Flow Cytometry in the Differential Diagnosis of Lymphocyte-Rich Thymoma From Precursor T-Cell Acute Lymphoblastic Leukemia/Lymphoblastic Lymphoma

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Abstract

We compared the antigen expression profile of thymocytes in lymphocyte-rich thymoma with that of precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma (T-cell ALL/LBL) cells using 4-color flow cytometry. In all 15 thymoma cases, the thymocytes demonstrated 3 distinct subpopulations. The least mature cells (double-negative) expressed low-density CD2 and CD5, high-density CD7, CD10, CD34, and heterogeneous CD4 and CD8. They had the lowest density CD45 expression and were surface CD3–. The immature cells (double-positive) expressed CD2, CD5, CD7, CD4, CD8, heterogeneous surface CD3, and intermediate-density CD45. They were CD10– and CD34–. The mature cells (single-positive) expressed CD2, surface CD3, CD5, CD7, and CD4 or CD8. The heterogeneous expression of surface CD3, CD4, and CD8 also created a characteristic smearing pattern for these antigens. In all 15 T-cell ALL/LBL cases, the lymphoblasts formed a tight cluster without discrete subpopulations or smearing pattern. Of 5 double-negative cases, 4 demonstrated loss of CD2, CD10, or CD34 expression. Of 7 double-positive cases, 5 showed complete loss of surface CD3, CD2, and/or CD5; 4 were CD10+; and 2 were CD34+. Of 3 single-positive cases, 2 showed loss of CD2 and/or aberrant expression of CD34. Analysis of antigen expression pattern, the presence or absence of T cell–associated antigen deletion, and the expression of CD10 and CD34 by 4-color flow cytometry can help differentiate thymoma from T-cell ALL/LBL.

Thymocytes are immature T lymphocytes with varying degrees of maturation. They commonly are encountered in the study of anterior mediastinal masses and often are a significant component of hyperplastic thymus or lymphocyte-rich thymoma.[1-3] Precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma (T-cell ALL/LBL) also is a tumor frequently occurring in the anterior mediastinum.[4] When a large biopsy or resection specimen is available for review, the morphologic differentiation between thymoma and T-cell ALL/LBL, in general, is straightforward. Owing to its anatomic location, however, the initial study of a mediastinal mass often commences with fine-needle aspiration (FNA) or mediastinoscopic biopsy, severely limiting the quantity of material available for histologic diagnosis. Differentiation of thymoma from T-cell ALL/LBL based on histologic examination can be confounded further by a thymoma that has a predominance of lymphocytes.[5-8]

Flow cytometric immunophenotyping (FCI) is one of the standard ancillary study modalities in the evaluation of lymphoid lesions, including anterior mediastinal masses. Although it has been used extensively to study the immunophenotype of thymocytes in normal and hyperplastic thymus and in thymomas, its usefulness in the differentiation of lymphocyte-rich thymoma from T-cell ALL/LBL has not been very well described.[2,9-17] One reason for the lack of this information in the literature is that thymocytes and T-cell ALL/LBL cells share many of the same antigens, making flow cytometric differentiation difficult by simply analyzing the expression of these individual antigens by using 1- or 2-color flow cytometry. We performed a comprehensive analysis of antigen-expression profiles of thymocytes in lymphocyte-rich thymomas and T-cell ALL/LBL lymphoblasts to determine

whether the expression pattern of certain antigens can be used to distinguish these entities by using 4-color flow cytometry.

Materials and Methods

Case Selection

A prospective analysis of lymphocyte-rich thymoma and T-cell ALL/LBL cases at Emory University Hospital during a 20-month period (July 2000 to March 2002) was performed. Clinicopathologic data were obtained by reviewing medical records or from referring pathologists.

Morphologic Examination

FNA smears were air dried for staining with the Romanowsky method and/or fixed in alcohol for staining with the Papanicolaou technique. Cytocentrifuged slides of the body fluid specimen also were stained with the Romanowsky method. Excisional biopsy specimens were fixed in buffered formalin solution, sectioned, and stained with H&E. Peripheral blood and bone marrow aspirate smears were stained with the Wright and Giemsa method.

