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ORIGINAL ARTICLE

Cellular and humoral immune alterations in thymectomized patients for thymoma

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Abstract The aim of this study was to analyze the impact of thymectomy on kinetics of the immune reconstitution in thymoma patients. Nine consecutive patients with completely resected thymoma were enrolled. Immunophenotype analysis (total lymphocytes, CD3, CD4, CD8, CD19, NK subsets) and detection of autoantibodies at 6, 12, 18, and 24 months after thymectomy were planned. A prolonged inversion of CD4/CD8 ratio was present, due to a diminished number of CD4+ cells; CD8+ cell numbers remaining constantly normal at different time points; CD19+ cells remained for a long time understatement, achieving almost normal levels at 24 months; and NK cells always showed a normal amount. Autoantibodies against the muscle acetylcholine receptor were detected in four patients (44.4%) at the time of diagnosis, while antinuclear antibody were detected in eight patients (88.8%) at different time points during postthymectomy. A high incidence of multiple primary neoplasms was observed (66.6% of cases). Our study showed that cellular and humoral immune alterations are a common sequelae of postthymectomy. Further studies, a longer surveillance and a cooperative approach, due to the rarity of the disease, are necessary to define eventual implications of immune alterations on patient's outcome.

Keywords Thymoma · Thymectomy · Immunology · Antinuclear antibody · Immunosuppression

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Introduction

Thymomas are rare anterior mediastinal tumors arising from thymic epithelial cells, characterized by a histologic heterogeneity and a wide range of autoimmune and paraneoplastic syndromes which can be associated with it. Complete surgical resection, followed by radiation therapy in resected tumors with invasion of surrounding tissues, is the principal treatment modality for early stages of the disease.

Immune regenerative capacity of the thymus decreases with age, although a thymic function continues to be present in adults, even in older individuals [1–3]. Immune system age-related declining shows a hyporesponsiveness to mitogens [4] and a higher incidence of autoantibodies [5], due to T cell competence, with a reduced B cell proliferation induced by mitogens [6] mainly, not exclusively due to a decline of T cell function [5, 7].

Thymus has a crucial role in T cell ontogenesis. After maturation and selection in the thymus, T cells emigrate to peripheral lymphoid tissues and establish the peripheral T cell compartment. Alterations of immune system are often associated with thymomas. These neoplasms have intratumorous capacity of generating T cells, with an enrichment of autoantigen-specific T cells and an alteration of the peripheral T cell repertoire, causing thymoma-associated

paraneoplastic autoimmune disorders [8]. Moreover, the presence of epithelial cells with endocrine and neuroendocrine characteristics inside the thymic gland can explain the paraneoplastic syndromes associated with these tumors. In the present study, we analyze the impact of thymectomy on kinetics of immune reconstitution through immune phenotype analysis of peripheral blood lymphocytes and detection of autoantibodies in resected patients at different time points after thymectomy.

Materials and methods

Eligibility Patients with completely resected and histologically proven thymomas were reviewed. Baseline evaluation included individual's medical history and physical examination, chest X-ray, liver ultrasonography, computed tomography scan, bone scan, magnetic resonance imaging or X-ray when indicated, complete cell count, and biochemistry. Signed informed consent was requested.

Treatment Thymectomy was the primary treatment. Radiotherapy was planned in resected tumors with invasion of surrounding tissues.

Evaluation for myasthenia gravis Neurological examination, electrophysiologic evaluation with repetitive nerve stimulation and single-fiber electromyelography, and autoantibodies against the muscle acetylcholine receptor (AchRAb) analysis were done at the moment of first diagnosis or before surgery if myasthenia gravis was suspected. Pulmonary function testing was performed on the basis of symptoms. AchRAb levels were measured using commercially available assay kits according to the manufacturer's instructions. Technique assay is shown in Table 1.

