American Journal of Pathology, Vol. 129, No. 3, December 1987
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RAPID COMMUNICATION

Utilization of Monoclonal Antibody L26 in the Identification and Confirmation of B-Cell Lymphomas

A Sensitive and Specific Marker Applicable to Formalin- and B5-Fixed, Paraffin-Embedded Tissues

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Immunophenotypic analysis of paraffin-embedded tissues of lymphoproliferative disorders has been facilitated by recent developments of monoclonal antibodies that react with epitopes that survive histologic processing. Leukocyte common antigen (LCA) antibody has made a significant contribution to the immunocytochemical separation of non-Hodgkin's lymphomas from nonlymphoid neoplasms. However, a small percentage of lymphomas, particularly some large cell or immunoblastic B-cell tumors, will not label with LCA antibody. Other antibodies, directed against B lymphocytes, experience problems of specificity and a lack of sensitivity when applied to formalin-fixed specimens. The authors recently investigated a monoclonal antibody (L26) that demonstrates excellent specificity and sensitivity for B lymphocytes, and tumors derived from them, in formalin- and B5-fixed, paraffin-embedded tissue. The avidin-biotin peroxidase complex (ABC) technique was utilized for immunostaining 95 cases of malignant lymphoproliferative disorders and a variety of normal and neoplastic nonlymphoid tissues. When applied to sections of benign lymphoid tissue, the L26 antibody labeled germinal center cells, mantle zone and scattered interfollicular lymphocytes, but not histiocytes or plasma cells. L26 marked 100% (44/44) of the large cell and immunoblastic B-cell lymphomas, along with 1 case of pre-B cell lymphoblastic lymphoma. This included 8 cases that were LCA-negative. None of the T-cell lymphomas or plasma cell tumors studied demonstrated L26 immunostaining. No normal, benign, or neoplastic nonlymphoid tissues examined stained with this antibody. L26 successfully labels B lymphocytes and B-cell lymphomas in routinely processed tissues, often with greater sensitivity and intensity than LCA. This antibody should prove invaluable in the investigation of atypical lymphoid proliferations and the identification of B-cell derived lymphomas, when fresh or frozen tissue is unavailable for analysis. (Am J Pathol 1987, 129:415–421)

THE DEVELOPMENT of monoclonal antibodies[1-10] that label lymphocytes in routinely fixed, paraffin-embedded tissues has contributed greatly to the confirmation and immunophenotyping of non-Hodgkin's lymphoma when fresh or frozen tissue is not available for investigation. Immunolabeling of histologically processed tissues eliminates problems associated with the securing, storage, transportation (if necessary), and interpretation of frozen specimens.

The introduction of leukocyte common antigen (LCA) antibody (clones PD7/26 and 2B11) has provided the surgical pathologist with an excellent marker to immunocytochemically distinguish lymphoid from nonlymphoid tumors.[1,11] Unfortunately, some lymphomas do not label with LCA when formalin-fixed tissue is assayed.[9,11,12] Also, large cell lymphomas may present a problem in immunolabeling because of low antigen density.[11,13] The fewer molecules of antigen a cell has on its cell surface, the less

Accepted for publication September 22, 1987.
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likely it will label after formalin fixation and tissue processing.

Monoclonal antibodies reactive with B-cell and T-cell antigens in paraffin-embedded tissues have shown promise in identifying B- and T-cell lymphomas, respectively.[2-10,13] However, most studies indicate that sensitivity decreases when formalin-fixed tissue is utilized.[2,3,7,10] Many of these cell lineage-specific antibodies also suffer problems resulting from a lack of specificity. B-cell antibodies LN1 and MB2 have been shown to react with a variety of nonlymphoid cells and neoplasms.[2,3,6] Therefore, they must be used as part of a panel to confirm lymphoid origin in an undifferentiated neoplasm. Others, LN2, MB1, and MB3, are not restricted to their labeling of B lymphocytes.[2,4,9,10]

Because the majority of lymphomas exhibit B-cell differentiation, it would be ideal to have an antibody that would reliably, and specifically, label B-cell lymphomas in formalin-fixed tissues. Ishii et al[14] recently reported six different monoclonal antibodies reacting with six distinct B-cell antigen systems. One of these antibodies (L26) reacts with a cytoplasmic antigen found in the majority of B cells present in the peripheral blood and lymphoid tissues. L26 was shown not to react with T-cell, myeloid, monocytic, or erythroid cell lines. When we immunostained formalin-fixed, benign lymphoid tissues with this antibody, we observed intense labeling of known B-cell areas.

