Clinical and immunological associations in myasthenia gravis 2: cell-mediated immunity

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SUMMARY Myasthenic patients showed low absolute T cell counts and low spontaneous and phytohaemagglutinin-induced lymphocyte transformation responses. The abnormalities were particularly associated with late onset disease and female sex, but not with HLA phenotype or autoantibody production. Serum factors inhibitory to lymphocyte transformation occurred in some patients.

Myasthenia gravis is a disease characterised by the production of autoantibodies, some of which may be aetiologically significant; it has been suggested that such autoantibody production occurs because of a genetically linked defect in immunoregulation.¹

Cellular immune mechanisms play a key role in the regulation of the immune system, with T cells exerting both helper and suppressor actions on B cells.² In myasthenia gravis, several lines of evidence suggest that this cellular immune activity may be deranged. The thymus shows histological abnormalities in a large proportion of patients³ and there is a disturbance in the distribution of T and B cells within it.⁴⁻⁷ Serum IgA levels have been found to be low in 20% of myasthenics, an abnormality commonly associated with cellular immunodeficiency.⁸ Delayed cutaneous hypersensitivity reaction to 1-chloro-2-4-dinitrobenzene has also been reported to be reduced.⁹ Studies of the *in vitro* lymphocyte transformation response to phytohaemagglutinin (PHA) have yielded conflicting results, but an impaired response in myasthenia gravis has been shown in some studies.^{8 10-12}

In the previous paper, we presented evidence that myasthenia gravis is a heterogeneous disease. In this report, the cellular immune data of the same patients is presented, based on lymphocyte transformation responses to PHA and the numbers of circulating T and surface immunoglobulin-positive (Sig+) cells. The results are

analysed with respect to the clinical groupings described in the previous paper; these were based on age of disease onset, sex of the patient and presence of HLA B8.

Methods

1 Patients and controls

The 34 patients with myasthenia gravis are those described in the previous paper. The control group comprised 78 healthy hospital personnel, mean age $\pm$ s.d. 38.2 years $\pm$ 13.7; 35 were male. Neither patients nor controls were infected or anaemic, or were taking any drugs apart from anticholinesterases and atropine at the time of study. Blood samples from patients and controls were all taken before 10.30 am. All assays of each subject were performed using aliquots of the same venepuncture sample.

2 Lymphocyte subpopulations

T cells were enumerated by an E-rosetting technique. Sig+ cells were detected by indirect immunofluorescence, using rabbit anti-human Fab and FITC-conjugated goat anti-rabbit immunoglobulin. These techniques have been described previously.¹³

3 Lymphocyte transformation

This was performed in both autologous plasma and pooled human AB serum by a modification of the whole blood micromethod of Junge *et al.*¹⁴ When cultures were done in autologous plasma, whole blood, anticoagulated with preservative-free heparin (15 units/ml (Sigma, USA), was diluted 1:10 with RPMI 1640 Flow Labs., Irvine). One ml aliquots of the mixture were cultured in triplicate at final concentrations of PHA (Wellcome Labs., Beckenham) of 0, 0.1 and 1.0 μ g/ml. Cultures were maintained in 5% CO₂/95% air at 37°C for 72 hours.

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For studies in AB serum, one ml of heparinised blood was washed three times with RPMI and reconstituted to 10 ml with RPMI enriched with 5% pooled human AB serum. One ml aliquots were cultured and stimulated with PHA similarly to the cultures in autologous plasma. Two μ Ci of tritiated thymidine (specific activity 5Ci/mmol, Radiochemical Centre, Amersham) were added to each tube for the last six hours of the culture period. After treatment with 3% acetic acid to lyse red cells, the DNA was then precipitated with 5% trichloroacetic acid, decolorised with methanol, solubilised in 1M alcoholic sodium hydroxide and mixed with NE 260 scintillation fluid (Nuclear Enterprises, Edinburgh) prior to counting in an Intertechnique liquid scintillation counter. Results are expressed as $\log_{10}$ average counts per minute of triplicate cultures. Control cultures were routinely included in each experiment performed with patient cultures.

