Characterization of the Human Thymic Microenvironment: Lymphoepithelial Interaction in Normal Thymus and Thymoma

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Summary. Recent advances in tissue culture technology and molecular biology have extended our understanding of the functional morphology of the thymus. The importance of a crosstalk between lymphoid cells and stroma has been appreciated as a prerequisite for the normal development of both. The network of direct cellular interactions and soluble factors comprising part of the microenvironment is far from being elucidated but the highly ordered thymic architecture clearly plays a pivotal role in normal thymic function. Insight into the genetic control of stroma development is only emerging while knowledge on the genetic control of the various steps in T cell development is already advanced and rapidly expanding.

The present paper gives an overview on the cellular components and matrix molecules of the *human* thymic microenvironment and their development during ontogeny. The intrathymic cytokine network is shortly reviewed. Special emphasis is put on molecules mediating lymphoepithelial interactions that are necessary for the expansion and early selection of immature thymocytes from precursor cells and for the generation of an MHC restricted and self tolerant T cell repertoire by positive and negative selection. Considering these physiological mechanisms we summarize the molecular pathology of the microenvironment and lymphocyte/stroma interactions in thymic epithelial tumors (thymomas). Finally, a pathogenetic model for paraneoplastic myasthenia gravis is given. We suggest abnormal autoantigen-specific positive selection of naive T cells as the essential molecular mechanism by which thymomas contribute to the autoimmunization against the acetylcholine receptor and other muscle proteins.

The microenvironment of the normal human thymus is provided by a complex network of stromal elements: epithelial and mesenchymal cells and the extracellular matrix. To be optimally functional for thymopoiesis the components of the microenviron-

ment are arranged to form a unique architecture with distinct subcapsular, cortical and medullary compartments confined and partially separated by the capsule, interlobular septae and perivascular spaces. Within the microenvironment thymopoiesis requires communication between thymocytes and stroma by receptor/ligand mediated cell-cell and cell-extracellular matrix contacts, interleukines and possibly neuropeptides to provide an MHC-restricted and self-tolerant T cell repertoire. Thymomas are epithelial tumors that retain thymus-like features and exhibit an unrivaled frequency of paraneoplastic autoimmune phenomena among human neoplasms. The neoplastic transformation of the epithelial component in thymomas is associated with abnormalities of the microenvironment concerning architecture, cellular composition and thymocyte/stroma interactions. Microenvironmental abnormalities result in an abnormal intra-tumorous T cell development and have been considered to contribute to the pathogenesis of paraneoplastic autoimmunity, particularly myasthenia gravis. Here we focus on recent findings concerning interactions between epithelial cell and thymocytes in the normal human thymus and in thymomas and suggest a molecular mechanism explaining why thymoma-associated thymopoiesis is inefficient and might be non-tolerogenic with respect to a limited number of autoantigens.

Architecture and Constituents of the Microenvironment of the Normal Human Thymus

The normal thymic microenvironment consists of nonlymphoid stromal cells and the extracellular matrix. The majority of stromal cells are epithelial

cells derived from the endoderm of the 3rd pharyngeal pouch and the cervical vesicle that develops from the 2nd, 3rd and 4th branchial cleft ectoderm (VON GAUDECKER, 1986). In humans thymic primordia are first present at the 7th week of gestation (HAYNES, 1984) with a distinct organization into a peripheral and central cell layer at the 8th week of gestation (VON GAUDECKER and MÜLLER-HERMELINK, 1980). Thymic mesenchymal cells are derived from the embryonic mesoderm forming the capsule, interlobular septae and vessels (BOYD et al., 1993). As shown in the mouse, both epithelial and mesenchymal elements are necessary for T cell development (ANDERSON et al., 1993, 1994).

The first T cell progenitors colonize the human thymus at the 8th week of gestation (VON GAUDECKER, 1986). The nature of the T cell progenitor colonizing early human thymic primordia and adult thymuses has not yet been resolved (reviewed by SPITS et al., 1995). At present experimental findings favor the view that the earliest progenitor in humans is not the pluripotential stem cell typical of bone marrow but a multipotential committed stem cell with a $CD34^+ CD38^{low} CD45RA^+ Thy1^-$ phenotype and the potential

to give rise to T and natural killer (NK) cells (SANCHEZ et al., 1994; SPITS et al., 1995) and dendritic cells in mice (ARDAVIN et al., 1993) and man (RES, 1996). Colonization of the thymus primordia by T cell progenitors is necessary for the further development of the thymic microenvironment including the compartmentalization into cortex and medulla and the development of Hassall's corpuscles as detectable at the 15th week of gestation (HAYNES, 1984; HAYNES and HEINLY, 1995). The necessity for colonization is obvious in patients with particular forms of severe combined immunodeficiency (SCID) and SCID mice that exhibit lymphocyte-poor dysplastic thymic rudiments devoid of distinctive medullary areas and Hassall's corpuscles (NEZELOF, 1992). "Dysplasia" in almost all these cases does not arise from an epithelial dysfunction but from various intrinsic T cell defects (see below). As a consequence normal thymic architecture and function is achieved after wild type bone marrow transplantation. These findings support the concept that a cross-talk between thymocytes and epithelial cells is necessary for thymopoiesis and differentiation of the microenvironment though not for its initial formation (RITTER and BOYD, 1993; VAN

Fig. 1. Model of early T cell development in the human thymus as defined by multicolor FACS analysis (SPITS et al., 1995).