Table 1 Autoantibodies panel

Type of autoantibodies	Technique
Autoantibodies against the muscle acetylcholine receptor	Radioimmunoassay
Antinucleus (ANA)	Indirect immunofluorescence assay
Anti-DNA	ELISA
Anti-ENA	
Anti-ENA (SSA-Ab)	ELISA
Anti-ENA (SSB-Ab)	ELISA
Anti-ENA (Sm-Ab)	ELISA
Anti-ENA (RNP-Ab)	ELISA
Anti-ENA (Scl 70 Ab)	ELISA
Anti-ENA (J01-Ab)	ELISA
Rheumatoid factor	Immunoturbidimetric assay
Antineutrophile cytoplasmic Ab	Indirect immunofluorescence assay
Antimitochondrial Ab	Indirect immunofluorescence assay
Antismooth muscle Ab	Indirect immunofluorescence assay
Anticardiolipin Ab (IgG)	ELISA
Anticardiolipin Ab (IgM)	ELISA
AGA IgG	ELISA
AGA IgA	ELISA
Antipancreatic islet cell Ab	Indirect immunofluorescence assay
Antiparietal cell Ab	Indirect immunofluorescence assay
Anti-endomysium Ab	Indirect immunofluorescence assay
Antithyroglobulin Ab	ELISA
Antithyroperoxidase Ab	ELISA

ELISA enzyme-linked immunosorbent assay, *AGA* antigliadin Ab, *ENA* extractable nuclear antigen

Bone marrow aspirate and biopsy These procedures were planned to detect primary hematologic disorders or bone marrow cancer metastases.

Clonogenic assay Clonogenic assays from bone marrow aspirate products and induced myeloid (colony-forming unit granulocyte–macrophage), erythroid (burst-forming unit erythrocyte), and multilineage colony formation (colony-forming unit granulocyte, erythrocyte, monocyte, and megakaryorocyte) were planned.

Immune reconstitution analysis Patients were followed for immune reconstitution for 24 months after thymectomy with peripheral blood samples at 6, 12, 18, and 24 months. Peripheral blood samples for immune phenotypic analysis were collected and analyzed for surface markers within 4 h. Immune phenotypic analysis was performed using fluorochrome-conjugated monoclonal antibodies and flow cytometry. Cells were incubated with monoclonal antibodies anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-CD16, and anti-CD56, conjugated with fluorescein isothiocyanate and phycoerythrin. CD4+ T lymphocytes were defined as cells positive for both CD4 and CD3 and CD8+ T lymphocytes were defined as cells positive for both CD8 and CD3. B lymphocytes were defined as cells positive for CD19, while NK cells as CD3−, CD16+, and/or CD56+. The absolute number of cells in any lymphocyte subset was automatically calculated by multiplying the percentage of specific antigen-positive cells per absolute number of lymphocytes. Functional assays were not planned. Lymphocyte subset numbers were measured using commercially available assay kits according to the manufacturer's instructions.

Autoantibodies analysis Peripheral blood samples at 6, 12, 18, and 24 months after thymectomy for the presence of

several autoantibodies were planned, in order to assess their frequency and significance. Autoantibodies analyzed and techniques are shown in Table 1. Autoantibodies levels were measured using commercially available assay kits according to the manufacturer's instructions. Peripheral blood samples at 6, 12, 18, and 24 months after thymectomy for immunoglobulins IgG, IgA, and IgM, circulating immune complexes, C3, C4, protein C, protein S, beta(2)-microglobulin, and thyroid function tests were also planned.

Results

Patient characteristics From March, 2002 to July, 2004, nine consecutive eligible patients with completely resected thymoma were enrolled (four males, five females); median age was 66 years (range 50–73), Eastern Cooperative Oncology Group performance status score 1 (range 0–1). Three patients showed multiple neoplasms: multiple synchronous neoplasms were diagnosed in two cases. Two patients were previously diagnosed having asthma, one patient was previously diagnosed having Graves' hyperthyroidism and asthma, while one patient was diagnosed having Good's syndrome at the time of diagnosis of thymoma. Patient's characteristics are shown in Table 2.