4 Response of normal lymphocytes to inhibitory myasthenic sera

Ten blood samples (six patients) showed a greater rise in transformation response than any of the control samples when autologous plasma was substituted for AB serum. These samples were tested for inhibitory effects on normal lymphocytes. All serum samples were taken from clinically stable patients and stored at -20°C ; they were only removed from storage immediately before use. The same normal lymphocyte donor was used in all experiments.

An equal volume of test or control AB serum was added to heparinised whole blood and the mixture diluted 1/10 with RPMI. Triplicate one ml cultures were then stimulated with PHA as previously described. Autologous plasma was retained in the system to avoid errors due to a possible nutrient deficiency in the test serum; non-supplemented cultures were also performed. Results are expressed as $\log_{10}$ counts per minute.

5 Statistical analysis

There was lack of homogeneity of variances between certain distributions of data; the non-parametric Mann-Whitney two-tailed test was therefore used to compare patients and controls in all tests. Correlations between lymphocyte transformation responses and T cell counts were assessed using least squares linear regression with log transformation of the distribution of T cell counts, which was skewed.

Results

1 Lymphocyte subpopulations

The myasthenia gravis group as a whole did not show significant differences from controls in total, T or SIg+ lymphocyte counts (no/mm^3). Overall mean values $\pm$ SD were: total lymphocyte count 2386 ± 898 (controls 2446 ± 649); T cell count 1570 ± 694 (controls 1697 ± 434); SIg+

cell count 339 ± 149 (controls 314 ± 153). Thymectomised patients tended to have lower T cell counts (339, 546, 746, 2095) and total lymphocyte counts (1000, 1361, 1660, 2890). Significantly low T cell counts were, however, seen in myasthenia gravis of late onset (over 35 years of age) (table 1).

2 Spontaneous lymphocyte transformation (fig 1)

Both patients and controls showed a wide range of lymphocyte transformation responses. The spontaneous lymphocyte transformation responses in autologous plasma of patients with myasthenia gravis were slightly but significantly lower than those of controls ($p < 0.01$); the low responses persisted in normal AB serum ($p < 0.02$). These responses did not correlate with T cell count ($r = +0.27$, $p > 0.1$). The abnormalities of spontaneous lymphocyte transformation occurred mostly in females with late onset of disease (table 3). Thymectomised patients tended to show low spontaneous lymphocyte transformation responses.

3 PHA-induced lymphocyte transformation

At the lower PHA concentration, the myasthenic group as a whole showed significantly reduced lymphocyte transformation responses in autologous plasma compared with controls ($p < 0.005$;

Table 1 Comparison between young onset (35-) and late onset (35+) myasthenics and young controls (40-) and older controls (40+) in lymphocyte transformation response and subpopulation counts

	Myasthenia gravis		Controls	
	35+ n=16	35- n=18	40+ n=36	40- n=42
Current age (years)	54.4 (11.7)	36.3 (12.7)	52.1 (7.8)	27.7 (4.7)
Auto	3.01 (0.24)	3.02 (0.25)		3.11 (0.18)
Spontaneous AB	2.99 (0.28)	3.00 (0.22)		3.15 (0.14)
Auto	4.52* (0.59)	4.91 (0.54)	4.92 (0.32)	5.07 (0.25)
PHA 0.1 $\mu\text{g}/\text{ml}$				
AB	4.69* (0.58)	4.91 (0.40)	5.05 (0.25)	5.01 (0.21)
T cells/ mm^3	1283* (436)	1793 (780)	1686 (438)	1719 (442)
SIg+ cells/ mm^3	318 (116)	347 (169)		314 (153)
Total lymphocyte count/ mm^3	2082 (706)	2586 (1008)		2446 (649)

Results expressed as mean (standard deviation).