We immunocytochemically investigated 95 cases of malignant lymphoproliferative disorders and a variety of normal and neoplastic nonlymphoid tissues that had been fixed in formalin and/or B5 with L26 to establish the antibody's ability to reliably and specifically label B-lymphocytes and B-cell tumors. Also, we compared the L26 immunoreactivity in B-cell lymphomas with that obtained with LCA antibody to establish whether L26 may be a more effective marker when one is confronted with histologically processed tissue.

Materials and Methods

Specimens

Tissues utilized in this study were selected from Hartford Hospital's Surgical and Hematopathology tissue files. All tissues investigated were fixed in 10% buffered formalin or B5 fixative. Cases of non-Hodgkin's lymphoma having frozen tissue were previously analyzed for B-cell clonality with the use of polyclonal antibodies to immunoglobulins (Kallestad, Austin, Tex). Also, immunophenotyping was performed with monoclonal antibodies to specific lymphocyte differentiation antigens (T1, T3, T4, T6, T8, T10, T11, B1, B2, B4, I2, Coulter Immunology, Hialeah, Fla; OKB-cALLa, Ortho Diagnostic Systems, Raritan, NJ; DRC-1, Ki-1, Ki-67, DAKO Corporation, Santa Barbara, Calif). Cases without frozen tissue were immunophenotyped with a panel of markers applicable to paraffin-embedded tissues (LN1, LN2, Techniclone International, Santa Ana, Calif; Leu-22 "L60," Becton Dickinson, Mountain View, Calif; UCHL1, IgA,1 IgD, IgG, IgM, Kappa, Lambda, DAKO). Normal tissues were obtained from autopsy procedures. Nonlymphoid tumors were all immunocytochemically confirmed using antisera to a wide variety of antigens (cytokeratins, S-100, melanoma antigen, vimentin, desmin, muscle-specific actin), depending on the given diagnostic situation.

L26 and LCA Immunocytochemistry

Paraffin sections, 5 μ thick, were placed on glass sides coated with Poly-L-lysine (Sigma Chemical Co., St. Louis, Mo; P1524) to promote tissue adhesion.[15] Previously melted sections (60 C, 30 minutes) were deparaffinized in Americlear (American Scientific Products), rehydrated through a graded alcohol series, and rinsed with tap water. B5-fixed sections were placed into iodine and 5% sodium thiosulfate, respectively, for removal of mercury pigment. Prior to immunostaining, all tissue sections were placed in 3% hydrogen peroxide for 5 minutes for quenching endogenous activity and then washed in phosphate-buffered saline (PBS). The avidin–biotin peroxidase complex (ABC) technique[16] was utilized for detecting the presence of primary antibody attached to antigen. Sections were incubated with nonimmune horse serum (Vector Laboratories, Burlingame, Calif) for 10 minutes. Without washing, excess nonimmune serum was removed, and optimally diluted primary antibodies (L26-1:200,000, sample—DAKO; LCA-1:1000, DAKO) were applied. Slides were placed in humidity chambers and incubated overnight (15 hours) in a 4 C refrigerator. After brief washes in PBS, sections were incubated sequentially with biotinylated horse anti-mouse IgG (1:200, Vector) and preformed ABC (1:100 each of avidin and biotinylated horseradish peroxidase, DAKO) for 30 minutes each. The presence of antigen was visualized by incubating sections in 3-amino-9-ethylcarbazole (Sigma) for 20 minutes. After rinsing in tap water and counterstaining in Lerner-2 hematoxylin (Lerner Laboratories, New Haven, Conn), the sections were coverslipped with DAKO Glycergel. Appropriate positive and negative controls were run in parallel with each batch of slides being immunostained.