EWIJK et al., 1994; KISIELOW and VON BOEHMER, 1995; NEHLS et al., 1996)

The epithelial cells of the thymic microenvironment are very heterogeneous (BOYD et al., 1993). Distinct cell types occur in the subcapsular area, cortex proper, medulla and Hassall's corpuscles (Fig. 1). By electron microscopy six types (type 1-6) of epithelial cells have been discriminated (VAN DE WIJNGAERT, 1984) considering shape and electron-lucency. The subcapsular type 1 epithelial cells that comprise the vast majority of cells with neuroendocrine features (DARDENNE and SAVINO, 1994; MAG-GIANO et al., 1994) are usually MHC (major histocompatibility complex) class II negative (VON GAUDECKER, 1986). By contrast type 2-4 (cortical) and type 5 and 6 (medullary) epithelial cells are MHC class I and II positive (BOFILL et al., 1985; VON GAUDECKER, 1986). Thymic nurse cells (TNCs) (WEKERLE and KETELSEN, 1980) in humans are specialized type 2 or 3 cells forming lymphoepithelial complexes by emperipolesis (RITTER et al., 1981; VAN DE WIJNGAERT et al., 1983). Since TNC contain mostly immature CD4⁺ CD8⁺ thymocytes but also mature and mainly CD4⁺ thymocytes with syngeneic reactivity (in mice) (PENNINGER and WICK, 1992) TNCs might play a role in positive selection. There are no monoclonal antibodies specific for TNCs. By immunohistochemistry an even greater heterogeneity of thymic epithelial cells can be realized and antigenic overlaps between epithelial cells in the different thymic compartments have been detected (BOYD et al., 1993). Nevertheless, some antigenic determinants have been identified by monoclonal antibodies as characteristic of the morphologically defined thymic compartments (Table 1). Such monoclonal antibodies have proved valuable for the analysis of thymomas (WILLCOX et al., 1987; KIRCHNER et al., 1992).

Macrophages are bone marrow derived constituents of the thymic microenvironment and appear in thymus primordia together with T cell progenitors

(JORDAN and ROBINSON, 1981; VON GAUDECKER, 1986). Macrophages are phenotypically heterogeneous (BOYD et al., 1993) with variable expression of MHC class II antigens (RITTER and CRISPE, 1992; RUCO et al., 1993). They are found throughout the thymus but occur at higher density in the cortex.

Interdigitating dendritic cells (IDC) are also bone marrow derived but can be observed only around the 12th week of gestation when the thymus primordia become lobulated (KAISERLING et al., 1974; VON GAUDECKER and MÜLLER-HERMELINK, 1980). IDC are MHC class II positive, occur throughout the thymus but are concentrated along the cortico-medullary junction. In humans IDC are considered the main mediators of negative selection (clonal deletion) *in vivo* (VANDEKERCKHOVE et al., 1992; BACHETTA et al., 1993, 1994; SCHOLS et al., 1994).

Thymic B cells (ISAACSON et al., 1987; FEND et al., 1991) and myoid cells (KAO and DRACHMAN, 1977; WEKERLE et al., 1975, 1978) are constituents of the human thymic microenvironment restricted to the thymic medulla. Thymic B cell precursors are probably derived from immature multipotential progenitors as are thymic T, NK and dendritic cells (BARCENA et al., 1994; RES et al., 1996). They colonize the thymus very early during ontogeny (KIMOTO et al., 1989) and almost all maintain unmutated, fetal type IgVH genes after intrathymic maturation throughout adulthood (DUNN-WALTERS et al., 1995). Thymic B cells in mice might play a role in negative selection (MAZDA et al., 1991) but their function in humans has yet to be established (SPENCER et al., 1992). Myoid cells can be detected as early as the 8th week of gestation (VON GAUDECKER and MÜLLER-HERMELINK, 1979) and in all postnatal thymuses though in declining number with higher age (DRENCKHAN et al., 1979; KIRCHNER et al., 1986; SCHLUEP et al., 1988; GEUDER et al., 1992; BORNEMANN and KIRCHNER, 1996). Myoid cells like extraocular and most facial muscles are not of somitic origin but derivatives of the precordial mesen-

Table 1. Monoclonal antibodies defining epithelial antigen determinants confined to the distinct thymic compartments of subcapsular epithelium, cortex, medulla, and Hassall's corpuscles in normal human thymus

Antibody	Compartment	Reference
K-20	Subcapsular epithelium (Type 1)	TAKEUCHI et al., 1991
RFD, MR19	Subcapsular epithelium plus medullary epithelium	BOFILL et al., 1985 DE MAAGD et al., 1985
MR3, MR6	Cortical epithelium	DE MAAGD et al., 1985
HTS7	Medullary epithelium	IZON and BOYD, 1990
MR13, TE-8, 15, 16	Hassall's corpuscles	DE MAAGD et al., 1985

chyme (SEIFERT and CHRIST, 1990). Their function is unknown but they may be involved in the pathogenesis of non-paraneoplastic myasthenia gravis (WEKERLE and KETELSEN, 1977; GEUDER et al., 1992; MARX et al., 1996a, 1997). Both B cells and myoid cells are usually absent from thymomas (KIRCHNER et al., 1992; MÜLLER-HERMELINK et al., 1996).

