

Thymectomy and azathioprine have no effect on the phenotype of CD4 T lymphocyte subsets in myasthenia gravis

A Melms, G Malcherek, U Gern, N Sommer, R Weissert, H Wiethölter, H J Bühring

Abstract

The influence of thymectomy and long term immunosuppression on the phenotype of CD4 T lymphocyte subsets, which were defined by the restricted expression of CD45RA and CD45RO markers, was studied by double immunofluorescence in 29 patients in different clinical stages of generalised myasthenia gravis. In the acute stage of myasthenia, before thymectomy and immunosuppression, no differences in CD4 subsets were observed in the peripheral blood from nine patients and 21 matched controls. Four to seven weeks after thymectomy, there was a slightly decreased proportion of CD4+CD45RO+ (UCHL1+) memory cells ($p < 0.05$, paired t test). Patients on steroids showed a more pronounced decrease of CD4+CD45RO+ cells suggesting, in addition, a drug-related effect. CD4 subsets (CD45RA, CD45RO, and CD29 positive) in the peripheral blood compartment remained largely stable over 18 to 24 months thereafter. In addition, CD4 subsets were examined in 20 patients with myasthenia gravis who had had a thymectomy between two and 17 years before. With the exception of patients on steroids, there were no differences in CD4 subsets in patients on or off azathioprine. These data did not show any relation of CD4 T cell subsets to the clinical course of myasthenia, or significant changes due to thymectomy, or immunosuppression with azathioprine. These results also complement the authors' clinical experience that thymectomy in adults does not leave a deficit in cell-mediated immunity. The slight change associated with steroid treatment might deserve further attention.

Department of
Neurology, University
of Tübingen,
Tübingen, Germany
A Melms
G Malcherek
U Gern
N Sommer
R Weissert
H Wiethölter

Department of
Medicine, Section of
Transplantation
Immunology,
University of
Tübingen, Tübingen,
Germany
H J Bühring

Correspondence to:
Dr Arthur Melms,
Department of Neurology,
University of Tübingen,
Hoppe-Seyler-Str. 3, 7400
Tübingen, Germany.

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Myasthenia gravis is a neuromuscular disease characterised by exertional weakness of striated muscle. Autoantibodies against the nicotinic acetylcholine receptor present in the serum of more than 90% of patients with generalised myasthenia are bound at the neuromuscular junction and have been shown to impair neuromuscular transmission.¹⁻³

Although the pathogenetic mechanisms ini-

tiating the autoimmune response against the acetylcholine receptor are not known, the thymus is suspected to be of major importance.^{4,5} In most of the patients, the thymus shows either lymphofollicular hyperplasia or neoplastic changes, and tends to accumulate acetylcholine receptor-specific T lymphocytes^{6,7} with features of T helper cells.^{8,9} Despite much knowledge on functional interactions in the immune system, the cellular explanations for the clinical benefit of thymectomy are not clear. Randomised clinical trials to evaluate the influence of thymectomy in myasthenia have proven difficult.¹⁰ There is good agreement among most clinicians, however, that thymectomy followed by immunosuppression is leading to clinical improvement in some 80% of patients with generalised myasthenia, especially in those cases of onset at a young age.¹¹

T cells of the CD4 subpopulation play a central role in immunoregulation, inducing antibody production by B lymphocytes or cytotoxic cells and, perhaps, suppressor responses mediated by CD8 T cells. Two functionally distinct CD4 T cell subsets can be identified by the restricted expression of different isoforms of the leukocyte common antigen (LCA T 200) termed CD45.¹²⁻¹⁴ The monoclonal antibody 2H4 defines suppressor inducer cells (CD45RA+) and monoclonal antibody UCHL1 defines helper-inducer cells (CD45RO+) among CD4 T cells. The distribution of these subsets has been studied in lymphatic tissues during ontogeny¹⁵⁻¹⁸ and in inflammatory lesions.¹⁸⁻²⁰ In particular, naive unprimed T cells and T cells emigrating from the thymus express the CD45RA variant. This phenotype is lost after antigen recognition, hence activated and memory T cells acquire the CD45RO moiety on the cell surface.¹⁴

Alterations among functionally distinct subsets of regulatory CD4 T lymphocytes have been reported in some (auto-) immune mediated disorders²¹⁻²⁴ and it has been speculated that these alterations would reflect a general disturbance of immunoregulation.