Flow Cytometric Immunophenotyping

FCI was performed on peripheral blood, bone marrow aspirate, body fluid, or fresh excisional biopsy tissue samples and/or FNA specimens collected in RPMI 1640 culture medium. Specimens were processed routinely, and single-cell suspensions were stained with various 4-fluorochrome–conjugated antibody combinations (fluorescein isothiocyanate, phycoerythrin, peridinin chlorophyll protein, and allophycocyanin) according to the lymphoma panel protocol routinely used in our laboratory. The antibodies in the panel included those against leukocyte common antigen CD45, B-cell antigens (CD19, CD20, CD22, κ and λ light chains), T-cell antigens (CD2, CD3, CD4, CD5, CD7, CD8), myeloid antigens (CD11b, CD11c, CD13, CD14, CD15, CD33, CD34, CD117), and CD10, CD16, CD25, CD36, CD38, CD56, CD103, and HLA-DR (Becton Dickinson Biosciences, San Diego, CA). Testing for terminal deoxynucleotidyl transferase was performed in permeabilized cells from selected cases. Approximately 10,000 events were acquired on a flow cytometer (dual-laser FACSorter, Becton Dickinson Biosciences) and analyzed using the CellQuest computer software program (Becton Dickinson Biosciences).

Immunohistochemical Analysis

Immunohistochemical staining for cytokeratin AE1/3 was performed on thymoma cases with paraffin-embedded tissue samples or cell blocks by using the avidin-biotin complex method.

Results

In this prospective study, we analyzed the surface antigen expression profiles of thymocytes in lymphocyte-rich thymoma and lymphoblasts in T-cell ALL/LBL. Of the 15 thymoma cases, 6 were FNA specimens and 9 were excisional biopsy specimens. Of 6 FNA specimens, subsequent tissue biopsy specimens were available for 2. Of 15 T-cell ALL/LBL cases, 4 were tissue biopsy specimens, 5 were bone marrow aspirates, and 2 were peripheral blood samples; for 1, both FNA and tissue biopsy specimens were available; and 1 was a pleural fluid sample. For 2 other cases, both peripheral blood and bone marrow aspirates were submitted for FCI analysis. The clinicopathologic data are summarized in **Table 1**.

The age of the patients diagnosed with thymoma ranged from 12 to 80 years (Table 1). The mean age was 53 years. The male/female ratio was 2:1 (male, 10 [67%]; female, 5 [33%]). Twelve patients with thymoma had mediastinal masses. Of the 12, 8 underwent biopsy, 3 had FNA, and 1 had FNA with a subsequent tissue biopsy of the mediastinal masses. In the remaining 3 patients, 1 had a left-sided chest wall mass and underwent excisional biopsy, 1 with a lung mass had FNA only, and 1 with a paraspinal mass had both FNA and biopsy. The presence of neoplastic thymic epithelial cells was confirmed in all cases by immunohistochemical staining for cytokeratins.

In contrast, patients diagnosed with T-cell ALL/LBL were much younger. Their ages ranged from 3 to 58 years, with a mean age of about 23 years. The male/female ratio was the same as that for the patients with thymoma. Of 15 patients, 7 had mediastinal masses and 1 of them had concurrent pleural effusion. Among the 7 patients with mediastinal masses, 2 underwent biopsy of the mediastinal mass, 4 had peripheral blood drawn or bone marrow aspiration performed, and 1 underwent thoracentesis. Of the remaining 8 patients, 2 had lymphadenopathy and underwent lymph node FNA and/or biopsy, 1 had a tonsillar mass and underwent tissue biopsy, and 5 had abnormal peripheral blood findings without a mediastinal mass or lymphadenopathy. The latter 5 patients had either peripheral blood drawn and/or bone marrow aspiration performed for FCI owing to leukocytosis or pancytopenia (Table 1). Of the 7 patients with a mediastinal mass, 4 (cases 17, 23, 28, and 30) were found to have peripheral blood or bone marrow involvement. Another patient with lymphadenopathy had later recurrence involving the breast (case 21). Morphologically, T-cell ALL/LBL blasts were indistinguishable from immature thymocytes on the FNA smears **Image 1**.

