

Evidence for Distinct Mechanisms in the Shaping of the CD4 T Cell Repertoire in Histologically Distinct Myasthenia Gravis – Associated Thymomas

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The major histocompatibility complex (MHC) class II is involved both in thymocyte maturation and peptide presentation and might thus play a key role in the pathogenesis of paraneoplastic myasthenia gravis (MG) in thymomas. To further investigate this issue, we analyzed and scored the expression of epithelial class II expression in 35 thymomas (medullary, MDT; mixed, MXT; cortical and well differentiated thymic carcinoma, CT / WDTC) and correlated it with the histological tumor subtype, prevalence of MG and thymocyte maturation, which was analyzed by flow cytometry and RT-PCR. Our results show that both MHC class II expression and thymocyte maturation are highly dependent on the histological tumor subtype. CT / WDTC retain features of the normal outer thymic cortex, namely substantial MHC class II expression together with normal early thymocyte maturation until late phases of positive selection, but disturbed terminal thymopoiesis. By contrast, MDT and MXT retain features of the normal inner cortex and the medulla with low to absent class II expression and highly abnormal early thymocyte maturation including impaired positive selection, while terminal T cell maturation in MXT appeared undisturbed. There was no correlation between MHC class II expression and MG status for a given tumor subtype. In conclusion, our results provide evidence for a different histogenesis of cortical thymomas and well differentiated carcinomas on the one hand and mixed and medullary thymomas on the other.

Decreased expression levels of MHC class II, although of crucial importance for abnormal intratumorous maturation, are not sufficient to explain the emergence of paraneoplastic MG.

Keywords: Cortical, epithelium, medullary, MHC class II, Myasthenia gravis, Thymoma, Thymus

INTRODUCTION

Myasthenia gravis (MG) is a neurological disorder characterized by autoantibodies against the acetylcholine receptors at the neuromuscular junction, resulting in generalized muscle weakness. The clinical presentation of MG is almost invariably associated with

pathological alterations of the thymus (Marx et al. 1997). About 10 % of MG cases are related to thymic epithelial tumors (thymomas), which have been subdivided into medullary (MDT), mixed (MXT) and cortical thymomas (CT) and well differentiated thymic carcinomas (WDTC) based on the morphological resemblance of the neoplastic to normal thymic

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epithelium (Kirchner *et al.* 1992, Müller-Hermelink *et al.* 1994).

Thymomas characteristically retain organotypic features of the normal thymus, especially the capacity to generate mature T cells. Thus, a maintained but non-tolerogenic intratumorous thymopoiesis plus export of mature T cells from thymomas to peripheral lymphoid organs have been proposed to be a prerequisite for the development of paraneoplastic MG (Müller-Hermelink *et al.* 1997). However, although thymomas have been shown to be enriched in autoreactive T cells (Sommer *et al.* 1990; Nenninger 1998, Schultz 1999), the molecular basis of autoimmunization by thymomas remains largely enigmatic.

In recent years, some consistent features of these tumors have been described, including 1) intratumorous overexpression of autoantigen – related epitopes (Mygland *et al.* 1997; Wilisch *et al.* 1997, Schultz *et al.* 1999) and 2) impaired intratumorous thymopoiesis, particularly of the CD4 lineage (Takeuchi 1995; Nenninger 1997, 1998). Cells maturing in the thymus pass two critical checkpoints, positive and negative selection. During positive selection the thymic cortical epithelium presents an evolutionary optimized set of thymic peptides (Chan *et al.* 1993) bound to major histocompatibility complex (MHC) class I and II molecules to immature thymocytes. Depending on the MHC class recognized by the T cell receptor (TCR), either CD4 or CD8 are engaged and help to further increase the surface expression of the TCR and CD3 (Davis *et al.* 1993). If the resulting avidity reaches a threshold level, the cell receives a survival signal. However, if the resulting avidity is too high, the cell is eliminated through apoptosis, a mechanism termed negative selection (Bevan 1997; Marrack and Kappler 1997; Williams *et al.* 1997; Jameson and Bevan 1998). Only cells surviving these two MHC-dependent checkpoints are allowed to complete maturation and leave the thymus (Janeway and Travers 1994). Although the MHC is thus critically involved in both thymocyte maturation and antigen presentation, it has not been clarified whether abnormal MHC expression levels are a consistent feature of thymomas (Willcox *et al.* 1987, Takeuchi *et al.* 1995, Nenninger *et al.* 1997) and whether alterations of MHC levels are

related to abnormal thymopoiesis. Therefore we tried to further dissect the role of this molecule in intratumorous thymocyte maturation and in the pathogenesis of paraneoplastic MG.

RESULTS

Reduced Epithelial Expression of MHC Class II is a Consistent Feature of Thymomas, but Does Not Correlate with the Presence or Absence of Myasthenia Gravis

A reduced expression of epithelial HLA-DR at least in a subset of MG-associated thymomas has been described previously (van der Kwast 1985, Chilosi *et al.* 1986, Willcox *et al.* 1987, Takeuchi *et al.* 1995, Nenninger *et al.* 1997). However, there were no conclusive data on the prevalence and extent of this alteration and its significance for autoimmunity. In this study, we scored the expression of HLA-DR, DP, DQ and invariant chain (Ii) semiquantitatively (0 to 3 points for negative, weak, intermediate and strong staining intensity of each isotype), and calculated the cumulative MHC class II expression level (MHC-score) in a given case as the percentage of the maximal possible score.