Results

Benign Nonlymphoid Tissues

Monoclonal antibody L26 was found not to react with any of nonlymphoid tissues presented in Table 1. As expected, scattered lymphocytes demonstrated immunoreactivity in many of the tissues examined.

Nonlymphoid Tumors

No immunostaining with L26 was observed in any of the 124 nonlymphoid tumors listed in Table 2, including cases of amelanotic melanoma (10), Ewing's sarcoma (3), neuroblastoma (2), undifferentiated small cell carcinoma of the lung (5), rhabdomyosarcoma (5), and seminoma (2), which can all be difficult to distinguish from lymphoma. Once again, scattered lymphocytes present in the tissue sections of these neoplasms labeled positively (Figure 3). When sections containing residual lymphoid tissue, such as lymph node with metastatic carcinoma, were immunostained, the majority of labeling was confined to known B-cell areas.

Table 1—Normal Nonlymphoid Tissues Displaying No Immunoreactivity With L26 Monoclonal Antibody

- Skin
  - Epidermis
  - Sebaceous glands
  - Hair follicles
  - Eccrine glands
- Thyroid
  - Follicular epithelium
- Lung
  - Pneumocytes
  - Bronchial epithelium
- Liver
  - Hepatocytes
  - Bile duct epithelium
- Pancreas
  - Acini
  - Intercalated ducts
  - Interlobular ducts
  - Islets
- Prostate
  - Epithelial glands
  - Stroma
- Kidney
  - Proximal convoluted tubules
  - Distal convoluted tubules
  - Glomeruli
  - Collecting ducts
- Uterus
  - Endometrium
  - Myometrium
  - Ectocervix
  - Endocervix
- Brain
  - Neurons
  - Glial cells
- Other
  - Endometrium
  - Fibroblasts
  - Mesothelium
  - Muscle (cardiac, smooth, skeletal)
  - Placenta

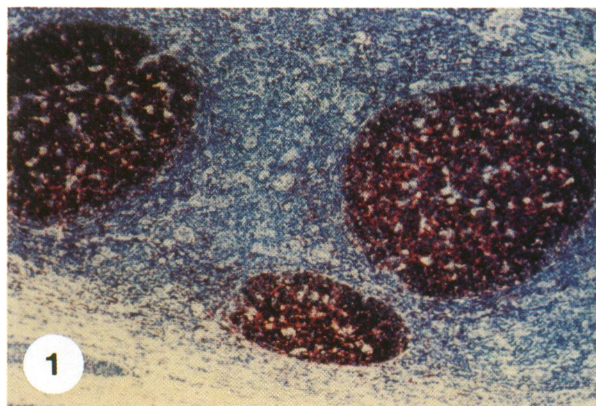
Table 2—Nonlymphoid Tumors* Displaying No Immunoreactivity With L26 Monoclonal Antibody

Diagnosis	Number of cases
Adenocarcinoma, breast	5
Adenocarcinoma, colon	5
Adenocarcinoma, lung	5
Adenocarcinoma, metastatic (no known primary)	4
Adenocarcinoma, pancreas	1
Adenocarcinoma, prostate	5
Adenocarcinoma, stomach	2
Ameloblastoma	1
Angiosarcoma	3
Astrocytoma	5
Carcinoid tumor	5
Cystoadenocarcinoma, ovary	2
Endometrial stromal sarcoma	3
Ewing's sarcoma	3
Fibroadenoma, breast	3
Ganglioneuroma	1
Hepatocellular carcinoma	3
Leiomyosarcoma	3
Malignant fibrous histiocytoma	5
Medullary thyroid carcinoma	3
Melanoma, amelanotic	10
Merkel cell tumor	4
Mesothelioma	5
Mixed mullerian tumor, uterus	1
Nasopharyngeal carcinoma	1
Neuroblastoma	2
Papillary thyroid carcinoma	2
Pancreactic neuroendocrine tumor	3
Parathyroid adenoma	1
Prolactinoma	2
Rhabdomyosarcoma	5
Salivary gland duct carcinoma	1
Seminoma	2
Small cell carcinoma (undifferentiated), lung	5
Squamous cell carcinoma	5
Thymoma	1
Thyroid carcinoma, anaplastic	1
Transitional cell bladder carcinoma	5
Wilm's tumor	1
Total	124

*Most tissue sections examined contained L26 immunoreactive lymphocytes.

