravis.

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