American Journal of Pathology, Vol. 128, No. 3, September 1987

Immunohistochemical Evidence of Active Thymocyte Proliferation in Thymoma

Its Possible Role in the Pathogenesis of Autoimmune Diseases

MARCO CHILOSI, ANTONIO IANNUCCI, FABIO MENESTRINA, MAURIZIO LESTANI, ALDO SCARPA, FRANCO BONETTI, LUCIANO FIORE-DONATI, BRUNO DIPASQUALE, GIOVANNI PIZZOLO, GIORGIO PALESTRO, GIUSEPPE TRIDENTE, and GEORGE JANOSSY

From Istituto di Anatomia Patologica, di Scienze Immunologiche ed Ematologia, Universita' di Verona, and Istituto di Anatomia Patologica, Universita' di Torino, Italy, and Department of Immunology Royal Free Hospital School of Medicine, London, England

Eight cases of human thymoma have been analyzed on cryostat sections with the monoclonal antibody Ki67, which reacts with cells in the proliferative phases of the cell cycle. The aim was to assess the proportion of proliferating thymocytes among lymphoid cells in the thymoma samples. In all cases a large number of cells (mean, 58.75%; range, 35–80%), recognized as thymocytes by morphology and lack of cytokeratin expression in a combined immunohistochemical assay, exhibited nuclear Ki67 staining. These findings differ from the reactivity pattern observed in age-matched nonneoplastic thymuses where lower growth activity of cortical thymocytes was observed (15–20% Ki67$^+$ cells). Intensive thymocyte proliferation in thymomas may represent one of the factors which lead to autoimmunity in myasthenia gravis and thymomas. (Am J Pathol 1987, 128:464–470)

HUMAN thymomas are neoplasms of epithelial origin that are frequently associated with an exuberant lymphoid component.[1] Recent studies have established that the epithelial and lymphoid components in thymoma closely mimick the cell organization observed in the normal thymus.[2–10] In most cases of thymoma with high lymphocyte/epithelial ratios neoplastic epithelial cells exhibit differentiation antigens of thymic cortical epithelium[3,11] and appear to be intermingled with lymphocytes exhibiting a "cortical" phenotype as observed in the normal cortex (TdT$^+$, T6$^+$, T4$^+$, *and* T8$^+$, etc.).[2,3,6,9–12] Residual areas of "medullary differentiation" are also present with mature T cells (TdT$^-$, T6$^-$, T3$^+$), and medullary epithelial cells as defined by their peculiar phenotype.[3,7] Cases showing predominantly medullary epithelial type have been also occasionally seen.[4,7] Thus, in lymphoid-predominant thymomas the following characteristic features are seen: a) an abundance of immature thymocytes and b) an abnormal neoplastic epithelial microenvironment which frequently exhibits hyperkeratinization and changes of HLA-DR expression.[3,11] Autoimmune phenomena are also frequently associated with thymoma. These include, most frequently, myasthenia gravis[1,7] and, occasionally, cytopenias, systemic lupus erythematosus, rheumatoid arthritis, thyroiditis, and other diseases.[13–15] It is not known whether the large numbers of thymocytes observed in these cases derive from an increased proliferation of cortical thymocytes or from an exaggerated accumulation of end-stage nondividing thymocytes. This is an important question because the increased proliferation is likely to be associated with an increased output of cells from the thymus into the

Supported in part by the Associazione Italiana per la Ricerca sul Cancro, Milano, and Progetto Finalizzato CNR "Oncologia", Italy.

Accepted for publication April 27, 1987.

Address reprint requests to M. Chilosi, Istituto di Anatomia Patologica, Universita' di Verona, Policlinico Borgo Roma, 37100 Verona, Italy.

periphery. For this reason, in our study we evaluated the replicating sets of thymocytes in 8 cases of thymoma using a quantitative immunostaining by the monoclonal antibody Ki67. This reagent binds to nuclear antigens expressed by cells in the proliferative phases G1, G2, M, and S.[16] These data were compared with the quantitative observations obtained with Ki67 staining on samples of normal thymuses.