The thymic microenvironment also comprises the extracellular matrix. This consists of collagens, glycosaminoglycans and glycoproteins, including laminin and fibronectin (SAVINO et al., 1993). Collagen type I is abundant in the thymic capsule and septae, while the basal lamina underlying type 1 epithelial cells in the subcapsular area and along perivascular spaces is composed of collagen type IV, fibronectin and laminin (BERRI et al., 1985; LANNES-VIEIRA et al., 1991). Destruction of the basal lamina is a typical feature of the thymus in myasthenia gravis-associated thymitis (KIRCHNER et al., 1986). A fine network of extracellular matrix composed of fibronectin, collagen type IV and laminin is also detectable by reticulin stains around epithelial cells in the thymic medulla but not in the cortex, where fibronectin but not the other two components of extracellular matrix can be detected only by immunohistochemistry (RUCO et al., 1993). As this difference is maintained after neoplastic transformation reticulin stains help to distinguish thymic epithelial tumors of medullary versus cortical differentiation (MÜLLER-HERMELINK et al., 1996). Receptors for the extracellular matrix are found on thymocytes suggesting a direct role of the extracellular matrix in thymopoiesis (see below). In addition receptors for the extracellular matrix are also expressed on human thymic epithelial cells (RUCO et al., 1993) supporting the idea that the extracellular matrix may also play an indirect role in thymopoiesis by maintaining the cellular network of the microenvironment (SAVINO et al., 1993; HAYNES et al., 1995).

The functional development of the thymus is not completed until after birth in the mouse (BOYD et al., 1993; SURH et al., 1993). Whether this is also the case in humans has not yet been evaluated.

Finally, the genetic control underlying the development and heterogeneity of the cellular components of the thymic microenvironment is in the beginning of being elucidated at least in mice (BOEHM et al., 1995). The *whn* gene that is characteristically inactivated in the nude mouse codes for a transcription factor that controls very early steps in the development of the thymic microenvironment prior to immigration of T cell progenitors (NEHLS et al., 1996). In contrast, absence of the *relB* gene results in developmental defects of the thymic medulla involving both a characteristic medullary epithelial subset and bone marrow derived

dendritic cells (WEIH, 1995; BURKLY et al., 1995). These genetic defects might be considered as "true thymic dysplasias" of the microenvironment in contrast to "dysplasias" in primary T cell defects. Human equivalents of these primary immunodeficiencies have yet to be detected though some subtype of Nezelof's syndrome (NEZELOF, 1992) may be a good candidate for a *whn* deficiency state (BOEHM et al., 1995). Likewise the role of both genes in the pathogenesis of thymomas awaits investigation.

Lymphoepithelial Interactions in the Normal Human Thymus during Early T Cell Development: The Pre-T Cell Receptor Complex, Cytokines, and Adhesion Molecules

Early T cell development comprises the entry of immature T cell progenitors into the thymus, and their proliferation and differentiation prior to positive selection. What governs the entry of thymocyte progenitors into the thymus is enigmatic so far (discussed by AURRAND-LIONS et al., 1996; PATEL et al., 1995). We summarize the phenotypic stages that can be delineated by flow cytometry starting from the earliest CD34⁺ CD3⁻ CD4⁻ CD8⁻ (triple negative, TN) precursors to the CD3/TCR^{low} CD4⁺ CD8⁺ (double positive, DP) subset in Figure 1 (SPITS et al., 1995) because characteristic quantitative alterations in these immature thymocyte subsets occur in thymomas (see below). Some of the molecular events involved in the expansion and differentiation of immature thymocytes have been elucidated in mice. Transition from the triple negative to the double positive stage requires expression of the T cell receptor (TCR) beta chain that has to associate with a 33kD invariant chain (preTCR) forming the preTCR complex (SAINT-RUF et al., 1994). This complex is expressed on the cell surface just before upregulation of CD4 while TCR alpha rearrangement is probably initiated only in the more mature CD3⁻ CD4⁺ CD8⁻ subset (SPITS et al., 1995). Recently the human preTCR has been cloned (DEL PORTO et al., 1995; RAMIRO et al., 1996). The preTCR complex can deliver signals to TN thymocytes resulting in the appearance of DP cells (LEVELT et al., 1993). Signal transduction triggered by the preTCR complex involves the tyrosine kinases *lck* (MOLINA et al., 1992) and *fyn* (VAN OERS et al., 1996). Furthermore, some of the genes coding for transcription factors that play important roles in the most early steps of T cell differentiation have been identified in mice (reviewed by TING et al., 1996). On the other hand it is so far unclear which ligand(s) in the thymic microenvironment trigger the preTCR

Fig. 2. Model of T cell development in the human thymus: T cell phenotypes as defined by multicolor FACS analysis just before and following positive selection (SPITS et al., 1995).

complex *in vivo* (OWEN and VENKITARAMAN, 1996).

In addition to preTCR complex signaling cytokines might play a role in human early T cell development (SPITS et al., 1995; LERNER et al., 1996). A wealth of cytokines have been identified in the human thymic microenvironment. IL-1, IL-3, IL-4, IL-6, IL-7, IL-8, G-CSF, M-CSF, GM-CSF, TNF- α , TGF- β -1 and SCF (C-kit ligand) were described as *in vitro* products of various epithelial subsets (HAYNES et al., 1990; DALLOUL et al., 1991; GALY et al., 1991, 1992, 1993; ROPKE and EIBROEND, 1992; MEILIN et al., 1992; RITTER and BOYD, 1993; FERNANDEZ et al., 1994). Of particular interest is the production of IL-7 but not of IL-2, 3 and 4 by cortical type human epithelial cell lines (FERNANDEZ et al., 1994). Several cytokines influence the production of each other and of adhesion molecules via cytokine receptors on thymic epithelial cells (reviewed by RITTER and BOYD, 1993). However, knock out experiments in mice and circumstantial evidence from humans (concerning IL-2, reviewed by SPITS et al., 1995) suggest that at least IL-2, 4, 6 and IFN- γ are not critical for thymic T cell and microenvironment development while lack of the common γ chain of the IL-2, 4, 7 and IL-15 receptor abrogates early thymopoiesis resulting in X-chromosome-linked SCID (VOSS et al., 1994). Based on this latter finding it can be concluded that IL-7 must be essential also for thymic T and NK cell