In the forward vs 90° right-angle flow cytometric plot, the thymocytes in all cases showed low light scatter properties, indicating a relatively small size and simple cytoplasmic

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Table 1
Clinicopathologic Data

Diagnosis/Case No./Sex/Age (y)	Clinical Manifestation	Anatomic Site	Specimen Type
Thymoma			
1/M/57	Mediastinal mass	Mediastinum	Biopsy
2/M/36	Left-sided chest wall mass	Chest wall	Biopsy
3/M/29	Mediastinal mass	Mediastinum	FNA
4/M/29	Paraspinal mass	Paraspinal area	FNA and biopsy
5/M/68	Mediastinal mass	Mediastinum	FNA
6/M/62	Mediastinal mass	Mediastinum	Biopsy
7/F/38	Mediastinal mass	Mediastinum	Biopsy
8/F/61	Mediastinal mass	Mediastinum	Biopsy
9/F/60	Mediastinal mass	Mediastinum	FNA and biopsy
10/F/42	Mediastinal mass	Mediastinum	Biopsy
11/M/74	Mediastinal mass	Mediastinum	FNA
12/M/73	Mediastinal mass	Mediastinum	Biopsy
13/M/69	Lung mass	Lung	FNA
14/M/12	Mediastinal mass	Mediastinum	Biopsy
15/F/80	Mediastinal mass	Mediastinum	Biopsy
T-cell ALL/LBL			
16/M/32	Leukocytosis	BM	Aspirate
17/M/20	Mediastinal mass	BM	Aspirate
18/M/58	Lymphadenopathy	LN	Biopsy
19/F/46	Pancytopenia	BM	Aspirate
20/M/19	Mediastinal mass	Mediastinum	Biopsy
21/F/22	Lymphadenopathy	LN and breast	FNA and biopsy
22/F/9	Mediastinal mass	Mediastinum	Biopsy
23/M/17	Mediastinal mass	PB	Aspirate
24/M/47	Leukocytosis	BM	Aspirate
25/F/12	Tonsillar mass	Tonsil	Biopsy
26/M/37	Leukocytosis	PB and BM	Aspirate
27/M/16	Leukocytosis	PB and BM	Aspirate
28/M/6	Mediastinal mass	BM	Aspirate
29/F/3	Mediastinal mass	Pleural cavity	Thoracentesis
30/M/7	Mediastinal mass	PB	Aspirate

BM, bone marrow; FNA, fine-needle aspiration; LN, lymph node; PB, peripheral blood; T-cell ALL/LBL, precursor T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma.

complexity. In the majority of thymoma cases, the thymocytes also displayed a distinct expression pattern of certain antigens. Of 15 thymoma cases, 14 exhibited a large population of thymocytes coexpressing CD4 and CD8 with tapering smaller populations of single-positive thymocytes exclusively expressing CD4 or CD8 **Image 2**. This in effect created a distinctive "smear" pattern in the CD4 vs CD8 dotplot diagram (Image 2D). A smear pattern also was observed