To test this hypothesis in another autoimmune disease, we studied CD4 T cell subsets in different clinical stages of myasthenia gravis to gain insight into the "milieu" that might have an influence on the activity of auto-reactive acetylcholine receptor-specific T and B lymphocytes, and which could reflect some influence of immunotherapy such as thymectomy and immunosuppression.

Table 1 Clinical data

Patient groups	n	Sex (F/M)	Mean age (years) (range)	Anti-AChR titre* (nM)	Thymus histology† (n)	AChE Inhibition treatment (-/+)‡	Immunosuppression			Clinical MG score (mean; range)
							CS	AZA	None	
Controls	21	15/6	38.3 (16–63)	-ve	ND	0/21	0/21	0/21	21/21	0.0
Myasthenia gravis Recent onset	9	5/4	35.3 (20–59)	0.6–98	LFH (7) BT (1)	7/2	2/9	0/9	7/9	1.5; 1.2/1.8
4–7 weeks after Tx	9	5/4		0.2–83	A (1)	7/2	4/9	1/9	4/9	0.6; 0.0–1.2
18–24 months after Tx	8	4/4		0.3–72		5/3	0/8	5/8	3/8	0.06; 0.0–0.3
Clinical remission	20	16/3	35.4 (18–60)	0.09–580		14/6	3/20	6/20	11/20	0.12; 0.0–0.8
2–5 years after Tx	11/20	9/2	31.9 (18–45)	5–163	LFH (10) TC (1)	7/4	1/11	4/11	6/11	0.07; 0.0–0.4
5–17 years after Tx	9/20	8/1	39.4 (25–60)	0.09–580	LFH (7) BT (1), A (1)	6/3	2/9	2/9	5/9	0.16; 0.0–0.8

*Normal less than 0.4 nM α -bungarotoxin binding sites per litre of serum.

‡Patients on choline esterase inhibitors (Mestinon) (see text).

†LFH = lymphofollicular hyperplasia; BT = benign thymoma; TC = thymus carcinoma; A = thymus atrophy.

AChR = acetylcholine receptor; AChE = acetylcholine esterase; CS = ; AZA = azathioprine; MG = myasthenia gravis; Tx = thymectomy; ND = no data.

Materials and methods

Patients

A summary of the clinical data including the current treatment is given in table 1. During 1989, nine patients with a recent onset of generalised myasthenia gravis (Osserman grade IIa/b) were scheduled for thymectomy and followed up for 18 to 24 months. Myasthenic signs and symptoms at onset were moderately severe according to a clinical rating scale.²⁵ All patients had antibodies against the acetylcholine receptor (more than 0.4 nM α -bungarotoxin binding sites per litre at onset). The compound muscle action potential showed a pathological decrement after repetitive nerve stimulation, which was ameliorated by edrophonium. Blood samples were taken before thymectomy, four to seven weeks later, and 18 to 24 months thereafter. Thymus histology revealed a lymphofollicular hyperplasia in seven of nine patients, and a benign thymoma and thymic atrophy in one patient respectively.

In addition, a second group of 20 patients who had undergone thymectomy for the treatment of generalised myasthenia gravis (Osserman IIa/b) two to 18 years before, were studied in "clinical remission". Three of 20 patients were on steroids (10–12.5 mg prednisolone every other day) because of poor tolerance of azathioprine. Six of 20 patients had been on azathioprine for up to seven years. Eleven of 20 patients were asymptomatic, without immunosuppression, seven still taking a low dosage of pyridostigmine (Mestinon, 40–100 mg per day). Immunosuppression had been discontinued in eight patients one year before. Three patients have never been treated with immunosuppressive drugs.

Twenty one individuals matched for age and sex were studied as controls including eight healthy volunteers and 13 patients with other neurological diseases without evidence of inflammatory or malignant disease: migraine (3); tension-type headache (2); cluster headache (1); low back pain (3); minor head trauma (1); intracranial haemorrhage (1); cerebellar atrophy (1); Parkinson's disease (1). All samples were obtained with informed consent.

