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# INFLUENCE OF ADULT THYMECTOMY ON IMMUNOCOMPETENCE IN PATIENTS WITH MYASTHENIA GRAVIS[1]

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**The influence of adult thymectomy on several parameters of immunocompetence in patients with myasthenia gravis (MG) was investigated. Since incomplete thymectomy may lead to the presence of thymic remnants, we determined the activity of a thymus-dependent factor in the sera of the MG patients. As measured by several parameters, MG patients showed a normal immunocompetence compared with healthy controls, except in the response to DNCB sensitization *in vivo*.**

**When tested at least 5 years after thymectomy, MG patients were found to have decreased response to mitogens, and a decreased cytotoxic T cell response in cell-mediated lympholysis. The response to challenge with DNCB *in vivo* was decreased both in thymectomized and nonthymectomized MG patients.**

**No difference was found in a) the percentage of circulating T, B, non-B/non-T cells; b) the response to allogeneic cells (MLR); c) the antibody-dependent lymphocytotoxicity; d) the production of immunoglobulins *in vitro* by pokeweed mitogen-stimulated cells; and e) the anamnestic response to antigens *in vitro*.**

**We conclude that adult thymectomy results in a decrease in the function of some subpopulations of lymphocytes.**

Both in mice and in humans, congenital aplasia of the thymus is associated with severe immunologic deficiencies, indicating the important role of the thymus in the acquisition of immunocompetence (1–3). Neonatal thymectomy in mice rapidly leads to atrophy of lymphoid organs and to well-characterized immunologic deficiencies (4). Adult thymectomy in mice, on the contrary, results only in subtle impairment of lymphocyte function (5–7). Little is known about the influence of adult thymectomy on the immunocompetence in humans, and the results obtained are rather conflicting.

Because thymectomy is a common therapy in myasthenia gravis (MG)[3] (8), the influence of adult thymectomy on lymphocyte function can easily be studied in these patients. To this end, we investigated several parameters of immunocompetence in nonthymectomized (non-Tx) and thymectomized (Tx) MG patients (post-thymectomy time up to 19 years). These findings were also compared with those obtained in healthy young donors.

The differentiation of stem cells into mature thymus-derived (T) lymphocytes is probably modulated by factors produced by the epithelial cells of the thymus (for review, see References 9 and 10). Because the existence of ectopic thymic tissue has been described and thymectomy may be incomplete, we determined the activity of a thymus-dependent factor (SF) in the sera of all the patients.

Previously, we have shown that SF stimulates cyclic AMP synthesis in thymocytes and induces thymocyte maturation (11–13). SF activity was found to be normal in the sera of MG patients, disappeared with thymectomy, and remained absent in most of the Tx patients (11, 14).

*Patients.* Clinical stage and several laboratory findings, including SF activity of the MG patients, are reported elsewhere (14). Tx patients were divided into groups according to their SF activity and time after thymectomy. Patients with low SF activity (<15 units) were considered to be completely thymectomized, and patients with normal SF activity after thymectomy were considered to have ectopic thymic tissue or tissue remnants (14). Because a small decline in most of the functions studied was observed in non-Tx patients with aging, only Tx patients younger than 50 years of age were investigated to study the influence of adult thymectomy.

## MATERIALS AND METHODS

*Determination of SF activity.* SF was isolated and assayed as previously described (12). In brief, serum separated from defibrinated blood was filtered through an Amicon CF 50 membrane (m.w. cutoff 50,000) (Amicon, Ooosterhout, The Netherlands) to remove high m.w. inhibitors. The ultrafiltrate was added to an equal volume of mouse (C57BL/6J) thymocyte suspension (final concentration $2 \times 10^6$ thymocytes/ml). After 5 min of incubation at 37°C, the cells were washed, lysed by

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[3] Abbreviations used in this paper: MG, myasthenia gravis; SF, thymus-dependent human serum factor; T lymphocyte, thymus-derived lymphocyte; Tx, thymectomized; B lymphocyte, bone marrow-derived lymphocyte; DNCB, dinitrochlorobenzene; ALS, anti-lymphocyte serum; PWM, pokeweed mitogen; CML, cell-mediated lympholysis; ADL, antibody-dependent lymphocytotoxicity; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

freezing in liquid nitrogen, and the intracellular cyclic AMP was extracted. Proteins, coagulated by boiling for 2 min, were spun down in the cold, and the amount of intracellular cyclic AMP in the supernatant was measured in duplicate with the Gilmans competition-binding procedure (15) with a cyclic AMP kit (T2K 432, The Radiochemical Centre, Amersham, U. K.).