Compared with adjacent thymic remnants and control thymuses, in which the average MHC-score was 88,5 %, class II expression was reduced in all instances regardless of the histological tumor subtype in a series of 35 thymoma cases. However, class II expression was lowest in those tumor types in which the prevalence of MG was lowest, namely medullary (average MHC-score 4 %, prevalence of MG 15,4 %) and mixed thymomas (average MHC-score 0,96 %, prevalence of MG 56 %). On the other hand, class II was significantly higher in those tumor types with high prevalence of MG, namely cortical thymomas and well differentiated thymic carcinomas (average MHC-score in cortical thymomas 21,2 %; prevalence of MG 75,8 %; average MHC-score in well differentiated thymic carcinomas 24,5 %; prevalence of MG 84,2 %) (Tab I). Within a thymoma subtype (e.g. among cortical thymomas), there was no correlation between the MHC-score and the presence or absence

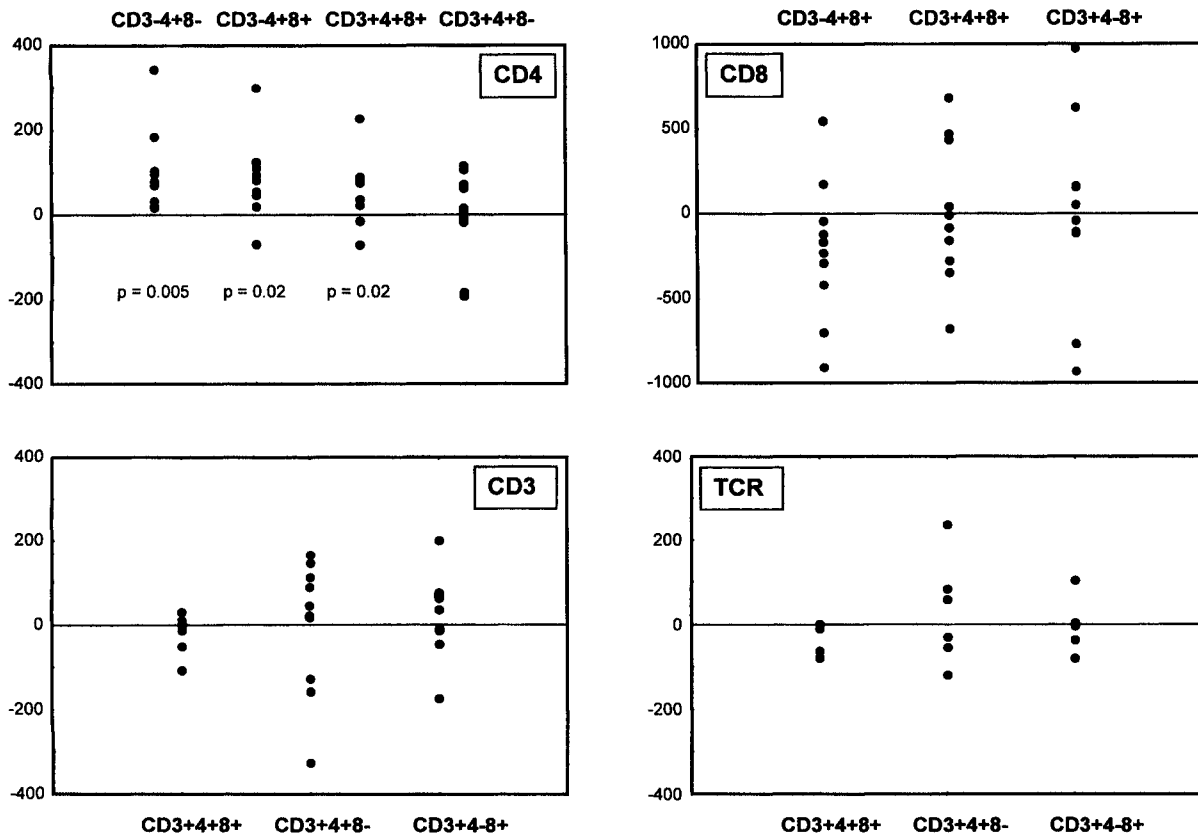


FIGURE 1 Intrapersonal comparison of median surface expression levels of CD4, CD8, CD3 and T cell receptor $\alpha\beta$ at different stages of thymocyte maturation in thymomas (flow cytometry). Dots indicate the difference between the value of the thymoma and the residual thymus of one individual patient. (4 mixed and 8 cortical thymomas / well differentiated carcinomas, 12 residual thymuses). As there were no statistically significant differences between mixed and cortical thymomas, all tumors were compared to the residual thymuses. In thymomas, surface CD4 was significantly increased on immature, pre-selective thymocytes, while there was no alteration in the surface expression of CD8, CD3 and the $\alpha\beta$ T cell receptor

of MG. Of note, there were no differences in the staining intensity of MHC class I isotypes between thymomas and residual and control thymuses. These

findings are in good agreement with earlier reports on reduced levels of MHC class II, but not class I, in thymoma epithelial cell lines (Papadopoulos et al. 1989).