Benign Lymphoid Tissues

Table 3 lists the observed L26 immunoreactivity in benign lymphoid tissues. Immunolabeling was concentrated in known B-cell areas (Figure 1) and was similar to that obtained previously with the lymphocyte differentiation antigen B1[17] (CD20) on frozen tissue sections. When serial sections were immunostained with L26 and LCA, B-cell areas consistently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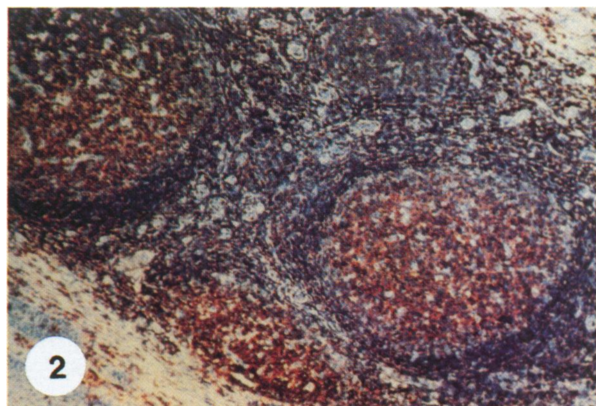
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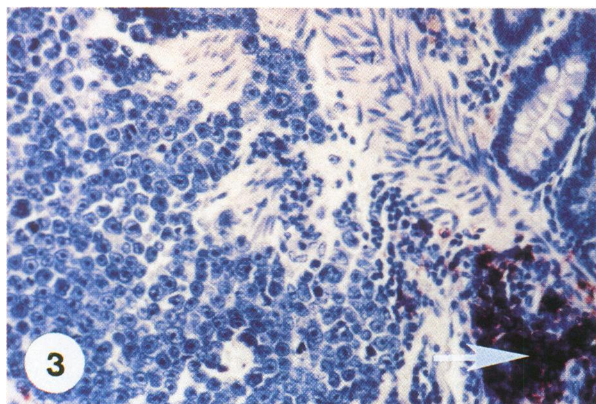
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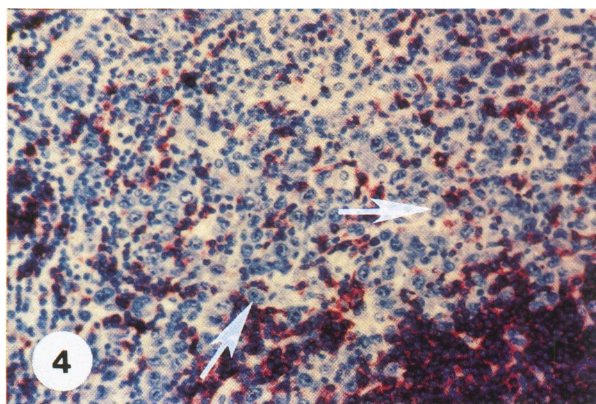
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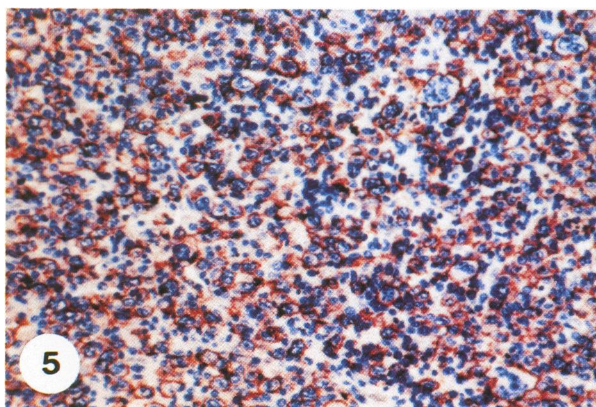
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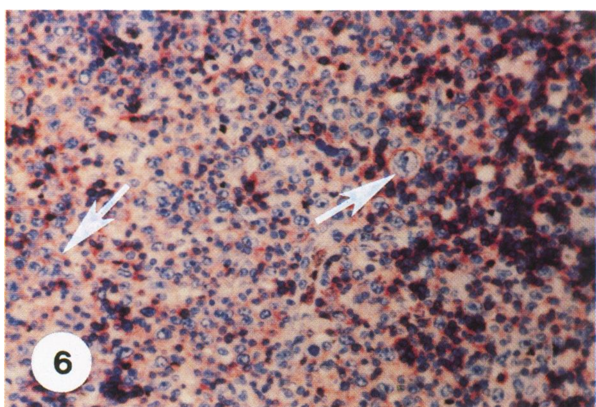
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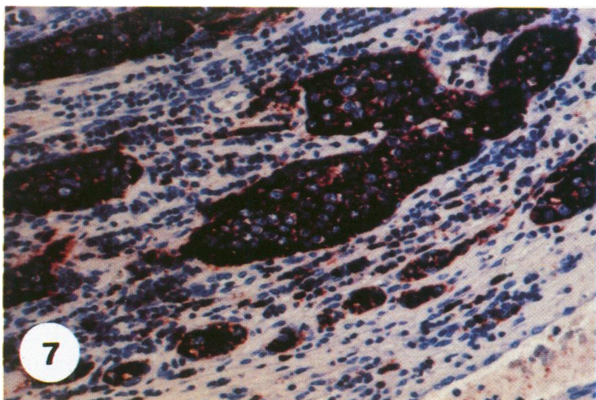
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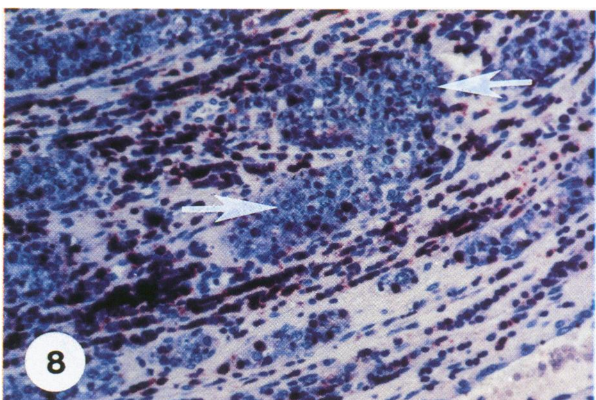
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Table 3—Distribution of L26 Immunoreactivity in Benign Lymphoid Tissues