development in humans (GRABSTEIN et al., 1993; HOZUMI et al., 1994). The molecular events triggered by IL-7 during early T cell development have still to be elucidated. It is also not clear whether the concerted action of IL-7 plus SCF that is necessary for optimal proliferation of triple negative thymocytes *in vitro* (MATSUZAKI et al., 1993) is also required in the human thymic microenvironment *in vivo*. Furthermore, it is unresolved whether epithelial-derived TGF- β regulates the transition of immature CD3⁻CD4⁺CD8⁻ thymocytes to double positive cells in humans as it regulates the respective development in mice (TAKAHAMA et al., 1994). Whether cytokines play a role in the T cell selection processes, particularly in negative selection, is presently under investigation (FOY et al., 1995; SPITS et al., 1995; LERNER et al., 1996).

Adhesion/costimulatory molecules are indispensable for the regulation of mature T cell activation (CLARK and LEDBETTER, 1994). Evidence that lympho-epithelial interactions by adhesion molecules (SINGER et al., 1990; MARX et al., 1994) including integrins (ZUTTER, 1991; RUCO et al., 1993), may contribute to early T cell development is restricted to *in vitro* mouse systems concerning the LFA-1/ICAM-1 (FINE and KRUISBEEK, 1991) and VLA-4/Fibronectin receptor/ligand pairs (UTSUMI et al., 1991). Other receptor/ligand lymphoepithelial interactions like CD2/LFA-3

obviously play no role during early thymopoiesis (KILLEEN et al., 1992) and VCAM-1, another ligand for VLA-4, seems to be restricted to macrophages and vessels in the normal human thymus (RUCO et al., 1993; MARX et al., 1994). The distribution of beta-1 (VLA) integrins other than VLA-4 has also been reported in the human thymus (LANIER et al., 1986; ZUTTER 1991; GIUNTA et al., 1991; RUCO et al., 1993; FERNANDEZ et al., 1994) and is summarized in Table 2. Integrins exhibit a stable expression during thymic ontogeny. However, their role in thymic microenvironmental development and human thymopoiesis has still to be defined (SAVINO et al., 1993; FERNANDEZ et al., 1994).

Positive and Negative Selection and Late Thymocyte Development: Lymphoepithelial Interaction by TCR/MHC, Costimulatory Molecules and Apoptosis-Related Proteins

The T cell repertoire is shaped by positive and negative selection (VON BOEHMER, 1994; NOSSAL, 1994). Positive selection is necessary for the maturation of double positive thymocytes into MHC restricted mature single positive thymocytes. Negative selection deletes most potentially autoaggressive T cells that efficiently recognized self antigens. Both positive and negative selection require interactions of the TCR (on thymocytes) with complexes composed of MHC molecules and self peptide that are exposed on cells of the thymic microenvironment. That the same receptor/ligand pair can mediate either sur-

vival signals (positive selection) or deletion by apoptosis (negative selection) is the result of different levels of avidity between TCR and MHC/peptide complexes (HOGQUIST et al., 1993; ALLEN, 1994; ASHTON-RICKARDT and TONEGAWA, 1994). High levels of avidity result in deletion of potentially autoaggressive thymocytes, while intermediate levels of avidity result in MHC restriction and rescue from apoptosis (positive selection) by survival signals.

Positive selection *in vivo* is mediated by thymic cortical epithelial cells while studies from bone marrow transplanted SCID patients demonstrate that negative selection requires dendritic cells (reviewed by SPITS et al., 1995). However, thymic epithelial cells may induce non-deletional tolerance by anergizing thymocytes (VANDEKERCKHOVE et al., 1992). Since this type of tolerance can be broken by IL-2 (SCHOLS et al., 1994) it is potentially important also for thymoma associated autoimmunity though this has yet to be investigated.

The signaling events involved in positive selection have partially been resolved in mice, and include CD45 (KISHIHARA et al., 1993), Zap-70 (NEGISHI et al., 1995), the p21ras and MAP kinase pathway (SWAN et al., 1995; ALBEROLA et al., 1995), calcineurin (WANG et al., 1995), and the Vav protein (FISCHER et al., 1995). Whether targets of these signaling elements are members of the Bcl2 family (MA et al., 1995) or transcription factors like Ets-1 (BORIES et al., 1995) has to be evaluated. In humans ZAP-70 is required for the positive selection only of CD8⁺ cells (ELDER et al., 1994; ARPAIA et al., 1994). How interaction of TCR

Table 2. Expression of very late activation (VLA) antigens (β 1 integrins) and other adhesion molecules relevant for thymocyte/stroma interaction in the normal human thymus (RUCO et al., 1993; MARX et al., 1994)

Adhesion molecule	Subcapsular epithelium	Cortical epithelium	Medullary epithelium	Macrophages	Endothelium
alpha 1 ^{1,2}	—	—	—	—	+
2	+	+	+	—	+
3	+	—	+	—	+
5	—	—	—	+	+
6	+	—	+/-	—	+
VCAM-1	—	—	—	+	+
ICAM-1	+/-	+	+	+	—
LFA-3 ²	—	—	+/-	+	+
B7-1(BB1)	—	—	+/-	—	—

¹VLA4 is expressed on immature and mature thymocytes strongly and moderately, respectively (MARX et al., 1994)

²Human cortical type thymic epithelial cells express all β 1 integrins and LFA-3 *in vitro* (FERNANDEZ et al., 1994)

with either an MHC class I or II molecule (plus peptide) elicits the differential development of either CD8⁺ or CD4⁺ single positive mature T cells ("lineage commitment") is still a matter of debate (LUCAS and GERMAIN, 1996).