TABLE I Histological subtypes and prevalence of myasthenia gravis in 164 thymoma cases and results of immunohistochemical analysis of MHC class II isotypes in 35 cases with available frozen material

Thymoma type	cases	MG+ (%)	IHC (MG+ / MG-)	MHC-II positive cases (MG+ / MG-)
medullary	13	2 (15,4)	5 (1 / 4)	1 (0 / 1)
mixed	41	23 (56)	8 (3 / 5)	1 (1 / 0)
cortical	91	69 (75,8)	10 (7 / 3)	5 (2 / 3)
well differentiated thymic carcinomas	19	16 (84,2)	12 (12 / 0)	7 (7 / 0)

MG: myasthenia gravis; IHC: cases available for immunohistochemical analysis of MHC class II isotypes, MHC-II positive cases (by IHC): MHC-score > 0 %.

Immature, Pre-Selective T Cells In Thymomas Show Selective Upregulation of Surface CD4

CD4 plays a crucial role in positive selection by enhancing the intrinsically low avidity of the TCR for its ligands (McCarthy *et al.* 1988; Zuniga-Pflucker 1989; Dutz *et al.* 1995, Groves *et al.* 1997). In consequence, any alteration of either the surface expression of MHC or the TCR that would affect the TCR:MHC interaction would also require adjustment of the CD4 level in order to achieve a survival signal. Therefore, we compared the median expression levels of CD4, CD8, CD3 and the $\alpha\beta$ T cell receptor on different thymocyte subsets in thymomas and residual thymuses by flow cytometry (Fig. 1).

Corresponding to the highly decreased expression of MHC class II on epithelial cells, thymoma-derived immature T cell populations of all histological tumor subtypes showed a significant upshift of surface CD4 compared to cells derived from residual and control thymuses. In particular, CD4 expression was significantly increased on CD3-4+8-, CD3-4+8+ and CD3+4+8+ cells, while this upshift was no longer detectable on mature CD3+4+8- cells. The MHC-class specificity of this alteration was underlined by the fact that CD8 surface expression did not show statistically significant alterations. Moreover, surface levels of CD3 and TCR $\alpha\beta$ were virtually identical in thymomas and residual thymuses.

The Shaping of the Mature CD4 T Cell Repertoire in Cortical Thymomas May Involve Other Factors than in Mixed Thymomas

It has been previously reported that thymocyte maturation in thymomas is quantitatively disturbed and that numbers of mature CD4 cells are significantly reduced, while the percentage of mature CD8 cells remains unremarkable (Takeuchi *et al.* 1995, Nenninger *et al.* 1997, Nenninger *et al.* 1998). As this constellation seemed to be related to the reduced MHC class II expression levels in thymomas, we tried to correlate our findings on subtype-specific reduction of class II with quantitative alterations in thymocyte maturation, which were assessed by three-colour flow

cytometry. As the pathogenesis of MG in medullary thymomas has been considered to be linked to the particularly high numbers of recirculated T cells found in these tumors (Nenninger *et al.* 1997), they were not included in this study. In mixed thymomas, MHC class II expression was generally completely lost on the sensitivity level of immunohistochemistry. Although these tumors displayed an abundant production of immature CD3-4+8- and CD3-4+8+ precursor cells as reported previously (Takeuchi *et al.* 1995, Nenninger *et al.* 1997, Nenninger *et al.* 1998), most cells apparently were not able to enter into positive selection and were lost during transition into the CD3+4+8+ stage (Fig. 2). This finding is in good agreement with the particularly low levels of MHC class II in these tumors, as T cell development is known to be blocked at this stage also in the thymus of MHC I° II° mice (Chan *et al.* 1993, Grusby *et al.* 1993) and in patients with MHC class II deficiency (bare lymphocyte syndrome, BLS) (van Eggermond *et al.* 1993). However, the few cells which entered into positive selection seemed to complete maturation towards the pre-emigrant stage successfully. This suggests that positive selection might be the most important single determinant in the shaping of the mature thymocyte repertoire in mixed thymomas.

By contrast, cortical thymomas and well differentiated thymic carcinomas in many cases expressed substantial amounts of variable class II isotopes. Although positive selection was also impaired in CT / WDTC, it seemed to be better preserved and the numbers of CD69+ cells were significantly higher than in mixed thymomas. However, unlike in the normal thymus and also in mixed thymomas, a major proportion of these positively selected cells was eliminated before reaching the pre-emigrant CD3+4+8- stage, downsizing the frequency of CD3+4+8- cells to that of mixed thymomas. Considering that epithelial MHC class II is not required during terminal thymocyte maturation (Vanhecke *et al.* 1997), our findings suggest that factors independent of epithelial MHC class II play a major and qualitatively abnormal role in the shaping of the mature T cell repertoire in cortical thymomas and well differentiated carcinomas, but not in mixed thymomas as compared to normal thymus.