*Isolation of lymphocytes and determination of subpopulations.* Lymphocytes of patients and normal donors were isolated by conventional buoyant-density-gradient centrifugation, with Ficoll-Isopaque (16) and preserved in liquid nitrogen as described (17). Cryopreservation allows testing of cell samples from different patients, collected at different times in one experiment, thus excluding technical variability between different experiments. Peripheral blood lymphocytes were classified as T cells by rosette formation with sheep red blood cells (SRBC) (E rosettes) (18) and as B cells (19) by staining with $F(ab)_2$ fragments of a rabbit IgG anti-human IgG $F(ab)_2$, labeled with fluorescein isothiocyanate (K-26 H-26 F, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service). The percentage of non-T/non-B lymphocytes, cells that neither form SRBC rosettes nor display surface immunoglobulins (Ig), was calculated by subtracting the number of T cells, B cells, and monocytes (evaluated by morphology) from the number of mononuclear cells.

*Lymphocyte transformation test.* Lymphocyte cultures were performed as described by du Bois *et al.* (20) by using as mitogens: phytohemagglutinin (PHA), concanavalin A (ConA) (21), anti-lymphocyte serum (ALS) (22), and pokeweed mitogen (PWM) (21). Results of each patient group are expressed as cpm ± S.D. and compared with the cpm ± S.D. of the control group of normal young donors run in parallel. Lymphocyte reactivity to allo-antigens was tested in the one-way mixed lymphocyte reaction (MLR) (20). Both the stimulator capacity and the responder capacity of patients lymphocytes were determined against three different (HLA-B8-negative) young donors. Results of each patient group are expressed as cpm ± S.D. and compared with those of young donors tested against each other.

The anamnestic response of the lymphocytes to antigens was tested in a microtiter system as described (23). Lymphocytes were tested with a mixture of the following antigens: purified protein derivative, Varidase, *Trichophyton*, *Candida albicans*, mumps.

*Cell-mediated lympholysis (CML).* CML was performed as described by du Bois *et al.* (20). In brief, effector cells to allogeneic target cells were generated in an MLR performed in microtiter plates. Each well contained $10^5$ irradiated (2000 rads) allogeneic stimulator cells and various numbers of responder cells in a final volume of 200 μl in HEPES-(see Abbreviations) buffered RPMI 1640 supplemented with 20% human serum. Target cells were prepared by culturing lymphocytes for 6 days without stimulants. Next, $10^6$ to $10^7$ cultured cells were incubated for 1 hr at 37°C with 100 μCi $Na_2{}^{51}CrO_4$ (spec. act. 50 to 400 mCi/mg Cr, The Radiochemical Centre). Target cells were washed three times, and $2 \times 10^4$ labeled cells were mixed with the effector cells in the original wells. After 8 hr incubation in a humidified atmosphere, $^{51}Cr$ release was measured. The extent of specific $^{51}Cr$ release was calculated by correcting for spontaneous and maximal $^{51}Cr$ release.

*Antibody-dependent lymphocytotoxicity (ADL).* A microassay system was used as described by Zeijlemaker *et al.* (24). In brief, mouse P815-$X_2$ mastocytoma cells ($2 \times 10^6$ cells in 0.2 ml medium 199 supplemented with 10% FCS) were incubated with 100 μCi $Na_2{}^{51}CrO_4$ solution (spec. act. 50 to 400 mCi/mg Cr, The Radiochemical Centre) for 60 min at 37°C. During the last 15 min, 10 μl of a rabbit anti-mastocytoma serum was added. Cells were washed and resuspended in bircarbonate-buffered medium 199 supplemented with 10% FCS. Cytotoxicity was performed in microtiter plates (Cooke, Cat. No. 220 M, 24 AR). Each well contained 200 μl of a suspension of $5 \times 10^3$ target cells and various numbers of effector cells. The plates were incubated for 180 min at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Each combination was tested in triplicate. The percentage of specific $^{51}Cr$ release was calculated as follows:

$$\text{Specific release} = \frac{\text{cpm test tube-cpm spontaneous release}}{\text{cpm maximal release-cpm spontaneous release}}$$