While positive selection may function by TCR/MHC interaction alone, thymocytes require more than TCR ligation with MHC/peptide complexes to undergo negative selection (reviewed by OWEN and VENKITARAH, 1996; LERNER et al., 1996). While thymocyte/stroma interactions via Fas/FasL (SINGER and ABBAS, 1994; ADACHI et al., 1996) are probably dispensable *in vivo*, CD30/CD30L (AMAKAWA et al., 1996) and CD40/CD40L (FOY et al., 1995) and CD28/B7-1 (KISHIMOTO et al., 1996) seem to be important (as shown in mice). The function of the Fas antigen intensively expressed on human normal and neoplastic thymic epithelial cells (ONODERA et al., 1996; KAWANAMI, personal communication) has still to be determined. Likewise, the significance of the intense cross-talk between thymocytes and the thymic microenvironment for thymocyte deletion involving various cytokines awaits clarification (LERNER et al., 1996). Only preliminary data are available about the signaling pathways mediating negative selection, again only in mice (CALNAN et al., 1995; LERNER et al., 1996; ANDERSON et al., 1996).

With respect to the pathogenesis of autoimmunity it has to be stressed that the predominant form of intrathymic cell death is not deletion (negative selection) but "death by neglect" of thymocytes that undergo neither positive nor negative selection in the

thymic microenvironment because of defective TCR/MHC interaction (SURH and SPRENT, 1994). Whether this type of apoptosis shares molecular signals with deletion-induced apoptosis is unknown (LERNER et al., 1996). Since "death by neglect" may be a tolerogenic mechanism for antigens/peptides not presented by MHC molecules in the thymus (MARX et al., 1990), a change of the thymic endogenous peptide pool or its presentation may contribute to the generation of autoaggressive T cells in thymoma (see below). The thymocyte subsets that can be distinguished by FACS

Table 3. Expression of VLA antigens and adhesion molecules in thymomas (RUCO et al., 1993; MARX et al., 1994; MARX et al., unpublished)

Adhesion molecule	Medullary thymoma	Mixed thymoma	Cortical thymoma*
alpha 1	—	—	—
2	+++	+++	+ / ++
3	+++	+++	- / +
5	—	—	—
6	+	++	- / +
VCAM-1	+	—	—
ICAM-1	+++	+++	+++
LFA-3	++ (focally)	- / +	+++
B7-1(BB1)	+	+ / +	+ / ++

*Similar expression in well differentiated thymic carcinoma (WDTC)

Table 4. Classification of thymic epithelial tumors (TET) according to MÜLLER-HERMELINK et al. (1995). Association with myasthenia gravis (MG) and expression of MG-associated 160 kD neurofilament NF-M containing AChR and titin epitopes (MARX et al., 1996 a, b)

Tumor	Cases investigated ¹	Frequency of MG	Tumors with NF-M	Tumors without NF-M
Organotypic TET				
Medullary thymoma	10	30%	0	3
Mixed thymoma	33	40%	1	14
Predominantly cortical thymoma	15	40%	6	5
Cortical thymoma	81	69%	14	1
Well differentiated thymic carcinoma	33	79%	11	0
Non-organotypic TET	24	0%	4	1
Tumors with MG			30	8
Tumors with MG			4	16

¹All cases available (frozen and paraffin)

Fig. 3. Histology of organotypic thymic epithelial tumors according to the “histogenetic” or “functional” classification (Table 4). **a.** Medullary thymoma with spindle shaped epithelial cells, characteristic cysts and few lymphocytes. **b.** Mixed thymoma with sharp transition between a lymphocyte poor spindle cell area and a lymphocyte rich area. The large epithelial cell nuclei and nucleoli that are characteristic of cortical thymomas are absent from mixed thymomas. **c.** Predominantly cortical (organoid) thymoma with well developed medullary and cortical areas. The position of the medullary area adjacent to the capsule in this tumor is a characteristic architectural abnormalities distinguishing this tumor type from the normal thymus. **d.** Cortical thymoma with lobular architecture, roughly balanced number of immature lymphocytes and epithelial cells with characteristic nuclear and nucleolar morphology (**Inset**). **e.** Well differentiated thymic carcinoma (WDTC) with characteristic lobular, solid architecture and prevalence of epithelial cells with slight nuclear atypia. **f.** Characteristic palisading of epithelial cells around a perivascular space in WDTC. Hematoxylin-eosin, a-d: $\times 100$, Inset in d: $\times 400$, f: $\times 250$

analysis after positive selection are given in Figure 2 (SPITS et al., 1995).