Materials and Methods

Patients

Fresh frozen tissue samples were obtained from 8 patients at surgery for the diagnosis and therapy of thymoma (Table 1). The patients received no immunosuppressive therapy before thymectomy. Four samples of normal thymus were also analyzed as controls. The antibodies applied for the study of epithelial and lymphoid antigens of the thymomatous samples are listed in Table 2.[3]

Methods

The samples were covered with OCT medium (Ames), snap-frozen in liquid nitrogen, and processed for immunohistochemistry as previously described.[17] Briefly, 5-μ sections were cut in a cryostat, dried onto glass slides covered with polylysine adhesive (Sigma), and fixed in cold chloroform/acetone mixed in a 1 : 1 ratio. A modification of the original technique for demonstrating Ki67 antigen[16] was used for improvement of the fixation of the nuclear antigens. This procedure included a second fixation step with 10% calcium–formalin for 10 minutes. After fixation the slides were washed in buffered saline (PBS) and labeled with Ki67 by means of the alkaline phosphatase anti-alkaline phosphatase method (APAAP).[18] A gentle hematoxylin counterstaining was performed for assessing the morphology and percentage of Ki67$^+$ cells. Ki67 antibody (Code M722) and the APAAP reagent (Code K670) were purchased from Dakopatts, Denmark.

Additional sections were also stained using a combined immunocytochemical staining for Ki67 and cytokeratin to confirm the nature of the Ki67$^+$ cells in the given samples. In addition to the staining for Ki67 (APAAP with red chromogen), the samples were simultaneously incubated with a rabbit anti-keratin antibody (Dakopatts, Code A575) followed by anti-rabbit serum and peroxidase anti-peroxidase (PAP) complex (Dakopatts, Code K548) with diaminobenzidine as a brown chromogen.

The samples were first viewed with the two-color immunochemical method and also with labeling for

Table 2—Conventional and Monoclonal Antibodies Used for Immunohistochemical Study

Immunologic reagent		Specificity	Source
OKT4 (CD4)	M	Helper T cells	Ortho
OKT8 (CD8)	M	Suppressor T cells	Ortho
OKT3 (CD3)	M	T cells	Ortho
OKT6 (CD1)	M	Cortical thymocytes	Ortho
TdT	R	Cortical thymocytes	(Ref 11)
Keratin	R	Epithelial cells	Dako
Ki67	M	Replicating cells	Dako
HLA-DR	M	B cells, etc.	Becton Dickinson
MR3	M	Thymic epithelium (cortex)	(Ref 19)
RFD4	M	Thymic epithelium (medulla)	(Ref 11)

R, rabbit; M, monoclonal.

Table 1—Evaluation of Replicative Fractions of Thymocytes in Thymomatous and Control Samples

Cases	Sex	Age	Histology	Associated disease	%Ki67$^+$
1	M	40	Thymoma	Myasthenia	40%
2	F	61	Thymoma	Myasthenia	70%
3	M	69	Thymoma	Myasthenia*	35%
4	M	41	Thymoma	Myasthenia	80%
5	M	42	Thymoma	Myasthenia	60%
6	M	19	Thymoma	None	70%
7	M	31	Thymoma	Myasthenia	45%
8	F	54	Thymoma	Myasthenia*	70%
Age-matched control thymuses					
9	M	37	Normal thymus	Thyroid adenoma	20%
10	F	36	Normal thymus	Hyperparathyroidism	15%
Control thymuses					
11	M	2	Normal thymus	Cardiopathy	60%
12	M	15	Normal thymus	Hodgkin's disease	48%

*Ki67$^+$ percentage confirmed on smear preparations (Case 3, 41%; Case 8, 68%).

RFD4 antigen present on medullary epithelium. The overall distribution of cortical and medullary areas has been determined. Fields with predominant epithelial components and with signs of medullary differentiation (such as Hassal corpuscles) were not included.

In all cases fields with lymphoid predominance were viewed, and more than 2000 cells were evaluated for Ki67 nuclear staining with a 40× objective. Five different areas were selected in all samples, and the percentages of positive cells within the lymphoid cell population were counted.

In 2 cases fresh cell suspensions were also available, and cytospin preparations were obtained. Immunocytochemical staining on these preparations allowed the precise evaluation of the proportions of $Ki67^+$ thymocytes within the TdT^+, $T6^+$ cortical thymocyte populations and comparison of these findings with the observations on cryostat sections.