Tissue	n*	L26 immunoreactivity
Lymph node/tonsil	8/12	
Germinal center cells		+
Mantle zone lymphocytes		+†
Interfollicular and medullary areas		−‡
Histiocytes (IRC, DRC, SH)§		−
Thymus	3	
Medulla		−‡
Cortex		−
Spleen	4	
White pulp (B-cell-dependent areas)		+
Red pulp		−‡
Bone marrow	6	
Erythroid cells		−
Myeloid cells		−
Megakaryocytes		−

*Number of tissues studied.
†Most cells demonstrated immunoreactivity.
‡Scattered positive cells identified.
§IRC, interdigitating reticulum cells; DRC, dendritic reticulum cells; SH, sinus histiocytes.

labeled more intensely with L26 than with LCA (Figures 1 and 2).

L26 and LCA Immunoreactivity in Non-Hodgkin's Lymphomas

L26 and LCA immunoreactivity in non-Hodgkin's lymphomas is presented in Table 4. Of the 95 cases investigated, 68 had been immunophenotypically confirmed as to B- or T-cell origin with the use of polyclonal and monoclonal antibodies and frozen tissue sections. The remaining 27 cases were confirmed by subjecting paraffin sections to a panel of antibodies (LN1, LN2, Leu-22, UCHL1, heavy and light chains) demonstrating immunoreactivity to lymphocyte antigens in processed tissue. The majority of cases without frozen tissue had been received in consultation from outside hospitals. Immunolabeling with L26 and LCA ranged from focal and weak to diffuse and