Thymomas

Thymomas are thymic epithelial tumors—in contrast to thymus-derived T or B cell lymphomas (ROSAI and LEVINE, 1976; MÜLLER-HERMELINK et al., 1996). “Organotypic” (i.e. “thymus-like”) morphological and functional features (KIRCHNER et al., 1992) distinguish thymomas as unique thymic tumors from other tumors also arising in the thymus but not unique to it like category II thymic carcinomas, germ cell neoplasms or mesenchymal tumors (reviewed by ROSAI, 1996; MÜLLER-HERMELINK et al., 1996). The most characteristic functional feature considered as “organotypic” is the capacity of thymomas to provide homing for immature T cell detectable by anti-CD1 or CD99 antibodies (KIRCHNER et al., 1992; CHAN et al., 1995). In addition, only thymomas but not other thymic neoplasms elicit myasthenia gravis or other autoimmune diseases.

Thymoma classification has been one of the most controversial fields in pathology as discussed recently (ROSAI, 1996; MÜLLER-HERMELINK et al., 1996). MÜLLER-HERMELINK and coworkers have proposed a “histogenetic” or “functional” classification (Table 4, Fig. 3) considering the morphological resemblance of the various neoplastic epithelial cell types to their counterparts in the normal thymus and the differential functional capacity to provide homing for or promote proliferation and differentiation of immature thymocytes (MARINO and MÜLLER-HERMELINK, 1985; MÜLLER-HERMELINK et al., 1996). Compared to earlier classifications (reviewed by ROSAI, 1996) this novel classification has been proven to be highly reproducible (CLOSE et al., 1995), to be of prognostic relevance (QUINTANILLA et al., 1993), to correlate with the occurrence of autoimmunity (Table 4), immunohistological features (MARX et al., 1996a) and intratumorous thymocyte differentiation (NENNINGER et al., 1996).

Abnormal Composition of Thymoma Microenvironments

The neoplastic transformation of the epithelium in thymomas is the basis for the disturbance of the normal compartmentalization into subcortical, cortical and medullary areas and of abnormal epithelial cell properties (see next paragraph). However, neoplastic epithelial transformation is associated also

with alterations of the other cellular constituents of the microenvironment. In particular, there is a dramatic paucity of thymic B cells in thymomas (FEND et al., 1993) but it is unknown whether thymic B cell deficiency compromises tolerance induction (SPENCER et al., 1992). S100⁺ interdigitating dendritic cells (IDC) (a minority of which is CD1a⁺) are thought to be the main physiological effectors of negative selection (SPITS et al., 1995). IDC seem also to be reduced in some but not all thymomas (CHILOSI et al., 1984; LAURIOLA et al., 1984; MÜLLER-HERMELINK, 1986; WILLCOX et al., 1987; TAKEUCHI et al., 1995). An association between a reduced number of IDC and autoimmune phenomena has not been found (LAURIOLA et al., 1984; KRAUS et al., 1988) though this may require further analysis (LERNER et al., 1996). Finally, thymic myoid cells, expressing the myasthenia gravis-relevant autoantigen acetylcholine receptor (AChR), are usually absent from thymomas (KIRCHNER et al., 1986), though rare benign thymomas (reviewed by ROSAI, 1996) and also high grade thymic carcinomas (unpublished) may exhibit increased numbers. Since it is not known, whether myoid cells normally play a role in AChR-specific negative selection, the significance of their absence for paraneoplastic autoimmunity remains to be investigated. Not only the cellular components of the neoplastic thymic microenvironment, but also constituents of the extracellular matrix exhibit abnormalities in thymomas. As a general tendency there is increased deposition of fibronectin, laminin and collagen type IV in mixed and medullary thymomas as compared to the normal thymus while the expression of these matrix molecules in cortical thymomas is very low as it is in the normal cortex (RUCO et al., 1993). Whether these abnormalities contribute to paraneoplastic autoimmunity is unknown (VON BOEHMER, 1994).

“Homing molecules” expressed by endothelial cells, the extracellular matrix or perivascular stromal cells (like VLA-6, CD44R or Vanin-1, reviewed by PATEL and HAYNES, 1994; AURRAND-LIONS et al., 1996) have not yet been investigated in thymomas.

Future investigations of the abnormal microenvironment of neoplastic thymuses will have to consider the so far neglected cytokine network of thymomas.

Abnormal Epithelium in Thymomas

The terms “medullary thymoma” or “cortical thymoma” applied by the functional/histogenetic thymoma classification (Table 4) could suggest that the various thymoma subtypes are neoplastic expan-

sions of one of the various thymic epithelial cell subsets encountered in the normal thymus. However, this is not the case, although some morphological similarities between neoplastic epithelial cells and their normal counterparts exist (MARINO and MÜLLER-HERMELINK, 1985). By contrast, epithelial cells of thymomas more closely resemble thymic epithelial cell subsets encountered at various maturational stages during thymus ontogeny (KRAUS et al., 1988). Antigenic heterogeneity in a given tumor has been explained by suggesting maturational arrest at various developmental stages of the progeny of a common (clonal) tumor stem cell (WILLCOX et al., 1987). A common and characteristic theme probably related to the heterogeneous state of "immaturity" might be the finding that features defining the distinct medullary and cortical compartments in the normal adult thymus are combinedly expressed by epithelial cells of thymomas (MÜLLER-HERMELINK et al., 1996). This finding concerns morphological features like the expression of keratins (MOKHTAR et al., 1984; CHILOSI et al., 1984; MÜLLER-HERMELINK, 1986; WILLCOX et al., 1987), neurofilaments (MARX et al., 1996b), adhesion molecules (RUCO et al., 1993), and costimulatory ligands for thymocyte receptors (MARX et al., 1994; GILHUS et al., 1995) but particularly functional features; in spite of partial medullary type immunohistochemical properties most thymomas support the development of immature thymocytes like the normal cortex (CHILOSI et al., 1984; MÜLLER-HERMELINK, 1986; WILLCOX et al., 1987). Vice versa epithelial cells derived from cortical thymomas can present soluble antigen to mature CD4⁺ T cells (MARX et al., 1994; GILHUS et al., 1995) though this is a property restricted to medullary type epithelium in the normal thymus (KASAI et al., 1996).