Peripheral blood mononuclear cells

These were isolated from heparinised venous blood samples drawn between 8 and 10 am and were immediately separated by density gradient centrifugation (800 \times g, 25 minutes, 20°C on Lymphoprep gradients (Nygaard, Oslo). Aliquots were cryopreserved in freezing medium containing 50% fetal calf serum, 10% dimethyl sulfoxide (Sigma, Germany) and 40% RPMI 1640 medium (GIBCO) and were stored in liquid nitrogen. Viability of thawed cells was 85–95% according to the trypan blue exclusion test. Serial samples were analysed in pairs.

Monoclonal antibodies

Anti-T3 (CD3; mature T cells), anti-T4 (CD4; 'helper-inducer' T cells), anti-T8 (CD8; 'suppressor/cytotoxic' T cells), anti-T11 (CD2; pan T cell marker), and B1 (CD20; mature B cells) were from Coulter Immunology (Krefeld). HLA-DR (L243) and Leu7 (cytotoxic cells, natural killer cells) were from Becton Dickinson (Heidelberg). Anti-human interleukin-2 receptor antibody (CD25) was from Biotest (Frankfurt). A monoclonal antibody binding to human MHC class I molecules (W6/32 HL) was used as a positive control, antibody variant W6/32 HK recognising non-translated MHC molecules as negative control.

Single immunofluorescence

Half a million peripheral blood mononuclear cells in 50 μ l Hank's balanced salt solution (HBSS, GIBCO) supplemented with 0.1% bovine serum albumine (Sigma) and 0.1% azide were incubated for 30 minutes on ice with saturating amounts of the first antibody. After washing in HBSS, labelled cells were stained with affinity-purified fluorescein isothiocyanate conjugated with the F(ab)₂ fragment of goat-anti-mouse IgG (Dianova, Hamburg).

Double fluorescence staining

This was performed as described.²⁶ Anti-T4 (biotinylated, Coulter) and fluorescein isothiocyanate streptavidin was used in combina-

Table 2 Frequencies of lymphocyte subsets

	Controls n = 21	MG in remission n = 20	MG pre-Tx n = 9	MG post-Tx* n = 9
CD2	83.8 ± 4.5	81.7 ± 8.0	86.8 ± 4.2	85.1 ± 6.9
CD3	76.8 ± 7.7	73.6 ± 10.4	76.8 ± 8.2	78.9 ± 8.0
CD4	50.7 ± 7.0	48.7 ± 9.8	49.2 ± 7.2	50.5 ± 8.7
CD8	25.2 ± 5.2	23.8 ± 5.7	25.7 ± 6.2	25.2 ± 5.9
CD20	7.3 ± 2.7	9.8 ± 4.3	6.3 ± 3.1	6.9 ± 3.6
HNK1	10.3 ± 4.7	10.5 ± 5.5	Not tested	Not tested
CD4+/DR+	1.5 ± 0.5	2.0 ± 1.0	1.4 ± 0.9	1.5 ± 0.8
CD8+/DR+	1.0 ± 0.6	1.0 ± 0.5	0.6 ± 0.5	0.8 ± 0.4

(% positive cells in the lymphocyte gate ± SD).

* 4-7 weeks after thymectomy.

MG = myasthenia gravis; Tx- thymectomy.

tion with HLA-DR-PE (phycoerythrin PE-conjugated, Becton Dickinson) or 2H4-RD1 (CD45RA) or 4B4-RD1 (CD29; both PE-conjugated, Coulter Immunology). UCHL1 (CD45RO; a generous gift of Dr Beverley, London, UK) was used in combination with fluorescein-conjugated goat-anti-mouse IgG and biotinylated anti-T4 (detected with PE-conjugated streptavidine).

Cells (one million peripheral blood mononuclear cells in 50 µl HBSS) were incubated with the first antibody (conjugated with fluorescein or biotinylated) as described above. After washing in HBSS, fluorochrome conjugate was added, if appropriate, to label the first antibody. Double staining was completed by adding another fluorochrome conjugated antibody and conjugated streptavidine in case of a biotinylated antibody.