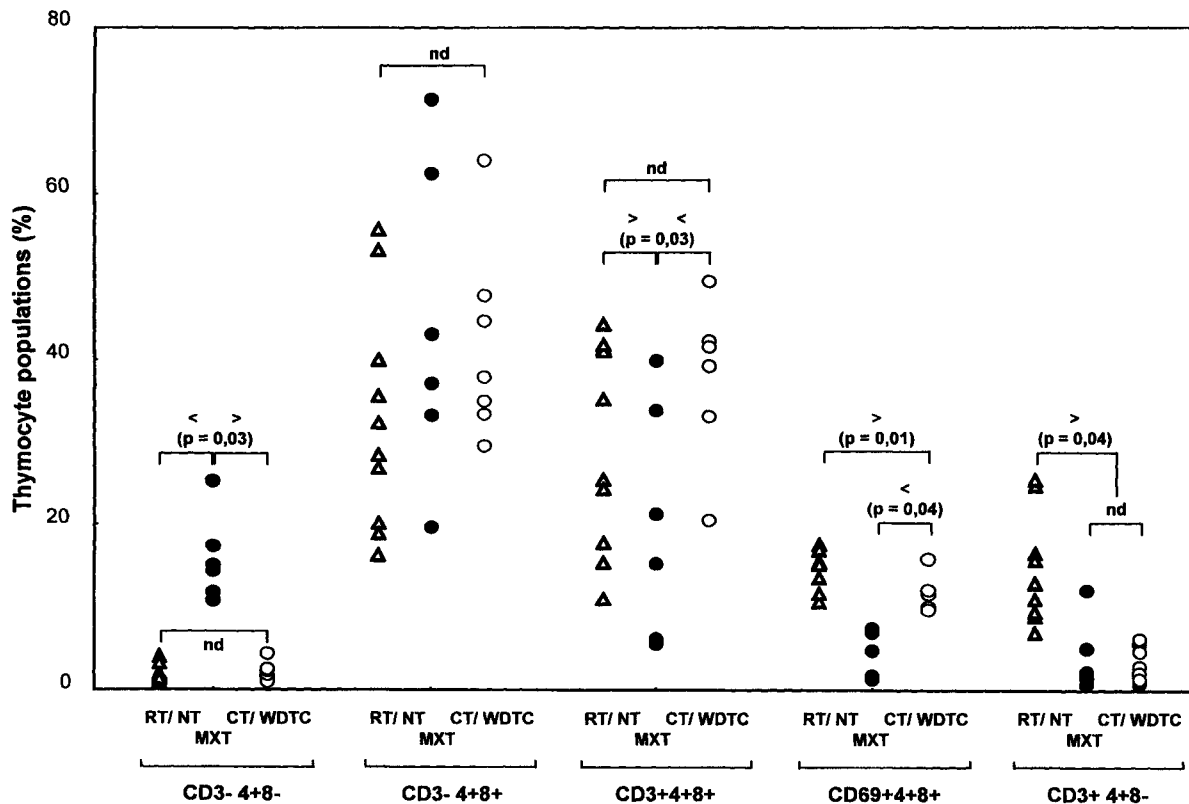


FIGURE 2 FACS analysis of main T cell subsets revealed significant differences in thymocyte maturation between mixed (MXT) and cortical thymomas / well differentiated carcinomas (CT / WDTC). In mixed thymomas, the majority of cells was lost before entry into positive selection, i.e. at the transition from the CD3-4+8+ to the CD3+4+8+ stage. However, the few positively selected (CD69+4+8+) cells in these tumors seemed to reach the mature CD3+4+8- stage. In cortical thymomas and well differentiated carcinomas, the numbers of positively selected cells were significantly decreased but still roughly comparable to residual and control thymuses (RT / NT). However, most of these cells were obviously eliminated before reaching the CD4^{int} stage. Maturation of the CD8 lineage in all thymomas was undistinguishable from control thymuses (not shown). (nd: not statistically different)

Reduced MHC Class II Expression in Thymomas Does Not Skew the T Cell Receptor Repertoire of Mature CD4 Cells

The interaction of the TCR with peptide / MHC class II complexes has been reported recently to play a predominant role in the shaping of the mature TCR repertoire (Sant'Angelo et al. 1997). To investigate the impact of reduced class II expression on the relative frequencies of TCR V β gene usage, we applied RT-PCR with a panel of 24 TCR V β family primers on highly purified, mature CD4 cells isolated from thymomas, residual thymuses and peripheral blood of the same patients. Medullary thymomas were

excluded from this study for the same reasons as stated above. The overall TCR repertoire was not qualitatively affected neither in mixed nor in cortical thymomas and was not significantly different from residual thymuses and peripheral blood. Among the most prevalent families were TCR V β 5, 6, 8 and 13, all of which have been reported to be the predominant TCR V β segments also in normal (Baccala et al. 1991, Jores and Meo 1993) and non-neoplastic MG-associated thymuses (Truffault et al. 1997; Navaneetham et al. 1998). Minor, statistically non-significant alterations in the peripheral blood were attributed to confounding factors in this compartment, such as

nonrandom homing to lymphatic tissues, different life spans etc. (Jores and Meo 1993). The analysis of CDR 3 length polymorphism did not reveal any differences between thymomas and residual and control thymuses. Thus, obviously neither intratumorous clonal deletions nor expansions do occur, as inferred from earlier studies based on TCR rearrangement studies (Katzin *et al.* 1988; Tesch *et al.* 1989; Scarpa *et al.* 1990).