Treatment All the patients performed transsternal total thymectomy. Seven received postoperative radiation therapy. One affected by synchronous thymoma and colon cancer received adjuvant chemotherapy for six courses with folinic acid and 5-fluorouracil (Mayo Clinic regimen) for colon cancer; one patient with a synchronous thymoma, gastrointestinal stromal tumor (GIST) of the stomach, and breast cancer was treated with thymectomy and postoperative radiation therapy for thymoma, wide resection for the GIST, and conservative surgery and postoperative radiation therapy and endocrine therapy with tamoxifen for breast cancer.

Autoantibodies against the muscle acetylcholine receptor AchRAb were found in four patients (44.4%) at the time of histologic diagnosis of thymoma and were negative in other patients during follow-up.

Bone marrow aspirate and biopsy Seven patients had bone marrow aspirate and biopsy, two refused the procedure. No primary hematologic disorders or bone marrow cancer metastases were diagnosed.

Clonogenic assay Clonogenic assays from bone marrow aspirate products did not show any impaired myeloid, erythroid, or other lineage colony formation.

Follow-up At median time of follow-up of 61 months (range 48–75), all patients were free from relapse from thymoma or other previous or synchronous primary neoplasms. However, a high incidence of new primaries postthymectomy was recorded. One patient showed tonsil cancer 31 months after diagnosis of thymoma and was successfully resected; one patient developed spinal cord myelitis as long-term radiation-related toxicity 18 months after postthymectomy radiation therapy and successively diagnosed to have metastatic nonsmall cell lung cancer 31 months after diagnosis of thymoma and died of neoplastic disease; one patient developed myocardial infarction and diagnosed with metastatic primary unknown 38 months after diagnosis of thymoma and died of that disease. A common observation of new primaries diagnosed after thymoma was their extremely rapid evolution. Genetic disorders in patients enrolled in the present study affected by multiple neoplasms were investigated, resulting without evidence of any genetic mutation.

Immune reconstitution analysis All patients had a 24-month follow-up of immune reconstitution analysis. Patients referred at our institution from different hospitals after thymectomy or during or after postoperative radiation therapy. There was no significant difference in the absolute number of peripheral blood lymphocytes before and after resection. We investigated the immune phenotypic analysis after primary treatment.

Data regarding the median total lymphocyte, T cell (CD3+), CD4+ cell, CD8+ cell, CD19+ cell, and NK cell count at 6, 12, 18, and 24 months after thymectomy are shown in Table 3. The CD3+ cell had had lower numbers, although nearly normal values, at 6 and 18 months.

During the postthymectomy period, the CD4+ cell subset had a dramatic decrease after thymectomy, with a numerically limited increase at 24 months. During the period of immune surveillance, the CD4+ cell subset always had a lower number than normal. Absolute counts of CD8+ cells were normal at 6, 12, 18, and 24 months after thymectomy.

The mean number of circulating CD19+ B cells were extremely low, slowly increasing at 6, 12, and 18 months and reached almost normal values only 24 months after thymectomy. One patient with Good's syndrome developed severe hypogammaglobulinemia and B cell lymphopenia in the postthymectomy period, with recurrent infectious diseases requiring antibiotics. After the start of immunoglobulin therapy, the patient did not experience any relevant infectious disease.

NK cells had normal values at different time points, with a transient increase 12 months after thymectomy. An inversion of CD4/CD8 ratio of peripheral blood T cells was observed throughout the postthymectomy period