With respect to the triggering of paraneoplastic autoimmunity abnormal expression of molecules mediating lymphoepithelial interactions in thymomas have attracted much attention. Interestingly there is a tendency for upregulation of adhesion and costimulatory molecules in thymomas as summarized in Table 3. This latter statement concerns beta-1 integrins like VLA-2, 3 and 6 (RUCO et al., 1993), CD40 (SCHULTZ et al., 1996), ICAM-1, LFA-3 and B7-1 (MARX et al., 1994; GILHUS et al., 1995). While upregulation of costimulatory molecules could principally suggest a higher susceptibility of mature T cells for antigen-specific activation (CLARK and LEDBETTER, 1994; MARX et al., 1994), experimental evidence does not support intratumorous T cell activation as a mechanism of autoimmunization in thymoma-associated autoimmunity (SCHULTZ, 1997). On the contrary, extrapolation from mice suggests that upregulation

of CD40 (SCHULTZ et al., 1996) or B7-1 in conjunction with ICAM-1 in thymomas (MARX et al., 1994) may either enforce negative selection (FOY et al., 1995; KISHIMOTO et al., 1996) or entail inefficient positive selection (KISHIMOTO et al., 1996). Both effects would counteract autoimmunization (SCHULTZ et al., 1996).

On the contrary, downregulation of MHC class II expression by epithelial cells of most thymomas is a very characteristic though not constant finding (MOKHTAR et al., 1984; CHAN et al., 1984; WILLCOX et al., 1987; KIRCHNER et al., 1992). Although a clear-cut correlation between MHC class II expression and the occurrence of myasthenia gravis has not been established (CHILOSI et al., 1984; WILLCOX et al., 1987) this finding may only demonstrate that different mechanisms of tolerance breakdown may work in differently abnormal microenvironments (CHILOSI et al., 1986). In fact, we consider reduced levels of MHC class II expression as highly important for paraneoplastic autoimmunity as given in the pathogenetic model below.

Abnormal Lymphoepithelial Interactions in Thymomas ?

While many features of lymphoepithelial interactions in the non-neoplastic mouse thymus have been defined at the molecular level (see above; SPITS et al., 1995), respective investigations are scarce in the normal human thymus (PATEL and HAYNES, 1994; RAMIRO et al., 1996) and lacking in thymomas. Therefore, abnormal lymphoepithelial interactions can be deduced only indirectly from abnormalities of the intratumorous thymocyte populations as defined by FACS analyses or functional assays. As we found recently, all developmental stages of thymocyte differentiation as shown in Figures 1 and 2 can be identified in thymomas by flow cytometry, suggesting that intratumorous thymopoiesis is generally maintained (NENNINGER et al., 1996). However, enormous quantitative differences with respect to the various subpopulations exist between thymomas and normal thymuses concerning early stages of thymocyte differentiation, positive selection and terminal maturation (TAKEUCHI et al., 1995). Each histological thymoma subtype exhibits typical abnormalities of the thymocyte subset composition as given for mixed and cortical thymomas in Figure 4 (NENNINGER et al., 1996). In contrast to mixed and cortical thymomas, immature CD3⁻CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes are almost absent from medullary thymomas (not shown).

In our series of tumors there was a correlation between a decreased expression of MHC class II and inefficiency of positive selection and terminal maturation.

Fig. 4. Abnormal T cell development in thymomas of cortical and mixed type. 50% of mixed thymomas (a) exhibit more severe defects in positive selection than all cortical and the other mixed type thymomas (b) (NENNINGER et al., 1996). Well differentiated thymic carcinomas (*WDTC*) (KIRCHNER et al., 1992) exhibit features similar to cortical thymomas. Characteristically there is an increase of immature CD3⁻CD4⁺CD8⁻ thymocytes in these thymoma subtypes while mature thymocytes are decreased. In contrast, mature T cells prevail in medullary thymomas by far (not shown).

Fig. 5. Positive selection in the normal thymus (a) and in cortical type thymomas (b). Apoptosis can occur either by deletion because of too high an avidity between TCR and MHC/peptide complexes (+++) or because of "death by neglect" with lack of survival signals (---). Positive selection occurs at intermediate levels of avidity (+). In thymomas abnormally hyperexpressed endogenous neurofilaments (with AChR- and titin-like epitopes, WILSCH et al., 1996) in combination with decreased MHC class II levels should rescue neurofilament-reactive T cells (and crossreacting AChR-reacting T cells) from "death by neglect". In addition there should be inefficient positive selection of the usual T cell repertoire. Both predictions are supported by experimental evidence given in the text.