Results

The cases examined were encapsulated multinodular neoplasms with the typical histologic pattern of the cortical type of thymoma and were characterized by nodules of different size, mainly composed by lymphoid cells and minor proportions of epithelial cells. Epithelial cells had round–ovoid nuclei and loose chromatin structure. Lymphoid mitoses were common. In three cases areas of medullary differentiation with Hassall's corpuscles were present. In another 2 cases areas of medullary differentiation were only detected by immunohistochemistry, revealing mature lymphocytes (TdT^-, $T6^-$, $T3^+$) among remnants of an epithelial network exhibiting the medullary phenotype ($RFD4^+$) when studied on adjacent sections.

Epithelial Component

In all cases the neoplastic epithelial cells expressed cytokeratin and the antigen detected by MR3 monoclonal antibody. This reagent is one of the most specific reagents for cortical epithelium.[19] HLA-DR was absent or heterogeneously expressed on epithelial cells, unlike in cortical epithelium of the normal control samples. RFD4, an antibody specific for subcapsular and medullary epithelium, only reacted with small medullary-like areas as described earlier.[3]

Lymphoid Component

In all cases the large majority of lymphocytes (more than 60–80%) were of the T type and exhibited a "cortical" phenotype as defined by TdT and T6 positivity together with the simultaneous presence of T4 and T8 antigens. Similar proportions of $T6^+$, TdT^+ lymphoid cells (62% and 70%) were observed in the two samples which were studied on cell suspension.

The lymphocytes homing within the areas of medullary differentiation were mostly TdT and T6 negative and expressed either T4 or T8 antigen.

Ki67 Expression

In all the cases examined Ki67 immunostaining revealed the presence of a large number (range,

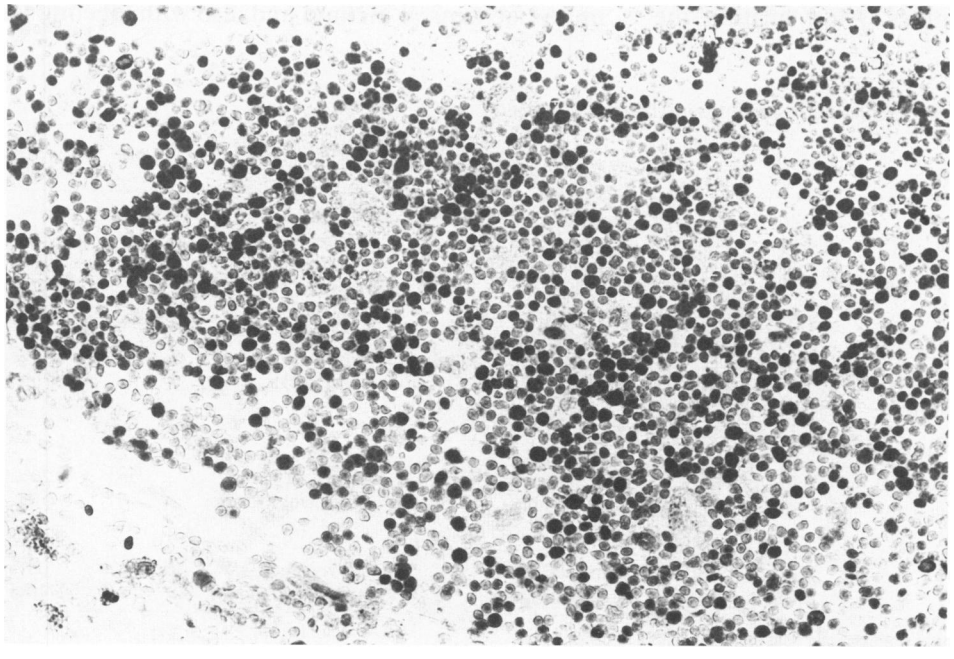

Figure 1—Cryostat section of thymoma of cortical type (Case 3). A proportion of thymocytes (evaluated as 35% on direct tissue examination) express the proliferative marker Ki67.

35–80%; mean, 58.75%) of positive cells with nuclear staining of heterogeneous intensity. The positive cells showed a lymphoid morphology (Figure 1). In double-stained sections keratin-positive epithelial cells (PAP, brown staining) were almost completely devoid of nuclear Ki67 staining (APAAP, red staining) (not shown).

In the 2 cases with areas of medullary differentiation these were characterized by a lack of (or few) Ki67-positive thymocytes and clearly appeared as negative islands within the tissue (Figure 2).