*Ig production in vitro.* Ig synthesis was tested *in vitro* with a modified assay as described by Waldman *et al.* (25). Lymphocytes were cultured in microtiter plates for 7 days in the presence or absence of PWM. Each well contained $40 \times 10^3$ lymphocytes in 150 μl of bicarbonate-buffered RPMI + 20% FCS. To each well, 20 μl of PWM (50 μg/ml) or 20 μl of RPMI were added. After 7 days of culture, the supernatants were collected, and the amount of IgM was measured by a solid-phase radioimmunoassay, performed essentially as described for IgE (26) with the following modification. Polystyrene plastic beads coated with rabbit anti-human IgM were first incubated with 50 μl culture supernatant. After the beads had been washed, a second incubation with $^{125}I$-labeled anti-IgM followed. The determinations were performed in duplicate. Measurements were made relative to a standard IgM preparation and expressed as ng IgM per $40 \times 10^3$ cultured lymphocytes.

*Dinitrochlorobenzene (DNCB) skin test.* Primary immune response was measured by DNCB skin testing (27). The test was performed as follows: on day 0, patients were sensitized for 48 hr with a patch containing 2 mg DNCB. On day 14, sensitization was tested by using three concentrations of DNCB (namely, 3, 10, and 30 μg DNCB) per patch. The test was evaluated as follows:

| | Score |
|---|---|
| Erythema | 1 |
| Erythema and induration | 2 |
| Erythema, induration and vesical | 3 |
| Erythema, induration and ulceration | 4 |

The final DNCB skin test score is the sum of the scores of each patch. The skin test is considered as positive with a score of 2 or more.

## RESULTS

Influence of adult thymectomy was studied by dividing the patients with low SF activity (50 out of 62) in four groups according to the time after thymectomy: less than 1 year after thymectomy; 1 to 5 years after thymectomy; 5 to 10 years after thymectomy, and thymectomy more than 10 years before. All the patients in the thymectomized group with normal SF activity (12 out of 62) had undergone surgery at least 1 year before blood collection.

*Lymphocyte subpopulation.* Table I shows the percentage of T, B, and non-T/non-B cells in these patient groups. The percentages of the different lymphocyte subpopulations were all within the normal range and comparable with those found in the non-Tx patients.

*Lymphocyte transformation test.* Lymphocyte responses to mitogens and alloantigens are shown in Table II. A normal response to mitogens was found in a) non-Tx patients, b) Tx patients with high SF activity, and c) patients who had under-

TABLE I

*Influence of adult thymectomy on the percentage of different lymphocyte subpopulations*[a]

| | T cells | B cells | Non-B/non-T cells |
|---|---|---|---|
| Normal donors | 69 ± 4.8 (n = 15) | 11 ± 0.6 (n = 15) | 20 ± 0.9 (n = 15) |
| MG non-thymectomized | 70 ± 1.6 (n = 30) | 9 ± 0.8 (n = 26) | 18 ± 1.9 (n = 18) |
| Thymectomized with low SF activity (total) | 68 ± 1.9 (n = 50) | 10 ± 0.8 (n = 26) | 20 ± 1.6 (n = 21) |
| <1 year after Tx | 66 ± 5.5 (n = 13) | 10 ± 1.2 (n = 6) | 20 ± 2.8 (n = 6) |
| 1-5 years after Tx | 68 ± 3.3 (n = 25) | 9 ± 1.1 (n = 8) | 21 ± 3.6 (n = 8) |
| 5-10 years after Tx | 75 ± 4.4 (n = 6) | 8 ± 1.0 (n = 4) | 18 ± 3.3 (n = 4) |
| >10 years after Tx | 65 ± 2.9 (n = 6) | 14 ± 0.9 (n = 3) | 21 ± 5.4 (n = 3) |
| Thymectomized with normal SF activity | 71 ± 4.1 (n = 12) | | |

[a] Results are expressed as mean percentage ± S.E. All patients were younger than 50 years.