Table 5. Morphological, functional, and epidemiological features different between thymitis-associated myasthenia gravis (MG) and thymoma-associated MG that might have a bearing for different mechanisms of MG pathogenesis

Feature	Thymitis-associated myasthenia gravis	Thymoma-associated myasthenia gravis
Myoid cells present	yes	no
AChR ¹ present	yes	no
Number of IDC ²	high	low
Activated T cells	yes	no
Number of B cells	high	low/none
Autoantibody production	yes	no
HLA/gender association	yes	no

¹AChR: acetylcholine receptor, ²IDC: interdigitating dendritic cells

Pathogenesis of Paraneoplastic MG in Thymoma Patients

Fig. 6. A pathogenetic model of paraneoplastic MG in thymoma patients. According to this model abnormal AChR- and titin-like epitopes provide the molecular basis for an autoantigen-specific abnormal positive selection that turns immature T cells (T_i) into mature and naive T cells ($T_{m/n}$). These T cells become activated ($T_{m/a}$) after export to the periphery, where they could stimulate B cells. The etiology triggering T cell activation is unknown.

tion (NENNINGER et al., 1996) while this was not so obvious in another study (TAKEUCHI et al., 1995). In both studies cortical and mixed thymomas harbored considerably fewer mature CD4⁺ T cells than normal thymuses. Nevertheless, functional studies revealed a higher frequency of T cells with specificity for the autoantigen acetylcholine receptor (AChR) in most mixed and cortical thymomas (SOMMER et al., 1991; MARX et al., 1996a). Relative to the number of CD4⁺ mature T cells, AChR-reactive thymocytes were increased up to 25 fold as compared to normal controls (SCHULTZ, 1997). No correlation between the increased number of potentially autoaggressive T cells and the occurrence of myasthenia gravis was found (SCHULTZ, 1997). In summary, these findings suggest an abnormal thymocyte/stroma interaction in thymomas.

Thymoma-Associated (Paraneoplastic) Myasthenia Gravis by Abnormal Lymphoepithelial Interactions ?

Myasthenia gravis (MG) comprises a group of autoimmune diseases characterized by muscle weakness due to autoantibodies against the acetylcholine receptor (AChR) (reviewed by MARX et al., 1996a). MG is heterogeneous with respect to clinical, immunological and pathological findings (VINCENT, 1994). Autoantibody production in MG is T cell dependent (HOHLFELD et al., 1984; MELMS et al., 1992). Remarkably, pathology of thymus is an almost constant finding. Thymitis, thymoma or thymic atrophy occur in 70, 20, and 10% of MG patients, respectively (MÜLLER-HERMELINK et al., 1996). While the pathogenesis of MG in thymic atrophy is obscure, MG in thymitis is considered the consequence of a T cell dependent humoral immune reaction that takes place completely inside the thymus and probably starts there (WEKERLE and KETELSEN, 1977; MEINL et al., 1991). The intrathymic immune reaction in thymitis encompasses the AChR (on myoid cells) as the (triggerring) autoantigen, IDC as antigen presenting cells, AChR-specific and activated CD4⁺ T cells and autoantibody secreting B cells. Thymectomy improves thymitis-associated MG by eliminating the main source of autoantibody production (reviewed by MARX et al., 1996a, 1997). This is not the case with paraneoplastic MG that differs from thymitis-associated MG in almost any respect (Table 5).

Paraneoplastic MG is also distinguished by the specific occurrence of autoantibodies against the muscle proteins titin and ryanodine receptor (AARLI et al., 1990; MYGLAND et al., 1995) and against neuronal proteins (MARX et al., 1992) in addition to

anti-AChR autoantibodies. With respect to the autoantigens of paraneoplastic MG it is a shared principle that AChR, titin and ryanodine receptors themselves are not expressed in thymomas but that crossreacting epitopes are (KIRCHNER et al., 1988; MARX et al., 1990; MYGLAND et al., 1995). Recently, we found that the AChR- and titin-like epitopes expressed in thymomas are constituents of neurofilaments that are abnormally expressed in thymomas (MARX et al., 1996b; WILISCH et al., 1996).

In our opinion the following findings await explanation by an appropriate pathogenetic model: 1) Paraneoplastic MG occurs only in organotypic thymic epithelial tumors that contain immature CD1⁺ thymocytes (MÜLLER-HERMELINK et al., 1996). 2) Thymomas with cortical differentiation and probably mixed thymomas (unpublished) abnormally express neurofilaments (with AChR- and titin-like epitopes) and ryanodine receptor-like epitopes and this abnormal expression is associated with autoimmunity against these autoantigens. 3) AChR- and neurofilament-reactive T cells occur in increased numbers inside mixed and cortical thymomas (SOMMER et al., 1990; MARX et al., 1996a) and these T cells are not activated *in situ* (SCHULTZ, 1997). 4) B cells producing autoantibodies are absent from thymomas (FUJII et al., 1984).

Considering evidence from mice that endogenous peptides are involved in positive selection (reviewed by ASHTON-RICKARDT and TONEGAWA, 1994) and that abnormal peptide loading on MHC class II molecules alters T cell selection (MARTIN et al., 1996) we hypothesize that abnormally expressed neurofilaments in cortical and mixed thymomas might cause "false-positive selection" of immature T cells by rescuing them from "death by neglect" (MARX et al., 1992). The characteristic abnormalities of the neoplastic thymic microenvironment—abnormal intraepithelial expression of neurofilaments with AChR and titin epitopes in a cortical microenvironment combined with decreased levels of epithelial MHC class II (Fig. 5)—would explain both the overall inefficiency of intratumorous positive T cell selection (TAKEUCHI et al., 1995; NENNINGER et al., 1996) and the increased number of AChR and titin-reactive T cells (SOMMER et al., 1990; MARX et al., 1996a). Since the autoantigen-reactive T cells are not activated inside thymomas (SCHULTZ, 1997) we assume that they become activated after export to the periphery and could stimulate the production of autoantibodies there (Fig. 6). The etiologic processes activating T cells after their export to the periphery have yet to be elucidated (VINCENT, 1994). Likewise, the pathogenesis of MG in medullary thymomas awaits further analyses.

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