DISCUSSION

In this paper, we tried to assess the effects of reduced epithelial MHC class II on thymocyte maturation and the development of paraneoplastic myasthenia gravis. Our results show that reduced class II expression is a general and consistent feature of thymomas. However, both the extent of this reduction and its effects on thymocyte maturation appear to depend on the histological thymoma subtype.

Unlike in other tumors, where downregulation of MHC is a common finding with tumor progression (Vegh *et al.* 1993; Lefebvre *et al.* 1999), class II reduction was most pronounced in medullary and mixed thymomas, which show a biologically benign behaviour (Marx *et al.* 1999). Moreover, the selective downregulation of class II isotypes pointed to one or several defects in the pathways that regulate class II expression, such as INF- γ , STAT1 / IRF-1, or CIITA (Darnell 1997; Piskurich *et al.* 1999) or any other of the involved regulatory factors or, alternatively, overexpression of suppressive factors, such as TGF- β (Piskurich *et al.* 1999). At the same time, medullary and mixed tumors show the lowest prevalence of myasthenia gravis among all organotypic thymomas, indicating that downregulation of MHC class II *per se* is not sufficient to explain the development of autoimmunity.

The effects of reduced class II expression on thymopoiesis seemed to be both qualitative and quantitative. Although maturation of the CD4 lineage was highly impaired, it was not altogether abrogated and thymomas always contained substantial numbers of phenotypically mature, pre-emigrant CD4 cells. This

finding was further confirmed by the fact that the T cell receptor repertoire of highly purified pre-emigrant CD4 cells isolated from both mixed and cortical thymomas and well differentiated carcinomas displayed the same restriction pattern as T cells isolated from control thymuses.

In qualitative terms, upregulation of surface CD4 was equally observed in both mixed and cortical type thymomas and suggested that immature thymocytes indeed reacted to the altered microenvironment. While CD4 seems to play only a minor role in positive selection in the presence of wild-type peptide repertoires (Takahama *et al.* 1994a; van Bergen *et al.* 1998), it has great effects on the recognition of altered, suboptimal ligands (Vignali and Strominger 1994, Madrenas *et al.* 1997, van Bergen *et al.* 1998). Decreased MHC expression by itself may restrict the wild-type self-peptide repertoire, as only dominant peptide:MHC complexes are displayed, while minor complexes fall below the threshold level required for positive selection (Grubin *et al.* 1997) (Fig. 3). This effect is the more important, as the self-peptide repertoire in thymomas is most probably biased and not identical with the wild type repertoire (Mygland *et al.* 1997; Wilisch *et al.* 1997, Schultz *et al.* 1999). Upregulation of CD4 on maturing thymocytes in thymomas might lead to the irregular positive selection of autoreactive precursors that would otherwise not have received a survival signal. As the affinity required for positive selection lies threefold below the affinity for a signal inducing negative selection (Alam *et al.* 1996), it is conceivable that positively selected, autoreactive T cells might escape thymic deletion (Fig. 4).

In quantitative terms, reduction of class II expression corresponded with marked numerical alterations of thymocyte subpopulations, again with subtype-specific variations.

In mixed thymomas, the observed subtotal loss of epithelial class II expression corresponded with a strong reduction of thymocyte numbers between the CD3-4+8+ and the CD4+8+69+ stage, indicating impaired initiation of positive selection in this tumor type. Indeed, the capacity to induce positive selection has been ascribed uniquely to MHC class II+ thymic epithelial cells (Anderson *et al.* 1994 and 1999; Ernst