Cell surface marker analysis

This was performed on a FACS IV cytofluorograph (Becton Dickinson) supported with an argon laser. Samples were run gated on 'lymphocytes' to exclude granulocytes and monocytes. Contamination with monocytes were usually 0-2.5% as detected with TM1, a marker for peripheral blood monocytes (a gift from Dr G A Müller, Tübingen). A total of 10 000 cells were analysed in single immunofluorescence. In double fluorescence analysis, data of 25 000 to 50 000 cells were collected and stored on floppy disks. A computer program designed for dual parameter FACS analysis written by M Tomuschat and K Schaut (University of Tübingen) was used on an IBM-XT personal computer. Evaluation was done without knowledge of the clinical status of the subjects.

Data from single fluorescence were presented as percentages of gated lymphocytes. The proportion of CD4 subsets were given as

the relative percentage of the CD4+ subset. Absolute cell numbers of CD4 subsets were calculated only in patients after thymectomy, because differential blood counts were lacking in a number of subjects. Statistical analysis of these data gave similar results as calculations with relative frequencies.

Statistical analysis of samples from patients before and after thymectomy were analysed by the two-tailed, paired *t* test. In other combinations, the two-tailed *t* test was applied.

Results

Immunophenotyping by single fluorescence

No significant differences in the frequencies of the major T cell subsets (CD2, CD3, CD4, CD8), B lymphocytes (CD20) or natural killer cells (HNK1) were found in patients with myasthenia gravis with recent onset of symptoms before and after thymectomy, or patients with myasthenia in clinical remission with regard to different treatments and controls (table 2).

Immunophenotyping by double fluorescence

T lymphocytes expressing activation markers such as HLA-DR molecules or the interleukin-2-receptor (IL2R) were found in very low frequencies without any differences among the various groups of patients. Approximately 2% of the proportion of CD4 T cells and 1% of CD8 T cells expressed HLA-DR molecules but in low density. A similarly small fraction (up to 4%) of CD4 cells expressed detectable amounts of the IL2R. This small amount was not due to the staining protocol, using either directly or indirectly labelled antibodies, because activated T cells from a T cell line, expressed high levels of both HLA-DR and IL2R (more than 95% of both markers, not shown) after stimulation in vitro. Double staining with antibody B1 (CD20) in fact showed that 95-99% of HLA-DR+ mononuclear cells in the lymphocyte gate (containing 0-2.5% monocytes) were mature B lymphocytes.

CD4 subsets

CD4 subsets analysed in this study are described in table 3. Two mutually exclusive CD4 subsets with different regulatory and maturational properties were defined by the restricted expression of the CD45 marker: 'virgin T cells' and 'suppressor-inducer' cells express the CD45RA+ isoform (defined by mab 2H4+); 'memory' T cells, recently activated T cells, and 'helper-inducer' cells express the CD45RO isoform as defined by mab UCHL1 as well as a high density of the marker CD29 defined by monoclonal antibody 4B4 (table 3 and fig 1). Subset CD45RO, CD45RA, and CD29 were determined with a specific antibody for internal cross reference. Only the results of the CD4+CD45RO+ (UCHL1+) subset are presented here.

Alterations of the CD4 CD45RO T cell subset ('helper-inducer' and memory phenotype) during the course of myasthenia is shown in fig 2. By statistical analysis, patients with a

Table 3 Combinations of monoclonal antibodies in double immunofluorescence studies

Antibody 1	Antibody 2	Subset analysed	Functional and maturational features of the subset
CD4 (T4)	CD45RA* (2H4)	CD4+CD45RA=	'Suppressor-inducer' T cells Naive, unprimed T cells
CD4 (T4)	CD45RO* (UCHL1)	CD4+CD45RO+	'Helper-inducer' T cells Previously activated T cells Memory T cells
CD4 (T4)	CD29 (4B4)	CD4+CD29+	'helper-inducer' T cells; memory cells
CD4 (T4)	HLA-DR	CD4+DR+	Activated T helper cells
CD4 (T4)	CD25 (IL2R)	CD4+CD25+	Activated T helper cells
CD8 (T8)	HLA-DR	CD8+DR+	Activated suppressor or cytotoxic cells
CD20 (B1)	HLA-DR	CD20+DR+	Peripheral blood B cells

*CD45RA and CD45RO define mutually exclusive subsets, i.e. CD4+CD45RA+ T cells are CD45RO- and vice versa.