Auto = autologous plasma AB = AB serum n = number of subjects.

*Late onset myasthenics show low PHA induced lymphocyte transformation in autologous plasma ($p < 0.0005$ against all controls; $p < 0.005$ against controls 40+) and AB serum ($p < 0.01$ all controls; $p < 0.05$ controls 40+); and low T cell counts ($p < 0.01$ all controls; $p < 0.02$ controls 40+).

Fig 1 Spontaneous lymphocyte transformation response in myasthenia gravis (MG) and controls (C). $\blacktriangle$ uncomplicated MG, $\triangle$ thymectomised MG, ∇ MG with thymoma. *p* values indicate the significance of differences from controls. Bracketed numbers indicate numbers of subjects.

Table 2 Serial studies of PHA (0.1 μ g/ml) induced lymphocyte transformation in three patients with myasthenia gravis

Patient	Autologous plasma	AB serum
1	3.07	4.01
	4.62	4.63
	3.18	4.49
	3.87	4.43
2	3.35	4.09
	4.94	4.81
	4.64	5.46
3	3.48	4.11
	4.98	5.11
	5.22	5.08

fig 2). In contrast to spontaneous lymphocyte transformation, substitution of AB serum was associated with a partial rise in this response (fig 3) such that patients and controls did not differ significantly ($p=0.1$). In late onset myasthenics, however, significantly low responses persisted in AB serum (table 1). This subgroup also accounted for virtually all the abnormalities of PHA-induced lymphocyte transformation in autologous plasma. The PHA-induced transformation responses in AB serum of the total myasthenic group correlated significantly with T cell count ($r=+0.52$; $p<0.01$). The PHA-induced lymphocyte transformation responses of thymectomised patients or patients with thymoma tended to be lower than those of other myasthenics. At the higher PHA concentration, no significant difference was seen between the lymphocyte transformation responses of myasthenics and controls.

Fig 2 PHA-induced lymphocyte transformation response in autologous plasma in myasthenia gravis. Symbols and abbreviations as fig 1.

Fig 3 PHA-induced lymphocyte transformation response in AB serum in myasthenia gravis. Symbols and abbreviations as fig 1.

4 Serum inhibitory effects

A rise in PHA induced (0.1 µg/ml) lymphocyte transformation response on substitution of AB serum for autologous plasma greater than in any of the controls was seen in six of the 34 patients on at least one occasion. Serial studies of three of these patients (table 2) showed that the serum effect in individual patients fluctuated considerably over a period of time. There was no relationship to disease activity since all three patients were clinically stable throughout. Addition of any of these "inhibitory sera" to normal lymphocytes was associated with a depression of PHA-induced (0.1 µg/ml) lymphocyte transformation; this depression was highly significant compared with the effects of the addition of AB serum ($p < 0.01$; fig 4). In contrast, spontaneous lymphocyte transformation was not inhibited by this myasthenic serum.

Table 3 Cellular immunity data analysed according to sex and age of disease onset in myasthenia gravis

		<i>Myasthenia gravis</i>				Controls n = 78
		Males		Females		
Spontaneous	Auto	35+ n=8	35- n=4	35+ n=8	35- n=14	
		3.15 (0.22)	3.02 (0.18)	2.87 (0.19)	2.99 (0.24)	3.11 (0.18)
	AB	3.12 (0.23)	3.06 (0.18)	2.85 (0.26)	2.98 (0.24)	3.15 (0.14)
		Auto	4.73 (0.48)	5.05 (0.43)	4.31 (0.65)	4.84 (0.57)
PHA 0.1 µg/ml	AB	4.95 (0.42)	5.14 (0.29)	4.44 (0.63)	4.84 (0.41)	5.04 (0.23)
		1419 (390)	1780 (588)	1100 (461)	1797 (847)	1697 (434)

Representation of results as table 1.