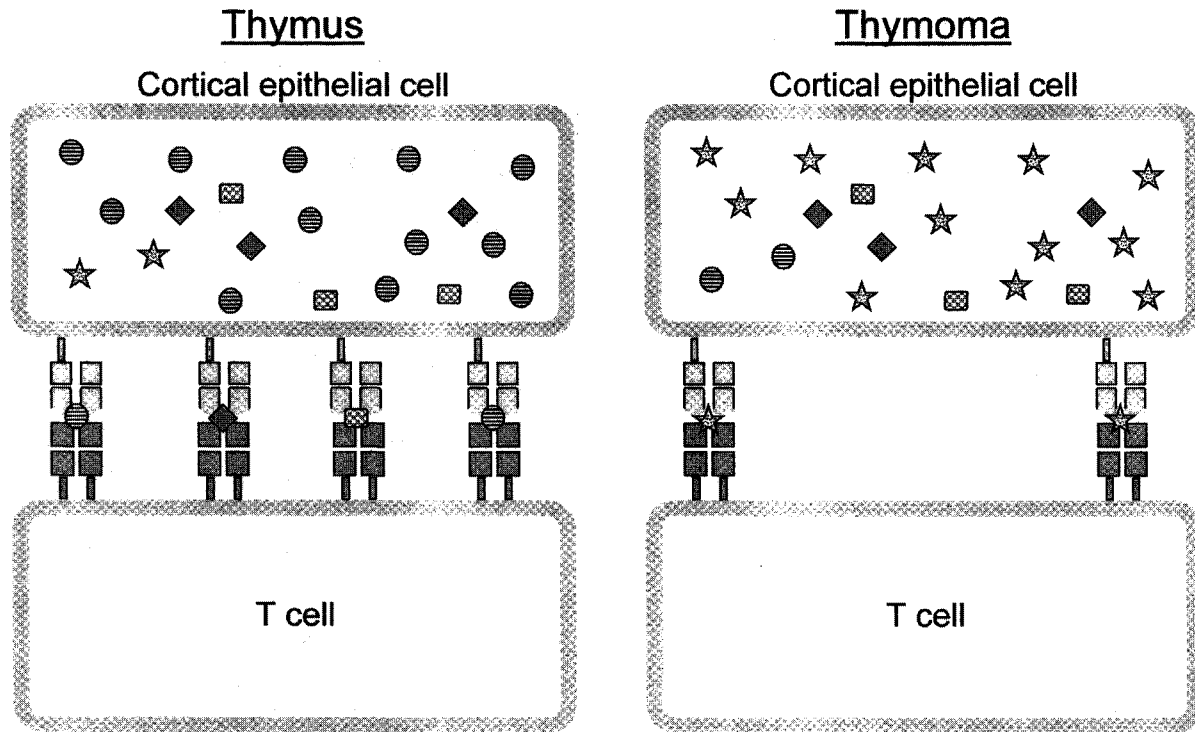


FIGURE 3 Hypothetical impact of reduced epithelial class II expression on peptide presentation: only frequent peptides are displayed, while minor peptides fall below the threshold level required for positive selection (Grubin et al. 1997). Moreover, due to overexpression of tumor-related antigens, the peptide repertoire presented by epithelial cells in thymomas is most probably biased and not identical with the wild-type repertoire

et al. 1996). However, MHC-independent factors, such as TGF- β , have also been reported to be rate-limiting in the maturation of CD4+CD8+ thymocytes in both mice (Takahama et al. 1994b, Plum et al. 1995) and humans (Mossalayi et al. 1995). Interestingly, TGF- β has also been found to be involved in negative regulation of MHC class II (Lee et al. 1997; Nandan et al. 1997). However, the few cells that reached the CD4+8+69+ stage in mixed thymomas seemed to complete maturation successfully, suggesting that positive selection might be the most important single determinant in the shaping of the mature thymocyte repertoire in mixed thymomas.

By contrast, in line with the generally higher MHC class II levels observed in cortical thymomas and well differentiated thymic carcinomas, positive selection seemed to be better preserved in these tumors and the

numbers of CD69+ cells were significantly higher than in mixed thymomas. However, unlike in the normal thymus and also in mixed thymomas, a major proportion of these positively selected cells was eliminated before reaching the pre-emigrant CD3+4+8- stage, downsizing the frequency of CD3+4+8- cells to that of mixed thymomas. We conclude from these findings that the initiation of positive selection in thymomas is crucially dependent on the expression of epithelial MHC class II. Although terminal maturation seems to be possible in the absence of MHC (Vanhecke et al. 1997), functional maturation still requires an intact thymic microenvironment (Dyall and Nikolic-Zigic 1995). Our findings thus suggest that factors independent of epithelial MHC class II play a major and qualitatively abnormal role in the shaping of the mature T cell repertoire in cortical thy-