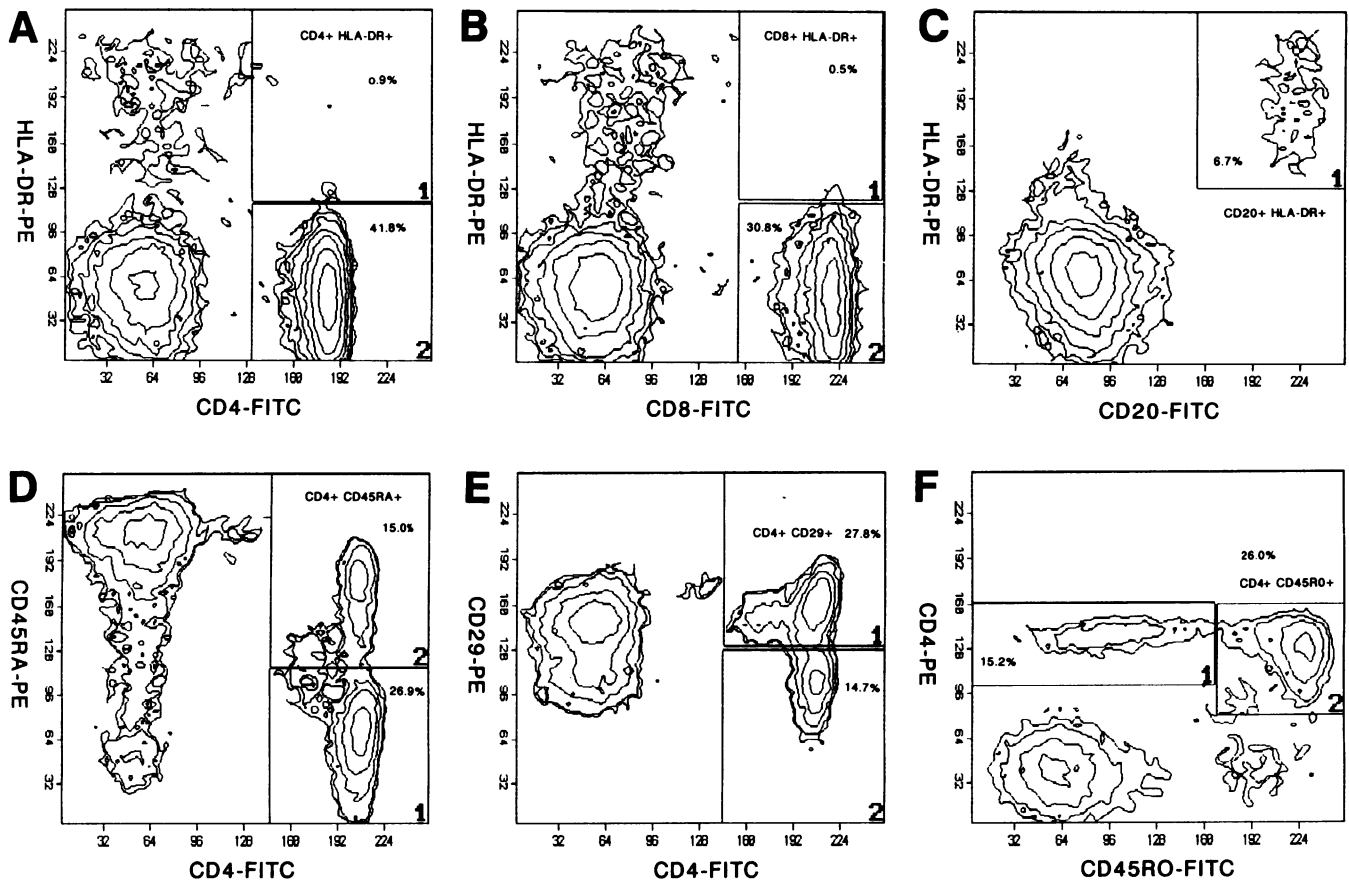


Figure 1 Dual parameter plot of lymphocyte subsets defined by double-immunofluorescence. Results from a patient in complete remission (clinical score zero) two years after thymectomy without any medications. (A) CD4/HLA-DR; (B) CD8/HLA-DR; (C) CD20 (B1)/HLA-DR; (D) CD4/CD45RA (2H4); (E) CD4/CD29 (4B4); (F) CD45RO (UCHL1)/CD4. The respective subsets are quantified as percentages of the total lymphocyte gate displayed.