Table 4—L26 and LCA Immunoreactivity in Non-Hodgkin's Lymphomas

Histologic subtype*	n†	n-IP‡	IP§	Immunoreactivity to L26	Immunoreactivity to LCA
Small lymphocytic (WDL/CLL)	9	6	B	8	9
Small cleaved (follicular and diffuse)	14	11	B	14	14
Mixed, small and large-cell (follicular)	2	2	B	2	2
Intermediate grade, large-cell	25	16	B	25	20
High grade, large-cell, immunoblastic	19	11	B	19	17
High-grade, small noncleaved	4	2	B	4	4
High-grade, lymphoblastic (pre-B)	1	1	B	1	0
(T-cell)	1	1	T	0	0
T-cell lymphoma	7	7	T	0	6
Other					
Plasmacytoma/plasma cell myeloma	9	8	B‖	0	2
Hairy cell leukemia¶	4	3	B	4	4

*International Working Formulation.
†Number of cases examined.
‡Number of cases examined with frozen section immunophenotyping performed. Cases without frozen tissue were confirmed utilizing a panel of antibodies reactive with lymphocytes in histologically processed tissue.
§Cell lineage of those cases examined with immunophenotyping.
‖Cytoplasmic immunoglobulin identified.
¶This category includes 1 case obtained by bone marrow biopsy.

Figures 1–8—All are ABC immunoperoxidase preparations of formalin-fixed, paraffin-embedded tissue. All sections were counterstained with hematoxylin. The original magnification is listed in parentheses. **Figure 1**—L26 immunoreactivity in tonsil. Staining is concentrated in and around germinal centers. (×80) **Figure 2**—LCA immunoreactivity in tonsil. (×80) **Figure 3**—L26 immunoreactivity in a case of metastatic breast carcinoma involving the ileum which was thought to be a lymphoma on frozen section diagnosis. Tumors cells are negative and residual lymphocytes (*arrow*) exhibit positivity. (×200) **Figure 4**—Diffuse large-cell lymphoma (T-cell immunophenotype) in a cervical lymph node immunostained with L26. Staining is confined to residual small lymphocytes. Tumor cells (*arrows*) are nonimmunoreactive. (×200) **Figure 5**—Section of immunoblastic lymphoma immunolabeled with L26. All tumor cells reveal positive staining. (×200) **Figure 6**—Same case as in Figure 5 stained with LCA. Only scattered tumor cell staining (*arrows*) is appreciated. (×200) **Figure 7**—Inguinal lymph node with diffuse large-cell lymphoma displaying intense L26 immunoreactivity. Tumors cells also displayed LN1 and LN2 positivity and were nonimmunoreactive for cytokeratins, S-100, and melanoma antigen. Clinically, the patient was thought to have diffuse carcinomatosis. (×200) **Figure 8**—Serial section stained for LCA. Small lymphocytes demonstrate positivity while tumor cells (*arrows*) are completely devoid of any immunoreactivity. (×200)

intense. In general, B5-fixed tissue provided greater and more intense staining with both antibodies than that seen with formalin-fixed material from the same case.

L26 immunoreactivity was observed in 99% (77/78) of the B-cell tumors (excluding the plasmacytoma/plasma cell myeloma category) examined, whereas 90% (70/78) marked with LCA. The 1 L26-negative case (small lymphocytic lymphoma with IgG/Lambda sIg) was received in consultation, and poor formalin fixation may have contributed to the lack of immunolabeling. Cases with infiltrates of chronic lymphocytic leukemia (CLL) in tissue (3 cases) tended to react in a scattered fashion, and less intensely than other cases of lymphoma. Large cell and immunoblastic lymphoma, with a B-cell immunophenotype, consistently labeled better with L26 (44/44; 100%) than with LCA (37/44; 84%) (Figures 3 and 4). Extremely limited LCA positivity was seen in 6 cases (1 mixed, 1 large cell, 4 immunoblastic) and may have been called nonimmunoreactive with different sampling or utilization of a less sensitive immunostaining technique. The 8 cases (5 large cell, 2 immunoblastic, 1 pre-B-lymphoblastic) that did not label with LCA demonstrated strong immunoreactivity with the L26 antibody (Figures 7 and 8). No L26 tumor cell staining was observed in any of the cases of plasmacytoma, plasma cell myeloma, or T-cell lymphoma studied (Figure 4). Four cases of hairy cell leukemia (1 bone marrow, 2 spleen, 1 spleen and liver) tested positive for L26 expression.