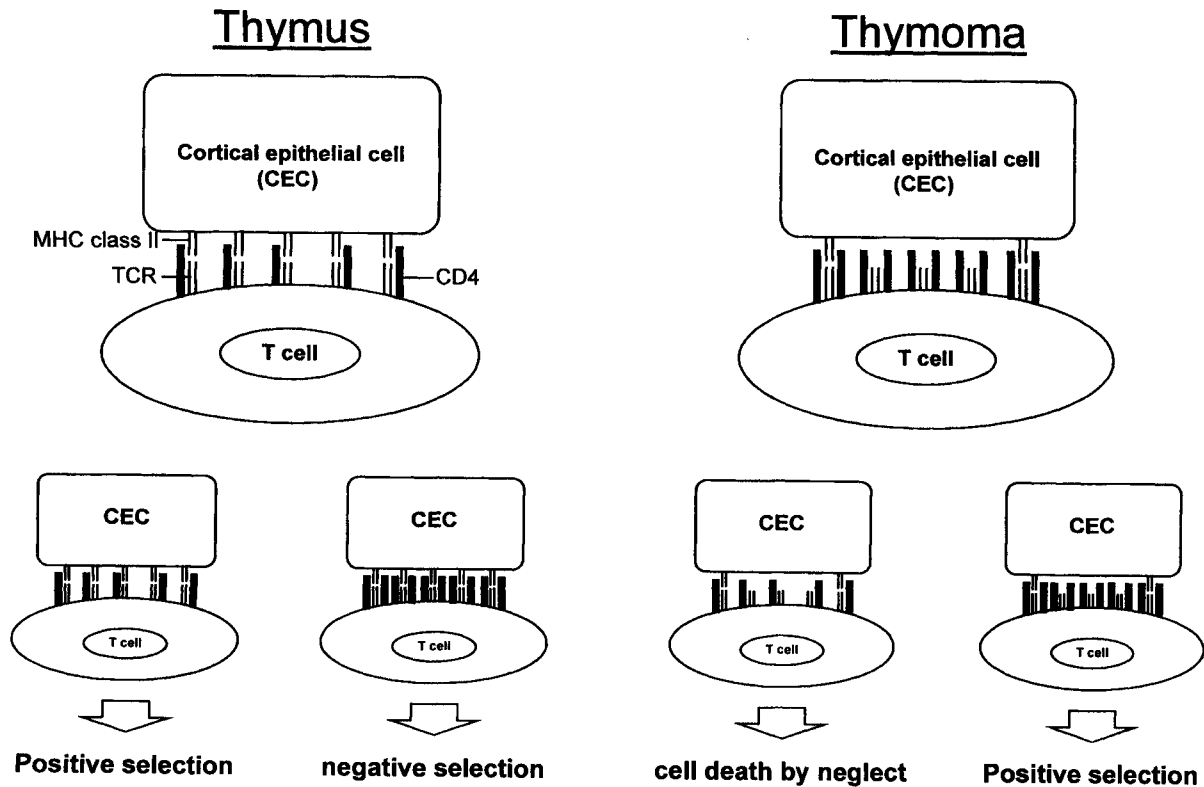


FIGURE 4 Potential selection advantage for pre-selection thymocytes with increased surface CD4 levels in thymomas with decreased MHC class II expression. In a microenvironment with low epithelial class II levels, upregulation of CD4 could convey a positively selecting signal, which under normal conditions would lead to the elimination (negative selection) of the cell. As the affinity required for positive selection is well below the affinity inducing negative selection, positively selected, autoreactive T cells might escape thymic deletion. Of note, CD4 upregulation was measured as median surface level and thus only a subset in a given thymocyte population in thymomas showed this alteration

thymomas and well differentiated carcinomas, but probably not in mixed thymomas as compared to normal thymus. Interestingly, we have previously reported on an relative stromal overexpression of B7, a costimulatory molecule involved in negative selection (Kishimoto *et al.* 1996; Amsen and Kruisbeek 1996), in cortical thymomas (Marx *et al.* 1994). Other factors influencing the shaping of the CD4 lineage at the post-selection stage could be the 1) action of dendritic cells (Robey and Fowlkes 1994), 2) lack of MHC-independent survival signals such as LFA 1 and 3 / ICAM (Jorgensen *et al.* 1996), or VCAM (Salomon *et al.* 1997), or 3) abnormally accelerated export of mature T cells into the peripheral compartment.

In summary, both MHC class II expression and thymocyte maturation are highly dependent on the histological tumor subtype. CT / WDTC retain features of the normal outer cortical epithelium, namely substantial MHC class II expression together with unremarkable thymocyte maturation until late phases of positive selection. By contrast, MDT and MXT show characteristics of the inner cortical / medullary epithelium with low to absent class II expression (von Gausecker *et al.* 1986) and highly abnormal early thymocyte maturation including impaired positive selection, while terminal T cell maturation appears undisturbed.

Our results thus provide further evidence for a different histogenesis of cortical thymomas and well differentiated carcinomas on the one hand and mixed and medullary thymomas on the other, as previously inferred from morphological observations (Müller-Hermelink et al. 1994).

While MHC class II expression and initiation of positive selection may be the central factors in the shaping of the T cell repertoire in mixed thymomas, positive selection is better preserved in cortical thymomas and well differentiated thymic carcinomas and additional, MHC-independent mechanisms seem to be predominant in these tumors. However, even heavy disturbances of thymocyte maturation and selection do not involve the T cell receptor repertoire of the few resulting pre-emigrant CD4 cells, at least as far as V β gene usage and CDR3 length polymorphism are concerned. Our failure to significantly correlate MHC II levels with MG status suggests that reduced epithelial MHC II expression by itself is not sufficient to explain the emergence of paraneoplastic myasthenia gravis.

MATERIALS & METHODS

Patients and Tumors

The tumors were subtyped according to the classification of Müller-Hermelink and coworkers (Kirchner et al. 1992; Müller-Hermelink et al. 1997). Cortical thymomas (CT) and well differentiated thymic carcinomas (WDTC), though morphologically distinct, are closely related in functional terms (Nenninger et al. 1997, 1998; Schultz et al. 1999). Therefore, they were considered as one group (CT / WDTC) to be compared with mixed thymomas (MXT). The diagnosis of MG was based on clinical findings and the detection of anti-AChR autoantibodies in the patient's serum.