recent onset of generalised myasthenia gravis (Osserman grade IIa/b) examined before thymectomy were no different from age-matched controls. Four to six weeks after thymectomy, a small change among the CD4 subset was observed. At that stage, CD4+CD45RO+ (UCHL1+) T cells showed a slight decrease ($p < 0.05$, paired t test). The CD4+ CD29+

(4B4+) phenotype did not change to the same extent (not shown). Patients (four of nine) receiving steroids during convalescence from thymectomy showed slightly more pronounced changes of the CD4+CD45RO+ subset (mean decrease -7.3%) than non-steroid-treated patients (mean decrease -4.0%) which was most likely related to the steroid treatment. Subsequently, 18 to 24 months after thymectomy, the proportion of this subset remained largely unchanged even though most patients were on azathioprine (2–3 mg per kg body weight) at that time ($p < 0.1$ compared with the proportion of CD4+CD45RO+ T cells before thymectomy; paired t test; not significant). All patients improved after thymectomy, and neither the frequencies of CD4 subsets before surgery, nor the degree of their changes thereafter were related to the clinical improvement.

The influence of long term immunosuppression after thymectomy was studied in 20 patients with myasthenia who had a thymectomy two to 17 years before and who were on different treatment schedules maintaining clinical remission (see above and table 1). Even though patients on steroids tended to have a lower proportion of CD4+CD45RO+ memory T cells (similar to the patients shortly after thymectomy), statistical analysis failed to demonstrate a significant difference compared with patients on other treatment regimes or controls. There was also no difference in CD4

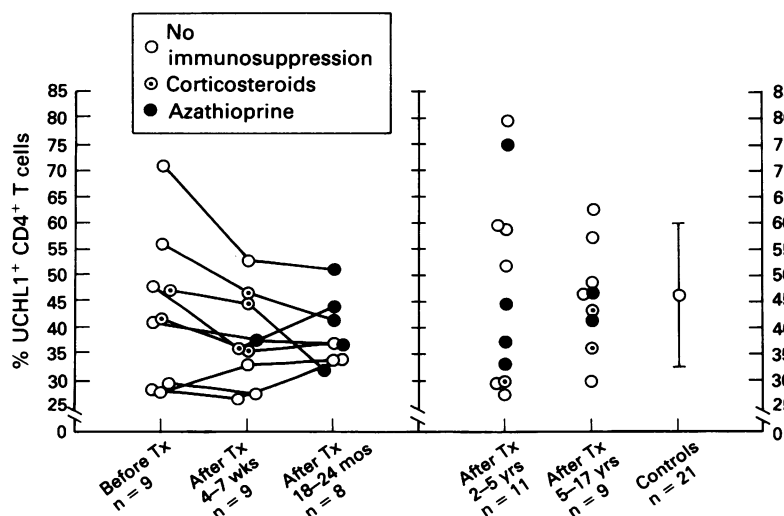


Figure 2 Frequencies of CD4+CD45RO+ T cells in the peripheral blood and the influence of thymectomy and immunosuppression. Results are expressed as the relative percentage of the CD45RO (UCHL1) positive T cells of the CD4 subset frequency. The symbols refer to the current immunosuppressive treatment (steroids, azathioprine, or none).

subsets whenever thymectomy had been performed.

Discussion

In this study, we analysed T cell subsets, especially of CD4 T cells, in different clinical stages of myasthenia gravis. As in earlier studies^{27,28} the major lymphocyte subsets (CD2, CD3, CD4, CD8, CD20) were not significantly altered in the peripheral blood of patients with a recent onset of myasthenia, both before and after thymectomy as well as in "clinical remission" along with appropriate therapy compared with patients with other neurological disorders and controls. In addition, activated T cells of the CD4 and CD8 lineage were detected in very low frequency in the peripheral blood of all subjects examined.

An alteration among functionally distinct subsets of regulatory T lymphocytes has been reported in some (auto-)immune mediated disorders such as systemic lupus erythematosus, rheumatoid arthritis, the post-polio syndrome and multiple sclerosis.²¹⁻²⁴ In particular, CD4+CD45RA+ (that is UCHL1 negative) T cells have been reported to decrease before an exacerbation in relapsing-remitting multiple sclerosis.²⁹ The selective loss of this suppressor inducer subset in the peripheral blood has been observed in patients with the chronic progressive course of multiple sclerosis^{22,30} which has a poor prognosis and responds less favourably to immunosuppression.