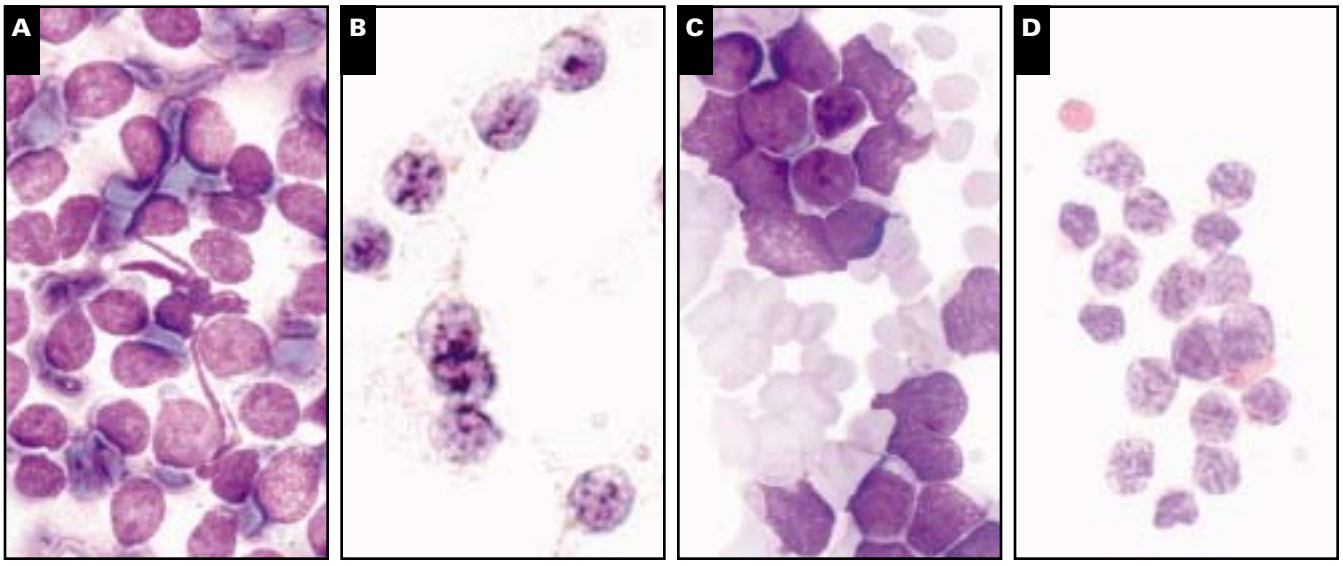

Image 1 Representative photomicrographs showing thymocytes (**A**, Romanowsky, ×500; **B**, Papanicolaou, ×500) and T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma lymphoblasts (**C**, Romanowsky, ×500; **D**, Papanicolaou, ×500). It is difficult to distinguish thymocytes from lymphoblastic lymphoma cells cytomorphologically.

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in surface CD3 vs CD4, CD8, and CD45 dot plots in the same 14 cases, with thymocytes displaying a spectrum of surface CD3 expression that ranged from high density to negative (Images 2A-2C). One case demonstrated CD4 and CD8 coexpression with no separation (case 13). In all 15 cases, the double-positive thymocytes lacked expression of CD10, CD34, and HLA-DR.

There was no loss of the T cell–associated antigens CD2, CD5, and CD7 in any of the thymoma cases studied. Interestingly, however, the density of their expression varied depending on the maturation stage of the thymocytes. The thymocytes in all cases, in fact, could be separated into 3 relatively distinct populations based on the expression of these T cell–associated antigens and CD45. The first population constituted 2% to 10% of the thymocytes. They had the lowest density CD45 expression and were positive for low-density CD2, low-density CD5, high-density CD7, CD10, CD34, and heterogeneous CD4 and CD8 **Image 3**. They were negative for surface CD3 (Image 3A), but were positive for terminal deoxynucleotidyl transferase in the 2 cases examined (data not shown). They represented the least mature double-negative thymocytes. The second population comprised the bulk of thymocytes. They expressed intermediate-density CD45 and were positive for normal-density CD2, CD5, and CD7. They were double positive for CD4 and CD8 but were negative for CD10 and CD34. The expression of surface CD3 among those cells was heterogeneous (Image 3). These cells represented the immature double-positive thymocytes. The last population constituted 5% to 15% of the thymocytes. They were positive for normal-density CD2, CD3, CD5, CD7, and CD4 or CD8 but were negative for CD10 and CD34 (Image 3). They constituted the majority of the mature, single-positive thymocytes.

The lymphoblasts in T-cell ALL/LBL cases displayed a drastically different antigen expression pattern. In the forward vs 90° right-angle flow cytometric plot, the lymphoblasts showed slightly increased light scatter properties compared with those of thymocytes, indicating relatively larger cell size and more complex cytoplasm. In 7 of 15 T-cell ALL/LBL cases, the lymphoblasts were double positive for CD4 and CD8. In the remaining 8 cases, 5 were double negative and 3 were single positive (2 CD4–CD8+ and 1 CD4+CD8–).