The quantitative evaluation of Ki67-positive thymocytes was performed on cryostat sections. Thymocytes were easily recognized by morphology and by lack of cytokeratin expression. The proportions of Ki67-positive thymocytes in thymomas ranged between 35% and 80% and were higher than those observed in the normal age-matched thymuses in our (15–20%) and Steinmann's (10%) series.[20]

In the 2 cases that were studied on cytospin preparations, the proportions of TdT^+, $T6^+$, $Ki67^+$ thymocytes could be carefully analyzed. In these 2 cases the

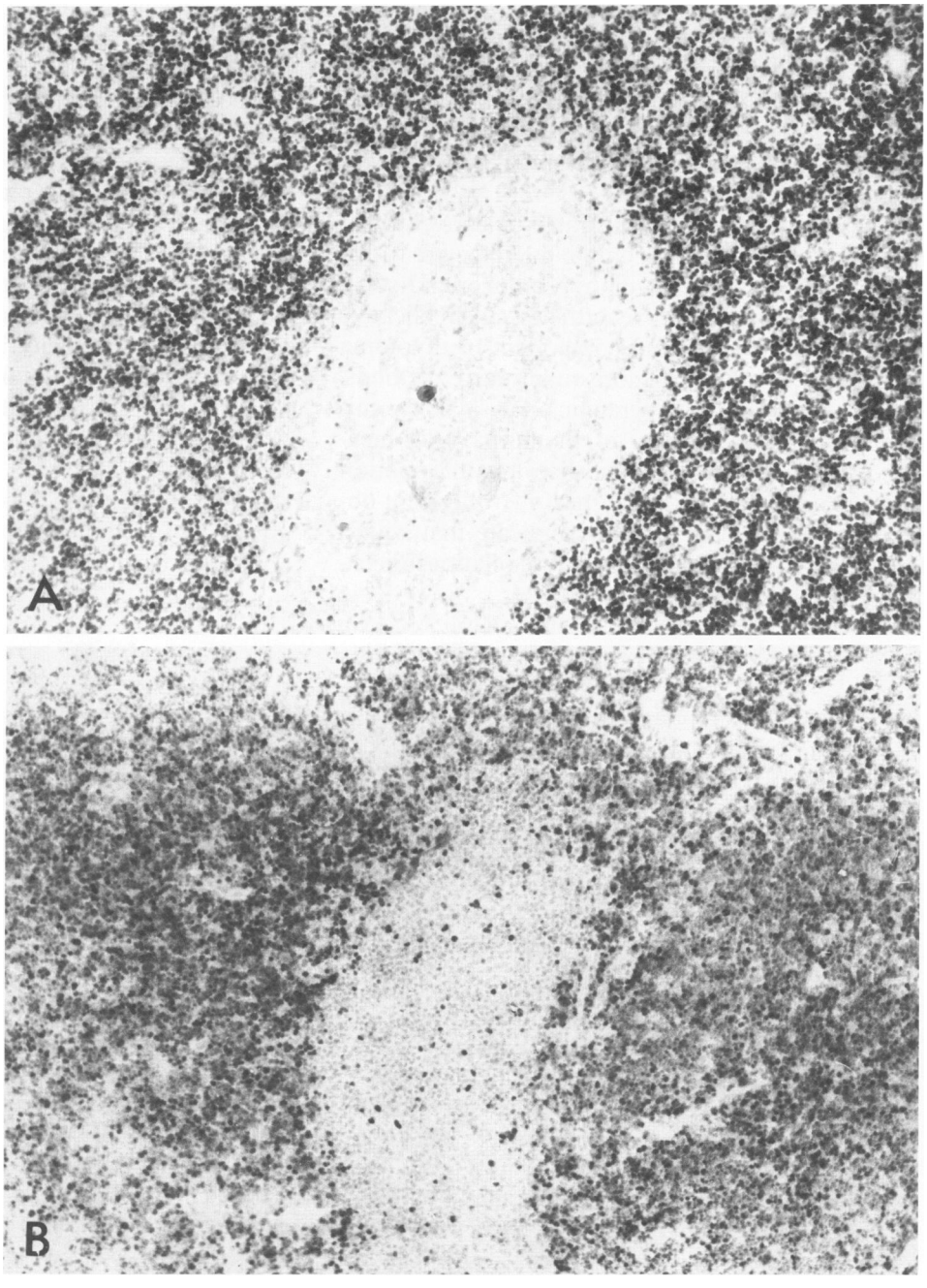

Figure 2—Cryostat sections of thymoma of cortical type with medullary differentiation (Case 2). A large number of TdT-positive "cortical" thymocytes are shown (**a**) around a small medullary area. In a serial section (**b**) most cortical cells express the proliferative marker Ki67. The medullary area is nearly devoid of positive cells.

results on cytospin and cryostat preparations were comparable (Table 1 and Figure 3).