TABLE II

*Influence of adult thymectomy on the lymphocyte response to mitogens, allogeneic cells and antigens*[a]

| | n | PHA | Con A | ALS | PWM | Allogeneic Cells (MLR) | Antigens | Age |
|---|---|---|---|---|---|---|---|---|
| Normal donors | 16 | 1.34 ± 0.40 | 0.97 ± 0.27 | 1.70 ± 0.62 | 1.22 ± 0.38 | 1.56 ± 0.57 | 0.53 ± 0.28 | 22 ± 2.0 |
| MG non-Tx | 32 | 1.34 ± 0.48 | 0.94 ± 0.38 | 1.61 ± 0.71 | 1.19 ± 0.71 | 1.30 ± 0.55 | 0.54 ± 0.46 | 28 ± 2.5 |
| Thymectomized patients | | | | | | | | |
| <1 yr | 8 | 1.53 ± 0.35 | 1.23 ± 0.31 | 1.85 ± 0.51 | 1.20 ± 0.56 | 1.18 ± 0.37 | 0.43 ± 0.25 | 24 ± 3.8 |
| 1-5 yr | 20 | 1.13 ± 0.38 | 0.82 ± 0.31 | 1.44 ± 0.58 | 0.99 ± 0.37 | 1.24 ± 0.45 | | 33 ± 2.0 |
| 5-10 yr | 8 | 0.86 ± 0.26[b] | 0.57 ± 0.19[b] | 0.96 ± 0.29[b] | 0.65 ± 0.37[c] | 0.99 ± 0.60[c] | 0.30 ± 0.26 | 31 ± 1.2 |
| >10 yr | 5 | 0.84 ± 0.30[b] | 0.58 ± 0.17[b] | 1.09 ± 0.37 | 0.62 ± 0.30[c] | 1.06 ± 0.30 | | 38 ± 3.9 |
| Thymectomized patients with high SF activity | 10 | 1.71 ± 0.60 | 0.94 ± 0.49 | 1.79 ± 0.63 | 1.14 ± 0.45 | 1.57 ± 0.52 | 0.34 ± 0.20 | 31 ± 4.1 |

[a] Lymphocyte reactivity is expressed as mean cpm ± S.D. × $10^{-4}$. All patients were younger than 50 years.
[b] Significantly decreased response compared with normal donors, non-Tx patients, Tx <1 year and Tx high SF activity; $p < 0.05$.
[c] Significantly decreased response compared with normal donors and Tx patients with high SF activity; $p < 0.05$.

gone thymectomy less than 1 year before testing. With the exception of the response to ALS, a decrease in the response ($p < 0.01$), correlated with the time after thymectomy, was observed for PHA, ConA, and PWM. Significantly lower responses to such mitogens were only found long after thymectomy.

No changes were found in the mitogenic responses of the lymphocytes of eight patients tested before and within a few months after thymectomy (data not shown).

At variance with Birnbaum and Tsairis (28), we observed no increased response to alloantigens in the MLR after thymectomy. We found normal responses in non-Tx patients and in the Tx patients with normal SF activity. The decline in the MLR response observed in the patients tested long after thymectomy did not reach significant levels (except for the patients 5 to 10 years post-Tx); furthermore, no difference was found in the stimulatory capacity of patients' lymphocytes before and after thymectomy (data not shown).

No significant change occurred in the anamnestic response to a mixture of several antigens, either in non-Tx patients or in Tx patients tested long after thymectomy. Nor were differences found in the patients tested before and after thymectomy.

*Cell-mediated lympholysis (CML).* The cytotoxic capacity of T lymphocytes sensitized to histocompatibility antigens were tested in the CML (Table III). A significantly lower response was found in patients thymectomized 5 or more years before testing ($p < 0.001$ with Tx group with high SF activity; $p < 0.01$ with non-Tx group, and $p < 0.02$ with Tx group tested less than 5 years after thymectomy.