In all 7 double-positive T-cell ALL/LBL cases, the lymphoblasts displayed a very tight cluster without a smear pattern on the CD4 vs CD8 plot **Image 4A**. Of the 7 cases, 3 showed uniform expression of surface CD3 without the smearing pattern seen in thymocytes, while the other 4 showed complete absence of surface CD3 expression **Image 4B** and **Image 4C**. Terminal deoxynucleotidyl transferase also was expressed in all 7 cases and demonstrated a similar expression pattern **Image 4D**. The lymphoblasts in all surface CD3– T-cell ALL/LBL cases uniformly expressed

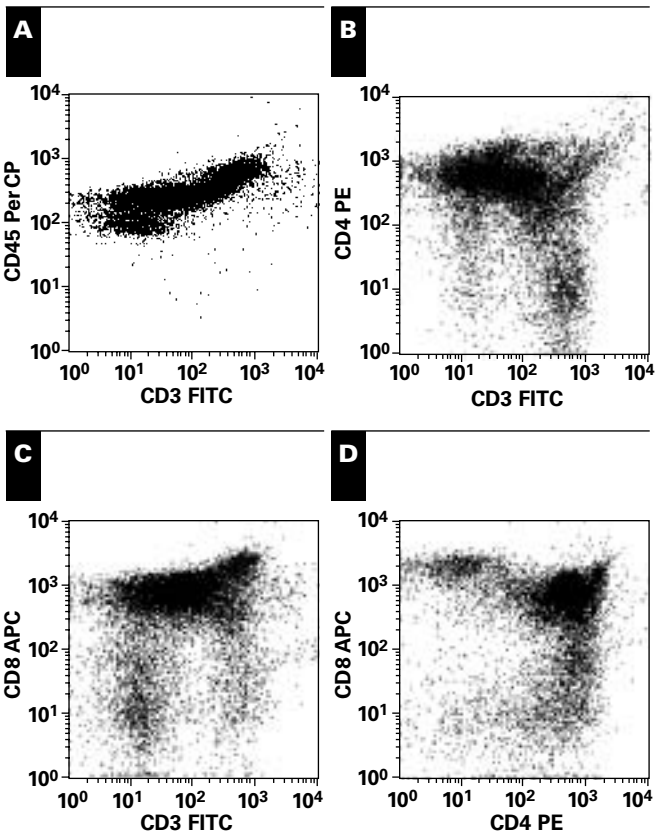

Image 2 Representative scatter plots showing a smear pattern of surface CD3, CD4, and CD8 expression in thymocytes (see "Results" for details). **A**, CD3 vs CD45. **B**, CD3 vs CD4. **C**, CD3 vs CD8. **D**, CD4 vs CD8. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

cytoplasmic CD3 (Image 4D). One double-positive case showed complete loss of CD2 and another loss of CD5 (not shown). Altogether, 5 of 7 double-positive T-cell ALL/LBL cases revealed loss of surface CD3, CD2, and/or CD5 antigen expression. There was no loss of CD7 expression. Among the 7 double-positive cases, 4 also were CD10+ and 2 were CD34+ **Image 4E** and **Image 4F**.

In the lymph node with partial involvement by double-positive T-cell ALL/LBL, the tight clustering and absence of smearing pattern for CD4 and CD8 expression clearly differentiated lymphoblasts from the CD3–, CD10+, CD34+ least mature thymocytes that always were accompanied by a larger population of more mature double-positive thymocytes in thymomas.

The lymphoblasts in the remaining T-cell ALL/LBL cases also were clustered tightly without a smear pattern for CD3, CD4, or CD8. They also exhibited abnormal antigen expression. Of the 5 double-negative cases, 2 showed complete absence of CD10 expression, 1 showed complete absence of CD2 and CD10 expression, and 1