Discussion

Ishii et al[14] previously reported on a monoclonal antibody directed against a cytoplasmic antigen, designated L26, which was shown to react with most B lymphocytes present in both blood and lymphoid tissues. When applied to formalin-fixed, paraffin-embedded sections of tonsils and reactive lymph nodes, we noticed that the L26 antibody produced excellent staining of lymphocytes localized in known B-cell areas. Although several other monoclonal antibodies reactive with B lymphocytes in processed tissue exist,[2,3,4,6,10] their applicability to immunodiagnosing lymphoma remains somewhat limited because of problems related to specificity and sensitivity. In order to ascertain L26's usefulness in identifying and confirming B-cell lymphomas in histologically processed tissues, we immunocytochemically investigated a variety of normal, benign, and malignant tissues.

L26 was found to be a specific and reliable marker for B lymphocytes in formalin- and B5-fixed tissues. In general, B5-fixed tissue resulted in better immunostaining with both L26 and LCA antibodies. No immunostaining was seen in any normal, benign, or malignant nonlymphoid tissue. All plasma cell tumors and T-cell malignancies were completely devoid of L26 immunoreactivity, except for scattered residual lymphocytes. In the majority of cases of B-cell lymphoma studied, L26 produced more intense labeling of tumor cells than LCA. Of the 44 cases of large-cell and immunoblastic lymphoma examined, 100% exhibited positivity with L26, whereas 84% demonstrated LCA immunoreactivity.

Our finding of 8 LCA-negative B-cell lymphomas is an important, and previously documented,[9,11,12] finding that can create a serious diagnostic dilemma when one is attempting to differentiate certain cases of large-cell lymphoma from epithelial and other mesenchymal tumors. We agree with Kurtin and Pinkus[11] that formalin fixation may contribute to "masking" of LCA. In all of our LCA-negative lymphomas, positive residual lymphocytes were present within the tissue section; and immunostaining for LCA on frozen tissue from a single case (diffuse large cell) revealed no immunoreactivity. Also, it is well known that large cell lymphomas can have lowered antigen densities on their cell surfaces; and, when immunocytochemically examined, this can result in variable staining intensity.[11,13] Therefore, we feel that immunocytochemical investigations of undifferentiated neoplasms should include L26 as well as LCA to rule out a lymphoid tumor of B-cell origin.

Three cases of hairy-cell leukemia localized in spleen and liver labeled positively with the L26 antibody. In addition, one consultation case consisting of a decalcified bone marrow biopsy demonstrated intense L26 staining. However, subsequent studies on bone marrow biopsy specimens processed at our institution reveal that decalcification may alter L26 immunoreactivity. This is presently under investigation.

From our immunocytochemical investigations we can conclude that the L26 monoclonal antibody is a specific and sensitive marker for benign and malignant lymphocytes of B-cell origin, applicable to routinely processed tissue. The antibody will contribute significantly to the identification and confirmation of B-cell lymphomas when fresh or frozen tissue is not available or presents problems in interpretation. At our institution, L26 has also aided in the investigation of atypical lymphoid proliferations and graft rejection on histologically processed tissues. Also, interesting information has been generated on L26 immunolabeling of Hodgkin's disease.[18]

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Note Added in Proof

Recent confusion has resulted concerning the use of Dr. Ishii's L26 and L27 monoclonal antibodies for immunolabeling B lymphocytes in paraffin-embedded tissues. We have found that the L26 antibody (IgG2a) produces immunoreactivity in paraffin tissue sections. Previous reports describing L27 immunoreactivity in paraffin-embedded tissues may in fact represent staining obtained with the L26 antibody.

Acknowledgments

We would like to thank DAKO Corporation for providing the sample of L26 monoclonal antibody used in this study (prepared in Dr. Ishii's laboratory). Also, we thank the Hartford Hospital Histology Laboratory for tissue section preparation, David R. Knibbs, MA (Electron Microscopy Division) for photographic assistance, and Wayne W. Hancock, MD, PhD (Surgical Research Division) for photographic assistance and useful discussions.