5 Relationships to age of disease onset and sex

Late onset (age over 35 years) myasthenic patients showed low PHA induced (0.1 µg/ml) responses in autologous plasma, and low T cell counts (table 1). These were significant compared not only to all controls, but also to age-matched controls and early onset (under 35 years) myasthenics. The low PHA induced (0.1 µg/ml) responses in AB serum were also significant. Spontaneous lymphocyte transformation responses were normal in the late onset group. Early onset myasthenics did not show any abnormalities of lymphocyte transformation responses or T cell counts. Table 3 shows the data analysed with respect to both sex and age of onset. In the early onset group, both sexes showed lymphocyte transformation responses and T cell counts similar to controls. In the late onset group females showed lower T cell counts than males and lower spontaneous and PHA-induced lymphocyte transformation responses, whether in autologous plasma or AB serum. Females with late onset disease accounted for most of the abnormalities of the myasthenic group as a whole.

6 Thymectomy

The results and clinical associations of the data remained significant if thymectomised patients were excluded from the analyses.

7 HLA phenotype and autoantibodies

Compared with HLA B8-positive patients, HLA B8-negative myasthenics showed slightly lower PHA (0.1 µg/ml)-induced lymphocyte transformation responses and T cell counts, but the differences were not significant. PHA(0.1 µg/ml)-

Fig 4 Effects of added myasthenic (MG) or AB serum (AB) on spontaneous and PHA-induced lymphocyte transformation in autologous plasma of one normal subject (C).

indicates the mean and standard deviation of 28 other assays performed on the same control subject on occasions other than those investigating the effects of added serum. p indicates the significance of the difference between added MG and AB serum.

induced lymphocyte transformation responses in autologous plasma were (mean $\pm$ SD) 4.86 ± 0.44 in B8-positive patients and 4.61 ± 0.69 in B8-negative patients; and in AB serum 4.92 ± 0.39 in B8-positive patients and 4.69 ± 0.62 in the B8-negative. T cell counts were 1623 ± 807 in the B8-positive group and 1501 ± 617 in the B8-negative. No difference was seen in any of the data between patients positive or negative for any of the autoantibodies previously examined (see previous paper).

Discussion

This study has confirmed the presence of cellular immunodeficiency in myasthenia gravis on the basis of both impaired lymphocyte transformation responses and reduced numbers of circulating T cells. Previous studies have investigated the lymphocyte transformation response to PHA in the disease but the results have been conflicting; although some have demonstrated impaired responses,^{8, 10-12} others have obtained normal results.^{4, 15-19} The reasons for this discrepancy are probably multiple and may involve differences in the culture system employed, the duration of culture, the concentration(s) of PHA used and the selection of patients for study. For example, it is possible that studies which employ separated lymphocytes,¹⁵⁻¹⁹ culture periods of more than three days,^{15, 16, 18} or a single concentration of PHA greater than used in this study,^{15, 18, 19} may fail to detect differences from controls because the culture systems are relatively insensitive.

Similarly, abnormalities may also be missed in patient groups which are small in number^{4, 15} or mixed with respect to thymic histology.^{4, 15, 17, 18} Conversely, falsely abnormal results may be obtained when patients are studied who are immunosuppressed¹¹ or coincidentally suffering from other diseases, many of which are themselves associated with impaired cell-mediated immunity.¹⁰ In this study, the culture period was optimal and the transformation responses were studied at two concentrations of PHA on the principle of the dose-response curve.²⁰ In addition, the effect of autologous plasma was investigated by the study of co-cultures in AB serum. The results thus obtained are in agreement with those of Simpson and colleagues,⁸ whose use of protein synthesis as an index of the lymphocyte response to PHA is probably more sensitive than the conventional H³ uptake. More recently similar results have also been reported by others.¹²