Immunohistochemical Detection and Semiquantitative Scoring of MHC Class II Isotypes

MHC class II epitopes were detected on frozen sections by a three-step immunoperoxidase technique as

described in detail previously (Kirchner et al. 1988). The primary antibodies used were: L243 recognizing a complex nonpolymorphic epitope on mature HLA-DR dimers (Shackelford et al. 1983), Tü36 recognizing a complex epitope on mature and immature (invariant chain (Ii)-associated) HLA-DR dimers and Tü22 recognizing a monomorphic determinant on HLA-DQ (Klohe et al. 1988). FA (B7/21) recognizes a complex epitope on HLA-DP dimers (Klohe et al. 1988) and SD3 253.74 recognizes a simple C-terminal epitope of the invariant chain (Ii) (amino acids 194–209) (Max et al. 1993). All primary antibodies provided by Dr. H. Kalbacher, Institute of Physiological Chemistry, Tübingen, Germany). Epithelial cells in thymomas and thymuses were identified using a monoclonal antibody against cytokeratin 19, which is known to be highly expressed in all subtypes of thymomas (Grommisch et al. 1997). Other MHC class II expressing cell types, such as B cells, macrophages and dendritic cells, were identified using monoclonal antibodies against CD22 (Dako, Hamburg, Germany), CD 16 (Dianova, Hamburg) and CD11c (KiM1) (Bachem Biochemica, Heidelberg, Germany), respectively. MHC class I expression was analyzed using an anti-human HLA A, B, C monoclonal antibody (Dako-HLA-ABC, Dako, Hamburg, Germany). Intensity of MHC class II staining was assessed using a semi-quantitative point score (0 to 3 points for negative, weak, intermediate and strong staining intensity, respectively). Staining intensity was expressed as percent of maximal possible score in a given case.

Flow Cytometry

Lymphocytes were immunophenotyped by three-color FACS analysis on a Becton Dickinson FACScan flow cytometer using the following monoclonal antibodies: isotype controls, anti-CD3, anti-CD4 (Quantum red labeled) (Sigma, Deisenhofen, Germany), anti-CD45RO (unconjugated), anti-CD25 (PE-labeled), anti-CD19 (FITC-labeled) (Dako, Hamburg, Germany), PE-conjugated F(ab')₂ fragment donkey-anti-mouse IgG, anti-HLA-DR (PE-labeled) (Dianova, Hamburg, Germany), anti-CD45RA (FITC-labeled) (Coulter Immunotech;

Hamburg, Germany) and anti-TCR $\alpha\beta$ (FITC-labeled) (Becton Dickinson, Heidelberg, Germany).

Semiquantitative RT-PCR

Freshly isolated CD3⁺4⁺8⁻ cells from native thymomas, residual thymuses and peripheral blood were isolated by a combination of depletion/selection procedures using cell isolation columns (Camon Labor Service, Wiesbaden, Germany) and immunomagnetic Beads (DynaL, Hamburg, Germany). The purity of the isolated subsets was controlled by FACS staining and was generally $\geq 95\%$. Total RNA was prepared from purified CD4^m (10^6) cells using the RNeasy Kit (Qiagen, Hilden, Germany). After cDNA synthesis with oligo-dT primers and MMLV reverse transcriptase (Gibco, Eggenstein, Germany), 1/20 of the reaction was amplified using Taq polymerase (Amersham, Braunschweig, Germany) and sequence-specific primers. A semiquantitative PCR was performed by adjusting all cDNAs to equal amounts of GAPDH transcripts. The T cell receptor V β (TCR V β) repertoire of a given sample was investigated using a panel of 24 TCR V β -specific primers and a fluorescent TCR C β -specific consensus primer as described previously (Genevee *et al.* 1992).

Quantitation was performed through digitalization of ethidium bromide-stained agarose gels and subsequent analysis using ONE-Dscan software (Scanalytics, Billerica, USA). Usage of a single TCR V β segment was expressed as percentage of the total product amplified by all the V β primers.

Evaluation of CDR3 Length Polymorphisms

Aliquots of the run-off products were subjected to electrophoresis using an ABI 373A sequencer (Applied Biosystems, Weiterstadt, Germany) in the presence of fluorescent ABI Rox-2500 marker. Evaluation of the CDR3 length polymorphisms was performed using the GenScan software version 1.2.2.

Statistical Procedures And Data Plotting

Analysis of the flow cytometric data and creation of histograms were performed using the Lysis II soft-

ware (Becton Dickinson). The density of different surface molecules was determined by using the median value provided by the software.

For all statistical analyses, data were computed using the Statistica software (StatSoft, Tulsa, USA) and tested for significant differences using the Mann-Whitney-U test (p value < 0.05, unless otherwise specified).

In this article, thymomas were subtyped according to the classification proposed by Müller-Hermelink *et al.* The thymoma subtypes used here correspond to the new World Health Organization classification, which distinguishes type A thymomas (medullary), type AB thymomas (mixed), type B thymomas (subclassified as type B1, predominantly cortical; B2, cortical; and B3, well differentiated thymic carcinomas) and type C thymomas (thymic carcinomas) (Rosai and Sobin 1999).

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