Table 2 Patient characteristics

Patient	Sex	Age at diagnosis	Surgical treatment	Diagnosis	WHO classification	Muller–Hermelink classification	Stage	Postoperative radiation therapy	AchR-Ab	Concomitant diagnosed diseases	Outcome thymoma	Other neoplasms	Other events	Survival
1	F	69	Total thymectomy	Thymoma	AB	Mixed	I	No	Yes	Asthma	FFR	No	None	Alive
2	M	70	Total thymectomy	Thymoma	AB	Mixed	I	No	No	Good's syndrome	FFR	Lower lip cancer[b] Colon cancer[c]	None	Alive
3	M	50	Total thymectomy	Thymoma	B2	Cortical	II	Yes	Yes	No	FFR	Tonsil cancer[b]	None	Alive
4	F	61	Total thymectomy	Thymoma	AB	Mixed	II	Yes	Yes	Asthma	FFR	Meningioma[b]	None	Alive
5	M	70	Total thymectomy	Thymoma	AB	Mixed	II	Yes	No	No	FFR	Nonsmall cell lung cancer[b]	Spinal cord myelitis	Died of lung cancer
6	F	66	Total thymectomy	Thymic spindle cell/papillary carcinoma[a]	C	Thymic spindle cell/papillary carcinoma	III	Yes	No	Graves 'hyperthyroidism', Asthma	FFR	No	None	Alive
7	M	55	Total thymectomy	Thymoma	B2	Cortical	II	Yes	Yes	No	FFR	No	None	Alive
8	F	73	Total thymectomy	Thymoma	B1	Predominantly cortical	IVa	Yes	No	No	FFR	Metastatic primary unknown	Myocardial infarction	Died of primary unknown
9	F	55	Total thymectomy	Thymoma	B2	Cortical	II	Yes	No	No	FFR	GIST[c] Breast cancer[c]	None	Alive

FFR free from relapse
[a] Spindle cell/papillary thymic carcinoma with neuroendocrine and medullary epithelial cell marker expression
[b] Metachronous
[c] Synchronous

Table 3 Kinetics of immune reconstitution

Per µl	+ 6 months	+ 12 months	+ 18 months	+ 24 months
Lymphocytes				
Median value	1,543	2,216	1,419	1,818
Range	756–2,500	980–2,837	1,166–2,068	1,128–3,876
CD3+				
Median value	1,057	1,500	1,184	1,325
Range	451–2,150	460–2,352	441–1,369	626–3,295
CD4+				
Median value	519	709	558	662
Range	213–925	309–807	260–747	247–1,124
CD8+				
Median value	552	706	515	625
Range	205–1,075	150–1,501	161–650	233–2,085
NK				
Median value	326	480	362	320
Range	175–449	419–584	60–583	59–496
CD19+				
Median value	79	61	111	184
Range	5–268	2–221	2–304	2–305
Ratio CD4/CD8				
Median value	0.89	1.15	0.9	0.9
Range	0.8–1.4	0.5–1.4	0.6–1.7	0.5–1.8

(median 0.9, range 0.85–1.8). This prolonged inversion of CD4/CD8 ratio was due to a diminished number of CD4+. Kinetics of immune reconstitution is shown in Table 3.

Autoantibodies analysis Antinuclear antibody (ANA) were detected in eight patients (88.8%) at different time points postthymectomy period. First detection of ANA occurred after 6 months in three patients (33.3%), at 12 months in two patients (22.2%), and at 24 months in three patients (33.3%). The incidence of anti-DNA and anticardiolipin was respectively 22.2% (two patients) with a first detection at 12 months and 11.1% (one patient) with a first detection at 24 months. One patient continuously remained without evidence of autoantibodies at 27 months of follow-up.

Other autoantibodies, apart from those against the muscle acetylcholine receptor, were not found. No rheumathoid syndrome (autoimmune-related) was observed.

Discussion

Apart from primary and HIV-related immunodeficiencies, different acquired conditions can cause T cell function alterations. Dose-intensive chemotherapy with hematopoietic growth factor support [9, 10] can lead to an immune damage, with a degree of depletion depending on regimens and drugs administered, as selective lymphotoxicity of fludarabine or immunosuppressive activity of cyclophosphamide [11]. Immune system may be seriously damaged by allogeneic or autologous hematopoietic stem cell transplant [12], as also pathology of the thymus showed [13, 14], and multiple courses of high-dose chemotherapy caused further damage to T lymphocyte pools and/or thymic microenvironment than a single course [15, 16]. In these patients, T cell reconstitution may involve two different progenitor pools: a thymic-dependent and a thymic-independent progeny [17], with a relevant role of the thymus in the CD4+ cell maturation [18].