*Antibody-dependent cytotoxicity.* The cytotoxic effector function of the lymphocytes was also tested in ADL of effector/target cell ratio from 2.5:1 down to 0.08:1. The cytotoxic capacity of the lymphocytes was not impaired at any of the ratios tested, even more than 10 years after thymectomy (e.g., effector/target cell ratio 2.5:1; net % $^{51}$Cr release ± S.E.: controls 59 ± 0.9; MG 51 ± 5.4; Tx <10 years 52 ± 7.8; Tx >10 years 42 ± 10.5).

TABLE III

*Influence of adult thymectomy on the cell-mediated lympholysis*[a]

| Donor | Effector/Target Cell Ratio | | | |
|---|---|---|---|---|
| | 0.625:1 | 1.25:1 | 2.5:1 | 5:1 |
| Normal donors (8) | 48 ± 5.7 | 57 ± 4.6 | 64 ± 3.3 | 65 ± 2.4 |
| MG non-Tx (32) | 48 ± 6.6 | 59 ± 7.3 | 65 ± 6.4 | 71 ± 6.7 |
| Tx low SF | | | | |
| 1-5 yr (20) | 30 ± 3.9 | 48 ± 5.4 | 56 ± 5.0 | 61 ± 4.7 |
| >5 yr (10) | 30 ± 6.5[b] | 38 ± 4.4[b] | 42 ± 4.5[b] | 44 ± 5.0[b] |
| Tx high SF (10) | 44 ± 7.4 | 61 ± 4.9 | 71 ± 4.7 | 73 ± 3.9 |

[a] Results are expressed as mean percentage of specific $^{51}$Cr release ± S.E. All patients were younger than 50 years.
[b] Significantly decreased response compared with normal donors, non-Tx patients and Tx high SF activity; $p < 0.01$ Fischer Transformation Test.

*Ig production in vitro.* Measurement of the Ig production *in vitro* was used to evaluate the helper function of T cells together with the ability of B cells to produce Ig. We found a mean IgM production of 197 ng/40 × $10^3$ lymphocytes (SE ± 54, n = 18) in the non-Tx patients and 253 ng/40 × $10^3$ lymphocytes (SE ± 69, n = 19) in the Tx patients. These values are not significantly different from those found in normal controls: 269 ng/40 × $10^3$ lymphocytes (SE ± 67, n = 12). No differences were found in the amount of IgM produced by lymphocytes of the different Tx patient groups (data not shown).

*DNCB skin test.* Primary immune response to DNCB was measured in 18 patients. All MG patients had a response to DNCB (mean score 2.8) lower than that of the controls. One out of seven non-Tx and two out of 11 thymectomized patients (post-thymectomy time 1 to 6 years) had a negative DNCB response (score lower than 2). No evidence for a decline of response to DNCB with time post-thymectomy was found.

TABLE IV

*Summary of the long-term effects of adult thymectomy on immunocompetence in human*

| Investigated Parameter | Effect of Tx |
|---|---|
| Percentage of: | |
| T cells | None |
| B cells | None |
| Non-B/non-T cells | None |
| Response to: | |
| PHA | Decreased |
| ALS | None |
| Con A | Decreased |
| PWM | Decreased |
| Allogeneic cells (MLR) | None |
| Cytotoxic capacity in: | |
| CML | Decreased |
| ADL | None |
| Production of immunoglobulins *in vitro* | None |
| Anamnestic response to antigens *in vitro* | None |
| Primary immune response to DCNB *in vivo* | None[a] |

[a] A response lower than that of the controls was found both in Tx and non-Tx patients with MG.

Table IV shows a summary of the influence of Tx on the parameters we investigated.

## DISCUSSION

It is generally accepted that the thymus plays a role in lymphocyte maturation before birth and undergoes involution during adult life (5–7, 29–32). In 1965, Taylor (33), Metcalf (34), and Miller (35) reported that adult thymectomy might also lead to changes in immunocompetence. Since then, several papers about the influence of adult thymectomy in animals and in humans have been published. However, because the results of these studies are rather conflicting, especially those performed in humans, we investigated immunocompetence in a large group of adult Tx MG patients. Results are summarized in Table IV.