Discussion

Human thymoma is a peculiar epithelial neoplasm where "abnormal" epithelium supports the differentiation of polyclonal T lymphocytes.[1-11,21] We recently confirmed the polyclonality of the lymphoid component of thymoma at the gene level using probes for the beta-chain of the T-cell receptor (Scarpa et al, manuscript in preparation).

The immunophenotypic studies have shown that most lymphocytes in thymoma are identical to normal cortical thymocytes.[2-11] In some cases minor proportions of mature T lymphocytes can be also found in islands which show "medullary differentiation." These areas are most frequently small, but normally organized.[3,6,7]

It has already been demonstrated by conventional histology that thymomas contain lymphocytes with a "stimulated" or "activated" appearance, and some mitoses are also seen.[1,21] Nevertheless, the replicative potential of the lymphoid component in thymoma needs further study with more quantitative methods. Unfortunately, autoradiographic analysis or the mitogen-responsive capacity of thymoma cells measured *in vitro*[22] may not give relevant information about their proliferative features *in vivo*. Recent observations with Ki67 antibody indicate that within the lymphoid lineage there is a close relationship between antibody reactivity and the cycling of the cell (in G1, S, or M phase).[16]

In this study we have determined the replicative fraction of thymocytes in thymomas using the immunohistochemical detection of Ki67 antigen.[16] We found a marked activity of thymocytes in all thymomatous samples. An internal control for each sample has been provided by the remnants of medullary areas where all cells were virtually devoid of Ki67 staining. The proportions of Ki67-positive thymocytes found in thymomas (35–80%) are similar to those observed in normal control thymuses from younger patients but significantly higher than those observed in the cortical areas of age-matched control thymuses in our study (15–20%) and in a series of cases recently reported by Steinman et al.[20] This observation suggests that in thymoma the accumulating thymocytes do not merely represent a population of long-lived end-stage cells which divide rarely. It seems that this population actively proliferates within the epithelial network, which has been shown to synthetize thymic hormones[23] and which, in normal thymus, may have a determinant role in the selection of immature T cells for self MHC specificity.[24] There is also evidence in both experimental animals and man that thymocytes acquire their antigen specificity within the cortex, where the rearrangement of genes coding for T-cell receptor takes place.[25,26] It appears from our study that the proliferating T-cell precursors in thymoma are part of a microenvironment constituted by an abnormal cortical epithelium, which is frequently