In our study, the impaired lymphocyte transformation responses in autologous plasma were partly caused by the inhibitory effects of myasthenic serum; this was shown by the large rise in transformation response which occurred when AB serum was substituted in the culture medium, and also by the inhibitory effects of myasthenic serum on the transformation of normal lymphocytes. These serum effects were intermittent and not related to disease activity. In AB serum, the transformation responses showed significant correlation with the numbers of circulating T cells which suggests that the demon-

strated reduction in T cell numbers might be largely responsible for these impaired responses. T cell counts have previously been noted to be low in the myasthenic thymus⁷ but, in the peripheral blood of myasthenics, the number of circulating T cells has been said to be normal.^{4 7 21-23} However, in none of these studies were the data compared between early and late onset myasthenics. In this study, in which such a division was made, significant abnormalities were found only in the late onset subgroup. The observed reduction in E rosetting cells could reflect either a generalised depression of T cell numbers or an underrepresentation of a functionally distinct subgroup of T cells. Such a disturbance in the distribution of T cell subpopulations in myasthenia gravis has been suggested from the observation of an increased percentage of IgG-binding T cells^{24 25} and of an increase in suppressor cell activity in the disease;²⁶ unfortunately the early and late onset forms of the disorder are not compared in these reports. Similar differences between early and late onset myasthenics, and between male and female patients, have however been reported from studies of the lymphocyte transformation responses to the acetylcholine receptor, the responses being lower in females and in patients with late onset of the disease.¹⁸ It seems clear that consideration should be given to these clinical subgroups in all immunological studies of the disease.

Several mechanisms could have led to the observed depression of cell-mediated immunity in late onset myasthenia gravis. Debility, immunosuppressive treatment and coincidental collagen vascular disease were all largely excluded in this study and cases with thymoma were distinguished from others. It therefore seems highly unlikely that any of the results can be attributed to such non-specific effects.

The particular association of the cellular immunodeficiency with the late onset group may be attributable to differences between the late and early onset groups in thymic histology. An overall reduction of T cell numbers may be associated with thymic involution in the late onset group whereas the early onset group may be those with hyperplasia.

T cells may be destroyed by lymphocytotoxins, which have been reported in myasthenia gravis²⁷ or, since lymphocytes carry cholinergic receptors,²⁸ they may be lysed by cross-reaction with acetylcholine receptor antibodies. Alteration of lymphocyte surface antigens, possibly by viral infection, may also lead to lymphocyte destruc-

tion; such altered antigens have been detected on the surface of lymphocytes of the myasthenic thymus.⁴ It has been suggested that autoimmune thymitis is important in the pathogenesis of myasthenia gravis;²⁹ this might lead to T cell destruction through the mediation of antithymus antibody.¹⁰ A widespread impairment of cell-mediated immunity may be caused by chronic antigenic stimulation, as in graft-versus-host disease,³⁰ and this could be associated with the high incidence of DNA antibodies reported in the previous paper. According to the theory of Simpson,¹ the immune tolerance of myasthenia gravis is genetically linked. In this study, no significant association was seen between the cellular immune deficiency and any HLA phenotype, but DRw antigens were not examined.

The relevance of the cellular immunodeficiency in late onset myasthenia gravis remains at present speculative. The late onset group tend not to have autoantibodies which suggests that the reduction in T cell numbers may play a protective role, perhaps through loss of their helper function. However, the relationship has not yet been established between the cellular immunodeficiency and the titre of antibodies to the acetylcholine receptor. Furthermore, some autoantibodies, such as skeletal muscle antibodies, occur more commonly in the late onset group.³¹

The differences in the cellular immune data between the early and late onset forms of the disease, and between male and female patients, does however lend further support to the concept that myasthenia gravis has at least two forms. The early onset group produce a wide range of autoantibodies but have relatively normal overall cellular immunity, whereas the late onset group show production of a few autoantibodies, such as skeletal muscle antibodies, but have a marked impairment of T cell number and function. It is suggested that these two forms differ in their pathogenesis. Further studies of these subgroups are required with perhaps an emphasis on their T cell subpopulations, in relation to the incidence and titre of autoantibodies, including those against the acetylcholine receptor.

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