Some investigators observed a decline in T cell numbers after thymectomy (28, 36–40). However, other authors failed to demonstrate such an effect (41–45). In our study we did not observe any difference in the percentage of B, T, and non-B/non-T cells. This might be due to the fact that our patients were tested at least 2 months after operation, and the reported fall in the number of lymphocytes was found to be only transient (36, 41). Scadding *et al.* (40) reported that a decreased percentage of E rosettes is found long after thymectomy; however, their values are not different from our findings ($68.3 \pm 9$ *vs* $65 \pm 7.1$), and both are within the normal range (46).

In contrast with the animal studies, mitogen-induced lymphocyte transformation has hardly been investigated in Tx humans. As reported for most of the animal studies (47–55), we also observed long after thymectomy a decreased response to some mitogens but not to others. This is in contrast with the findings of other authors (28, 40, 56), who did not observe differences in the responses to PHA in lymphocytes of Tx patients. The normal response to ALS may be explained by the observation that almost all T cells respond to this mitogen (57), whereas it is known that PHA, ConA, and PWM stimulate subpopulations of T cells. At variance with Birnbaum and Tsairis (28), we did not observe enhanced MLR response after thymectomy.

In contrast to the near-normal response in the MLR, the ability of cells primed *in vitro* to lyse allogeneic target cells in the CML decreased after thymectomy and was significantly lower in patients tested more than 5 years after thymectomy. Andersson *et al.* (47) reported that after adult thymectomy in mice, the killer cell precursors disappear sooner than the lymphocytes that react in the MLR. As far as we know, the dissociation we observed in the effects of adult thymectomy on the MLC and CML responses has not yet been reported for humans. This dissociation can be explained by a slow loss of precursors of cytotoxic T lymphocytes, a subpopulation that has been reported to be distinct from the lymphocytes responding in the MLR (58–61).

Kalden *et al.* (62) reported a decreasd ADL capacity of lymphocytes from patients with MG. In agreement with others (40), we observed no such impairment in the ADL capacity. No alteration of the ADL capacity was found after thymectomy, most likely because the cytotoxic lymphocytes, responsible for the lysis of antibody-coated target cells, are mainly non-T cells (63, 64), and this cytotoxic response is not regulated by T-helper and T-suppressor cells. No influence of adult thymectomy on the Ig production *in vitro* was found. These findings are in contrast with those from animal studies where decreased antibody production was measured long after thymectomy (33–35, 65), although test systems were used other than the one we used.

In agreement with Adner *et al.* (66) and Scadding *et al.* (40), we found no differences between Tx and non-Tx MG patients in the ability to respond to DNCB. We also noticed a significantly lower response in MG patients compared with normal donors. This may indicate that thymic function is not completely normal in MG as also suggested by the beneficial effect of thymectomy in MG (8).

It has been reported, both for animals (50, 65, 67–71) and for humans (28), that several parameters of immune competence change shortly after thymectomy, probably due to the disappearance of a small subpopulation of suppressor T cells with a short life span. However, other authors reported that this effect of adult thymectomy is only transient (47, 49, 72). Although we also found a slow disappearance of some lymphocyte functions after thymectomy, as shown by the diminished response to some of the mitogens and a lower cytotoxic capacity, we have no indication that the disappearing cells were suppressor cells. In addition, at variance with others (28), we found this lower response only long after thymectomy.

Because we found only small immunologic impairments also of primary immune response, after adult thymectomy, we have to conclude that most of the post-thymic T cells (i.e., cells that have been processed by the thymus) no longer require the presence of the thymus (at least for a period up to 20 years). This phenomenon might be explained by one of the three following possibilities: 1) There exists a population of self-maintaining post-thymic T-stem cells, which can generate T cells of every specificity; 2) another organ (e.g., spleen, lymph node) replaces some of thymic functions but not the humoral function of the thymus, as is reflected by SF, and either supplies T cells of every specificity or acts on prethymic bone marrow cells inducing their maturation to post-thymic T cells; 3) T cell clones with specificity for the whole repertoire of T-dependent antigens, generated in the presence of the thymus, do have a life span of at least 20 years, which is in agreement with findings indicating that small lymphocytes in human blood can have a rather long life span (73).

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