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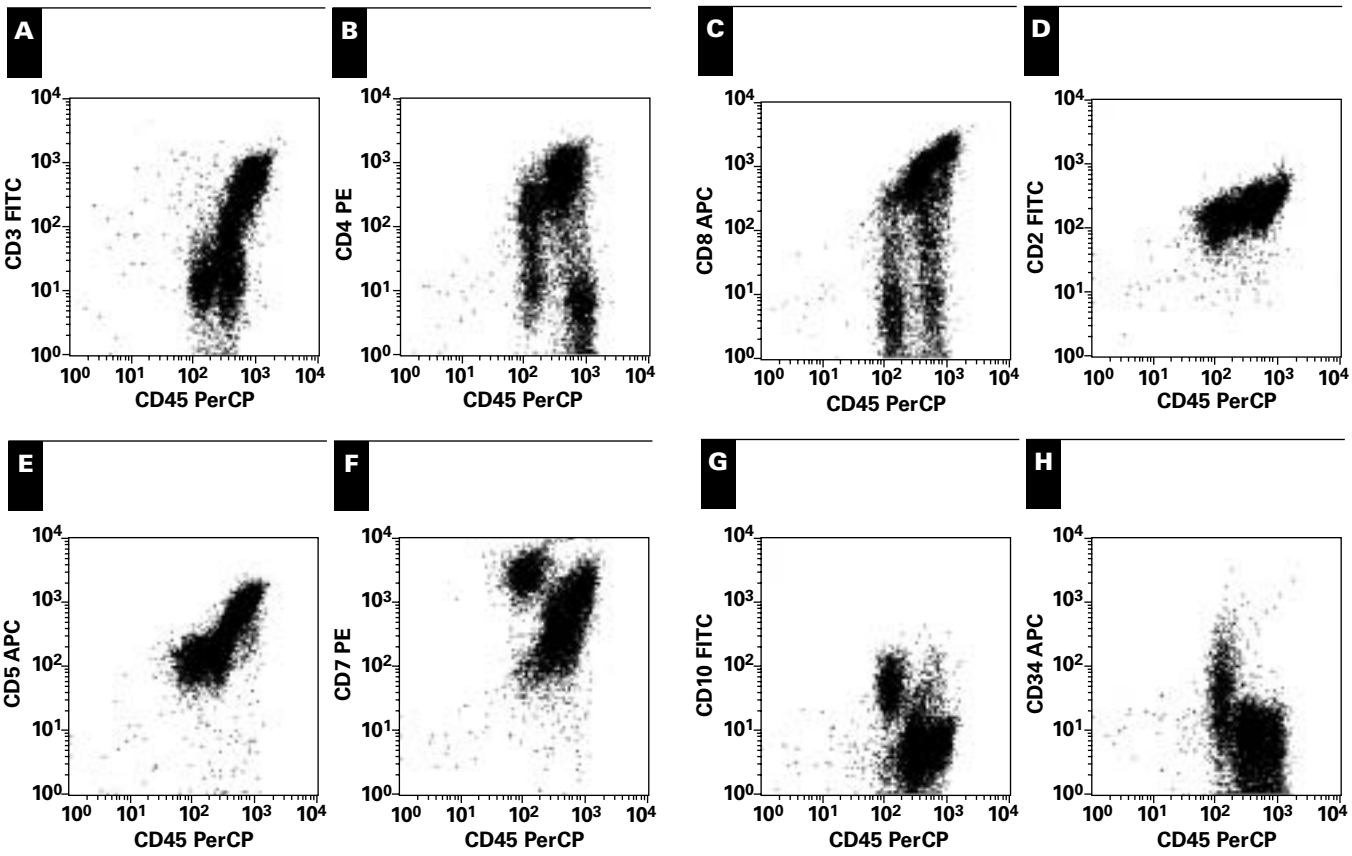

Image 3 Representative scatter plots showing the 3 distinct populations of thymocytes at different stages of maturation (see "Results" for details). **A**, CD45 vs CD3. **B**, CD45 vs CD4. **C**, CD45 vs CD8. **D**, CD45 vs CD2. **E**, CD45 vs CD5. **F**, CD45 vs CD7. **G**, CD45 vs CD10. **H**, CD45 vs CD34. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

showed complete absence of CD10 and CD34 expression. Of the 3 single-positive cases, 2 demonstrated aberrant expression of CD34, and 1 of them also demonstrated loss of CD2 expression.

Discussion

T cells originate from committed lymphoid progenitor cells, which themselves arise from the pluripotent stem cells in the bone marrow. Maturation of the T-cell precursors occurs in the thymus, at which point they are referred to as *thymocytes.* The differentiation process begins as CD4–CD8– cells in the cortex of the thymus, continues to differentiate to CD4+CD8+ double-positive cells predominantly in the cortex, and reaches final completion as CD4+CD8– or CD4–CD8+ cells in the medulla. The double-positive cells constitute the majority of thymocytes present in the thymus and represent the most characteristic immunophenotype.