If these changes among CD4 T cell subsets were common to autoimmune disorders, similar findings should also be expected during the course of myasthenia gravis. In the present study, however, CD4 subsets in patients with a recent onset of generalised myasthenia were not found to be different compared with age-matched controls. Thymectomy had only a minor and transient influence on the phenotype of CD4 T cells in these patients. Slight changes were observed four to seven weeks after thymectomy. At that stage, the CD4+CD45RO+ (UCHL1+) subset containing "helper-inducer" and memory T cells slightly decreased and remained largely stable over 18 to 24 months thereafter. Patients receiving steroids after surgery had more pronounced changes which we considered to be a drug effect. It is known that certain lymphocyte stages have a different sensitivity to deletion by steroids.³¹ On the other hand, non-selective immunosuppression by azathioprine, even for long periods apparently, had no influence on the CD4 phenotype. This minor change could hardly explain the benefit of thymectomy in our patients. It has been speculated that thymectomy removes autoimmune T helper cells accumulating or being trapped in the hyperplastic myasthenic thymus.^{6,7} Monoclonal antibodies defining subsets among the CD4 lineage, however, fail to detect changes in the frequency of acetylcholine receptor-specific T cells within the CD4 population. Moreover, the peripheral blood compartment harbours migrating T cells.

Therefore, CD4 subsets in this compartment do not necessarily reflect the distribution in the lymphatic tissue or in target organs of inflammation. In the peripheral blood compartment, we did not observe significant changes among CD4 subsets during the course of the disease. Neither thymectomy or immunosuppression with azathioprine grossly affected the frequencies of "regulatory" CD4 T cell subsets. These results also complement our clinical experience that thymectomy in adults does not leave a deficit in cell-mediated immunity. It remains to be shown if other cell surface markers that more precisely define certain T cell stages among the major T cell subsets will shed more light on both the pathogenesis of autoimmune diseases and the mechanisms of therapeutic benefits.

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Romberg's Sign

Moritz Heinrich Romberg of Berlin published his classic Lehrbuch in sections between 1840-6; Sieveking translated the "Manual" into English in 1853. The description of that most misspelled of all eponyms lies in his description of tabes dorsalis.¹

"Early in the disease we find the sense of touch and muscular sense diminished, while the sensibility of the skin is unaltered in reference to temperature and painful impressions. The feet feel numbed in standing, walking or lying down, and the patient has the sensation as if they were covered in fur; the resistance of the ground is not felt . . . The gait begins to be insecure . . . he puts down his feet with greater force . . . The individual keeps his eyes on his feet to prevent his movements from becoming still more unsteady. If he is ordered to close his eyes while in the erect posture, he at once commences to totter and swing from side to side; the insecurity of his gait also exhibits itself more in the dark. It is now ten years since I pointed out this pathognomonic sign, and it is a symptom which I have not observed in other paralyses, nor in uncomplicated amaurosis . . . in no case have I found it wanting."

Romberg gives a fulsome account of the muscular weakness, urinary frequency, retention and incon-

tinence, and the constricting pains that

"encircle the trunk like a hoop, and not infrequently renders breathing laborious . . . troublesome in sleep, causing them suddenly to start up and scream. Others complain of a heavy weight pressing upon the rectum and bladder, others again of colic and gastric pains; the majority suffer from pain shooting through the legs, and a sense of pricking, itching, burning, or cold in the skin of the lower as well as the upper extremities;"

The whole clinical spectrum is described, the natural history extending over several—as many as ten to fifteen—years. The wasting (tabes) of the cord and roots is related, but "as yet we possess no microscopic investigation of the atrophied portion."

Romberg also gives a brief but unmistakable description of the pupils to be described 16 years later by Douglas Moray Cooper Lamb Argyll Robertson. Curious how students add an undropped 'h' in one and insert a non-existent hyphen in the other.

JMS PEARCE

1 Romberg MH. *A Manual of the nervous diseases of man*, Vol 2, (EH Sieveking trans) London. Sydenham Society, 1853:395-401.