The CD4+ T cell function alterations can have relevant implications on B cell compartment, because a specific CD4+ cell seems to be able to inhibit the appearance of autoreactive cell clones [19]. Autoantibodies, in particular ANA, are frequently detected in aging adults, and a higher incidence of ANA is observed in patients with hematologic malignancies [20] or solid tumors [21] in respect of controls.

In thymectomized patients for thymoma, a paradoxical phenomena occurs due to three sequential events:

1. Thymoma can induce the onset of autoimmune disorders or immune deficiency.
2. Thymectomy causes T cell quantitative and qualitative alterations in lymphocyte population, particularly of CD4+ and CD19+ cells, with the onset of autoantibodies after surgery.

3. Patients with myasthenia gravis may receive immunosuppressive therapy after thymectomy, with further immunosuppression, and other clinical syndromes, apart from myasthenia gravis, requiring long-lasting corticosteroid therapy.

We monitored the impact of thymectomy through CD4+ cell counts. Durable quantitative and qualitative abnormalities in lymphocyte subset reconstitution characterize the postthymectomy immune recovery. An inversion of CD4/CD8 ratio of peripheral blood T cells was observed due to a diminished number of CD4+. Thymectomy damages T lymphocyte pools, but it does not seem necessarily lymphoablative. In the absence of thymus, T cell lymphopoiesis can take place in other lymphoid organs, mainly in the gut mucosa, as demonstrated by experimental models in athymic mice, generating mostly gammadelta T cells than alphabeta T cells (extrathymic pathway) [22]. In euthymic mice, the T cell precursors are firstly imprinted in the thymus and then colonize the gut proceeding with their differentiation in intraepithelial lymphocytes within the gut wall, where T cell receptor rearrangements can occur as result of local exposure to some cytokines such as interleukin (IL)-7 and IL-15 [22, 23]. The extrathymic pathway is shut off in normal conditions and can resume only when the thymopoiesis is defective [22, 24]. Thus, in thymectomized patients, the ontogeny of T cells can have several origin: from thymus, thymoma, and mucosa of the gut. Within the limitations of our small study of research, the results support the hypothesis that the development of CD4+ cells is due to peripheral T cell expansion.

Over CD4+ cells, thymectomy had a further secondary severe effect on residual B lymphocytes: CD19+ B cells deeply decreased after thymectomy, remaining extremely low at 6, 12, and 18 months, but reaching almost normal values at 24 months, except for the patient affected by Good's syndrome. Evaluations at planned time points after thymectomy did not give evidence of any decrease in NK cell subset. The thymus is not required for NK development [25] and experimental studies have given evidence that NK cells can differentiate from bone marrow progenitors in long-term bone marrow cultures [26, 27].

AchRAb were found in four patients (44.4%), with similar incidence as larger series [28]. A high incidence of antinuclear autoantibodies was found in thymoma patients (88.8%); however, apart from AchR, clinical symptoms correlated to autoimmune alterations were not recorded.

An increased rate of second primary malignancies in thymoma patients is reported [29, 30]. In our little series, a high incidence of multiple neoplasms (66.6% of patients) was observed. Cellular and humoral immune alterations occurring in patients affected by thymoma could have implications on patient's outcome, raising the question of the role of tumor immune surveillance.

The deep immune alterations observed after thymectomy must be taken in account in planning follow-up programs. Our study needs to be confirmed, and further investigations, a long-term surveillance and a cooperative approach, due to the rarity of the disease, are necessary to better define the clinical significance of long-lasting decreased CD4+ cell number and the onset of autoantibodies in thymectomized patients for thymoma. Moreover, future investigations should also include monitoring of T cell reconstitution through the phenotypical characterization of T cell receptor for a better understanding of the ontogeny of peripheral T cells in thymectomized patients.

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