Thymocytes occur in varying numbers in thymic lesions such as thymic hyperplasia and thymoma. Thymomas are the most common tumors of the anterior mediastinum. They typically consist of a well-demarcated tumor surrounded by a thick fibrous capsule. Microscopically, they are composed of a mixture of neoplastic thymic epithelial cells and nonneoplastic thymocytes, with the proportion of epithelial cells and thymocytes varying widely. Immunohistochemically, the neoplastic epithelial component of the thymoma expresses keratin and has been described to express epithelial membrane antigen.[5,8] In addition, thymomas often are associated with paraneoplastic syndromes. The prognosis depends on staging and the completeness of original excision, but, overall, the majority of cases have an excellent outcome.[18,19]

T-cell ALL/LBL arises from transformed precursor T cells that often can be identical to thymocytes immunophenotypically and, therefore, are referred to as having a "thymic" phenotype. T-cell ALL/LBL often similarly manifests as a mediastinal mass.[4] A small percentage of patients with T-cell ALL/LBL might have peripheral blood and bone marrow involvement, or the disease might manifest as acute leukemia without mediastinal enlargement or lymphadenopathy. In

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contrast with thymomas, patients with T-cell ALL/LBL are treated with chemotherapy once the diagnosis is established. Without aggressive treatment, T-cell ALL/LBL is almost always fatal.[20]

Thymomas occur for the most part in adult life; children rarely have this tumor. T-cell ALL/LBL, on the other hand, is a lesion that manifests in adolescents and young adults. With adequate sampling, the differential diagnosis between thymoma and T-cell ALL/LBL usually is uncomplicated, and confirmation frequently is by immunohistochemical analysis. In part because of the mediastinal location, however, the initial study of masses that can occur in both diseases commonly involves as minimally invasive a procedure as possible, such as FNA or mediastinoscopic biopsy. Although the specimens recovered from these techniques are extremely helpful, it sometimes can be difficult to differentiate thymocytes from malignant lymphoblasts histomorphologically, particularly when thymocytes predominate in the thymoma. Immunophenotypic analysis by immunohistochemical staining can be equivocal, especially in cell block preparations from FNA samples or small biopsy specimens from mediastinoscopy. Owing to the early stage of differentiation in thymocytes and T-cell ALL/LBL lymphoblasts, not only do these cells resemble each other cytologically but they also often share many similar immunophenotypic features, making flow cytometric differentiation problematic, particularly with 1- or 2-color flow cytometry.

Our prospective study using 4-color flow cytometry shows that FCI is indeed a useful ancillary tool in the differentiation of benign thymocytes and malignant T lymphoblasts. While the expression of similar surface antigens is inherent, careful examination of these antigen expression profiles permits reliable distinction between thymocytes and T-cell ALL/LBL lymphoblasts. In the majority of the thymoma cases examined in our study, the thymocytes displayed distinct expression patterns of certain antigens. Of 15 cases, 14 exhibited large populations of double-positive thymocytes with 2 tapering smaller populations of single-positive cells, which formed a distinctive smear pattern in the CD4 vs CD8 diagram. A similar pattern also was observed in the CD3 vs CD45 diagram in those cases. This characteristic pattern of surface CD3, CD4, and CD8 expression clearly contrasted with that of T lymphoblasts in the 7 double-positive T-cell ALL/LBL cases that showed a tight cluster without separation. Thymocytes in all 15 cases, including the case without a clear smearing pattern, could be separated into 3 distinct populations based on the expression of CD45 vs the T cell–associated antigens CD2, CD5, and CD7. This feature was not seen in any of the T-cell ALL/LBL cases in the present study. In addition, T-cell ALL/LBL lymphoblasts frequently showed deletion of the T