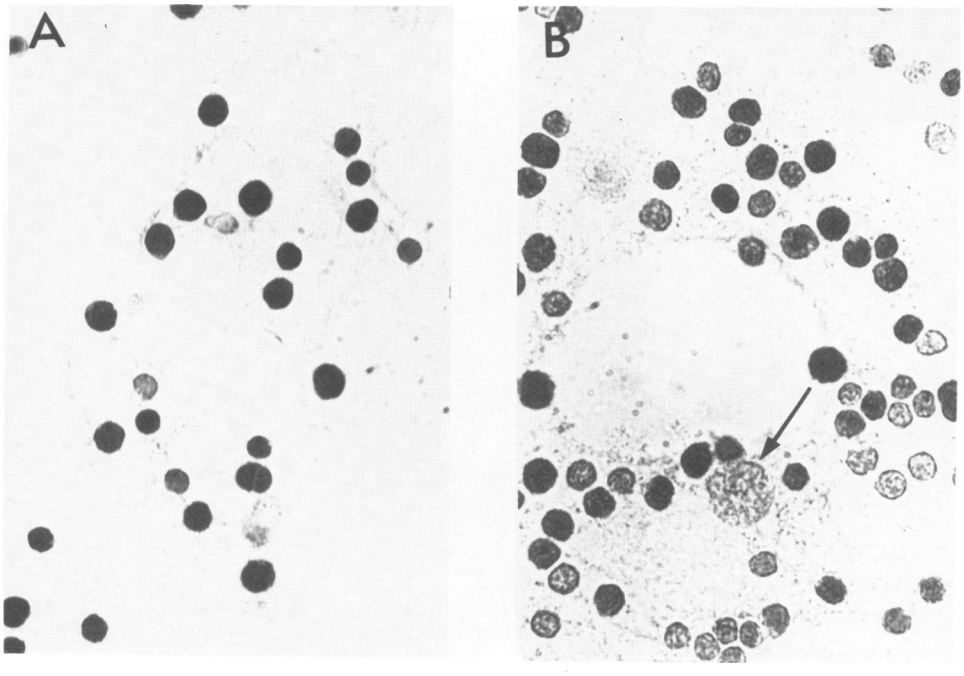

Figure 3—Smear preparations obtained from a cell suspension of thymoma of cortical type (Case 8). The majority of cells show the immature "cortical" marker TdT (**a**) and the proliferative marker Ki67 (**b**). A Ki67-negative epithelial cell is shown (*arrow*).

HLA-DR negative, as it has been demonstrated in another disease, the "bare lymphocyte" syndrome.[27] Education in such a microenvironment in thymoma may lead to the export of functionally handicapped T-cell clones. The search of such immature T cells in the peripheral blood of patients with thymoma is an ongoing project in our laboratories, but it is relevant here that lymphocytes of intermediate phenotype ($T4^+, T8^+, T6^-$) can be demonstrated in some patients with myasthenia gravis.[28]

In addition to the cortical overproduction of thymocytes, we have previously detected another important contributing defect in thymoma. Proliferating cortical thymocytes may avoid medullary influence exerted at the cortical-medullary junction prior to entry into the circulation. It has been recently demonstrated that medullary interdigitating dendritic cells induce self-tolerance.[29,30] These factors, taken together, may represent the key factors in the frequent development of autoimmunity in thymomas.