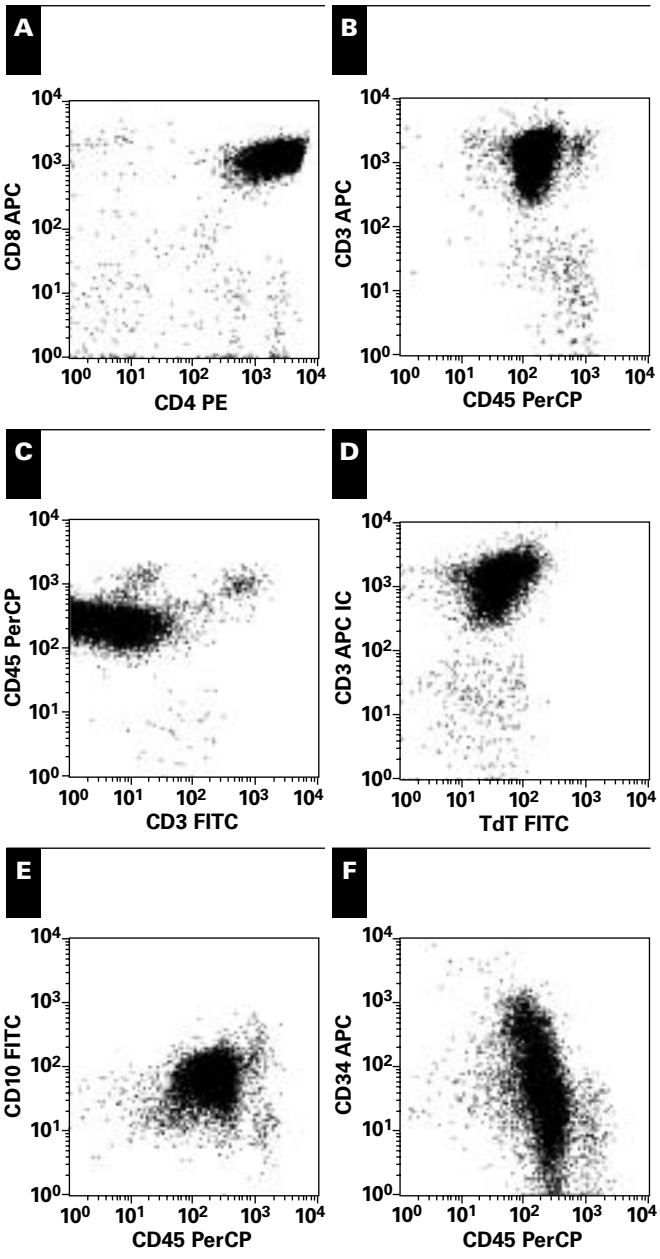

Image 4 Scatter plots showing expression of CD4 and CD8 (**A**), surface CD3 (**B**, **C**), intracytoplasmic (IC) CD3 and terminal deoxynucleotidyl transferase (TdT; **D**), CD10 (**E**), and CD34 (**F**) in T-cell acute lymphoblastic leukemia/lymphoblastic lymphoma lymphoblasts of selected cases. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin; PerCP, peridinin chlorophyll protein.

cell–associated surface antigens CD3, CD2, or CD5 and aberrantly expressed CD10 or CD34.

The relevance of the ability to distinguish benign thymocytes from malignant T lymphoblasts by FCI has many implications. With flow cytometry being the main method for immunophenotyping in FNA, FCI can be established definitively as a reliable technique to differentiate

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these 2 cell types as demonstrated in the present study. FNA obviates the need for surgery if a definitive diagnosis of T-cell ALL/LBL can be established for which chemotherapy is the conventional treatment modality. Patients with thymoma identified by FNA can later undergo surgical excision. Not all patients with T-cell ALL/LBL with mediastinal masses, however, have to undergo FNA or mediastinoscopic biopsy of the mediastinal mass itself. If they have stage IV disease or pleural cavity involvement, FCI of a peripheral blood sample, a bone marrow aspirate, or a pleural fluid sample can provide a rapid diagnosis and spare the patients from undergoing a more invasive procedure.

Immunophenotyping by 4-color flow cytometry can be a reliable technique to differentiate benign thymocytes from malignant T-lymphoblasts. This is particularly important when the pathologist must distinguish a lymphocyte-predominant thymoma from a T-cell ALL/LBL. These difficulties often are a result of the nature of the biopsy specimen because many mediastinal masses are examined primarily by FNA or mediastinoscopy. Diagnosis made by using these minimally invasive procedures can eliminate the need for invasive surgery in patients with T-cell ALL/LBL who can immediately begin chemotherapy.

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