References

1. Rosai J, Levine GD: Tumors of the thymus, Atlas of Tumor Pathology. Armed Forces Institute of Pathology, Washington, DC, 1976
2. Chan WC, Zaatari GS, Tabei S, Bibb M, Brynes RK: Thymoma: an immunohistochemical study. Am J Clin Pathol 1984, 82:160–166
3. Chilosi M, Iannucci A, Fiore-Donati L, Tridente G, Pampanin M, Pizzolo G, Ritter M, Bofill M, Janossy G: Myasthenia gravis: immunohistological heterogeneity in microenvironmental organization of hyperplastic and neoplastic thymuses suggesting different mechanisms of tolerance breakdown. J Neuroimmunol 1986, 11:191–204
4. Chilosi M, Iannucci A, Pizzolo G, Menestrina F, Fiore-Donati L, Janossy G: Immunohistochemical analysis of thymoma: Evidence for medullary origin of the epithelial cells. Am J Surg Pathol 1984, 8:309–318
5. Lauriola L, Maggiano N, Marino M, Carbone A, Piantelli M, Musiani P: Human thymoma: immunologic characteristics of the lymphocytic component. Cancer 1981, 48:1992–1995
6. Mokhtar N, Hsu SM, Lad RP, Haynes BF, Jaffe ES: Thymoma: Lymphoid and epithelial components mirror the phenotype of normal thymus. Hum Pathol 1984, 15:978–984
7. Muller-Hermelink HK, Marino M, Palestro G: Pathology of thymic epithelial tumors, The Human Thymus: Histopathology and Pathology. Edited by HK Muller-Hermelink. Berlin, Springer-Verlag, 1986, pp 207–268
8. Reddick RL, Jennette JC: Immunologic and ultrastructural characterization of the small cell population in malignant thymoma. Hum Pathol 1983, 14:377–380
9. Aisemberg AC, Wilkes B, Harris NL, Frist WH: The predominant lymphocyte in most thymomas and in nonneoplastic thymus from patients with myasthenia gravis is the cortical thymocyte. Clin Immunol Immunopathol 1985, 35:130–136
10. Eimoto T, Teshima K, Shirakusa T, Takeshita M, Okamura H, Naito H, Mitsui T, Kikuchi M: Heterogeneity of epithelial cells and reactive components in thymomas: An ultrastructural and immunohistochemical study. Ultrastruct Pathol 1986, 10:157–173
11. Janossy G, Bofill M, Trejdosiewicz LK, Willcox HNA, Chilosi M: Cellular differentiation of lymphoid subpopulations and their microenvironments in the human thymus,[7] pp 89–125
12. Janossy G, Thomas JA, Bollum FJ, Mattingly S, Pizzolo G, Bradstock KF, Wong L, Ganeshaguru K, Hoffbrand AV: The human thymic microenvironment: An immunohistological study. J Immunol 1980, 125:202–212
13. Souadjian JV, Enriquez P, Silverstein MN, Pepin JM: The spectrum of diseases associated with thymoma. Arch Intern Med 1974, 134:374–379
14. Verley JM, Hollmann KH: Thymoma: A comparative study of clinical stages, histologic features, and survival in 200 cases. Cancer 1985, 55:1074–1086
15. Rosenow EC, Hurley BT: Disorders of the thymus: A review. Arch Intern Med 1984, 144:763–770
16. Gerdes J, Lemke H, Baisch H, Wacker HH, Schwab U, Stein H: Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984, 133:1710–1715
17. Chilosi M, Pizzolo G, Fiore-Donati L, Bofill M, Janossy G: Routine immunofluorescent and histochemical analysis of bone marrow involvement of lymphoma/leukaemia: The use of cryostat sections. Br J Cancer 1983, 48:763–775
18. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KAF, Stein H, Mason DY: Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984, 32:219–229
19. De Maagd RA, MacKenzie WA, Schuurman HJ, Ritter MA, Price KP, Broekhuizen R, Kater K: The human thymus microenvironment: heterogeneity detected by monoclonal anti-epithelial cell antibodies. Immunology 1985, 54:745–754
20. Steinman GG: Changes of the human thymus during aging,[7] pp 43–88
21. Levine GD, Polliack A: The T-cell nature of the lymphocytes in two human epithelial thymomas: A comparative immunologic, scanning and transmission electron microscopic study. Clin Immunol Immunopathol 1975, 4:199–208
22. Musiani P, Maggiano N, Aiello FB, Larocca LM, Piantelli M: Phenotypical characteristics and proliferative capabilities of thymocyte subsets in human thymoma. Clin Immunol Immunopathol 1986, 40:385–392
23. Dalakas MC, Engel WK, McClure JE, Goldstein AL, Askanas V: Immunocytochemical localization of thymosin-al in thymic epithelial cells of normal and myasthenia gravis patients and in thymic cultures. J Neurol Sci 1981, 50:239–247
24. Kyewski BA: Thymic nurse cells: Possible sites of T-cell selection. Immunol Today 1986, 7:374–379
25. Royer HD, Acuto O, Fabbi M: Genes encoding the Ti beta subunit of the antigen/MHC receptor undergo rearrangement during intrathymic ontogeny prior to surface T3-Ti expression. Cell 1984, 39:261–266
26. Snodgrass HR, Kisielow P, Kiefer M, Steinmetz M, von Boehmer H: Ontogeny of the T-cell antigen receptor within the thymus. Nature 1985, 313:592–595
27. Schuurman HJ, Wijngaert FP, Huber J, Schuurman RKB, Zegers BJM, Roord JJ, Kater L: The thymus in "bare lymphocyte" syndrome: Significance of expression of major histocompatibility antigens on thymic epithelial cells in intrathymic maturation. Hum Immunol 1985, 13:69–82
28. Matsui M, Kameyama M: A double-label flow cytometric analysis of the simultaneous expression of OKT4

and Leu2a antigens on circulating T lymphocytes in myasthenia gravis. J Neuroimmunol 1986, 11:311–319
29. Kyewski BA: Thymic dendritic cells present blood-borne antigens to medullary thymocytes in vivo : A possible role in the generation of the T-cell repertoire, Haematology and Blood Transfusion. Vol 29: Modern Trends in Human Leukemia VI. Edited by Neth, Gallo, Greaves, Janka. Berlin, Springer-Verlag, 1985, p 486
30. Lo D, Sprent J: Identity of cells that imprint H-2-restricted T-cell specificity in the thymus. Nature 1986, 319:672–675

Acknowledgments

We thank Mrs. L. Montagna and Mrs. D. Toffali for their skillful technical assistance.