RAPID COMMUNICATION

Monoclonal Antibodies Marking T Lymphocytes in Paraffin-Embedded Tissue

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The authors investigated the ability of 70 monoclonal antibodies obtained from the Third International Workshop on Human Leukocyte Antigens (Oxford, 1986) to mark T lymphocytes in B5-fixed paraffin-embedded tissue. No staining occurred with 65 of the antibodies; however, 5 antibodies marked small lymphocytes in the T-cell areas of human tonsil. Two antibodies which strongly labeled lymphocytes, UCHL1 and T2/48, were used to examine 106 cases of non-Hodgkin's lymphoma, 29 cases of Hodgkin's disease, and a variety of normal and neoplastic tissues. UCHL1 and T2/48 each marked 86% (37/43) of B5-fixed T-cell lymphomas. Only 50% of formalin-fixed T-cell lymphomas were marked with these antibodies. UCHL1 marked 1.8% (1/56) of the B-cell lymphomas, compared with T2/48, which marked 19.6% (11/56) of the B-cell lymphomas. T2/48 had the interesting attribute of marking cells of the follicular mantle-zone and intermediate lymphocytic lymphoma, suggesting that the antibody recognizes a B-cell differentiation antigen. No Reed–Sternberg cells, epithelial neoplasms, sarcomas, neurogenic tumors, or normal nonlymphoid tissue were marked by either antibody. These antibodies successfully mark T cells in paraffin tissue sections and should aid in the investigation and characterization of abnormal lymphoid proliferations, "undifferentiated" malignant neoplasms, and immunologically mediated disorders. (Am J Pathol 1987, 127:1–8)

DURING the past decade monoclonal antibodies have played a key role in the immunophenotyping of lymphoid malignancies, and in the investigation of immunologically mediated disorders.[1] In particular, the study of fresh tissue by flow cytometry and frozen tissue sections by immunohistochemistry has elucidated the cellular origin of most lymphoid neoplasms and has defined stages of lymphoid differentiation.[2] A major impetus for these investigations has been the notion that the immunophenotype may have therapeutic implication in many diseases.[3–5]

A handicap to the immunophenotypic analysis of immune-mediated disorders and lymphomas is the requirement for fresh or frozen tissue. It has been particularly difficult to identify T lymphocytes in paraffin-embedded tissue. To determine whether any monoclonal antibodies from the Third International Workshop on Human Leukocyte Differentiation Antigens (Oxford, UK, 1986) might react with paraffin-embedded T lymphocytes, we systematically screened antibodies from the T-cell panel against paraffin-embedded sections of B5-fixed human tonsil. Two antibodies that strongly marked T cell dependent areas of tonsil are described in this report, along with their staining qualities against cases of non-Hodgkin's lymphoma, Hodgkin's disease, and a variety of other tumors.

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Materials and Methods

Immunohistochemistry

Fresh human tonsil was obtained immediately after excision and placed in B5 fixative. Tissue was processed routinely and embedded in paraffin. Five micron-thick sections were cut and placed on gelatin-coated slides. After rehydration with graded ethanol, the slides were washed with 0.1 M phosphate-buffered saline at a pH of 7.2 (PBS). Nonspecific binding of immunoglobulin was blocked by a 10-minute preincubation with normal horse serum. A separate section of tonsil was then incubated with each of 70 antibodies from the T-cell panel of the Third International Workshop on Human Leukocyte Differentiation Antigens (Oxford, 1986). Most of the antibodies were in murine ascitic fluid, and all were diluted to a titer of 1:200 before immunohistochemical studies. After a 30-minute incubation at 24 C with the test antibody, the slides were washed with PBS, and biotinylated anti-mouse immunoglobulin was applied (Vector Laboratories, Burlingame, Calif). After a PBS wash, endogenous peroxidase activity was blocked by a 5-minute incubation with 3% hydrogen peroxidase. The slides were again washed with PBS and then incubated for 30 minutes with avidin–DH horseradish peroxidase complex (Vector Laboratories). Antibody binding was then demonstrated with diaminobenzidine as a chromagen, against a light hematoxylin counterstain. The slides were examined with a light microscope, and the number of cells with positive surface staining was scored as follows: 0, negative; 1+, occasional scattered (<10%) cells staining; 2+, 10–75% of cells staining; 3+, greater than 75% of cells staining. The labeling intensity was graded as either weak (W) or strong (S).

Antibodies UCHL1 and T2/48 had strong labeling intensity of cells in T-cell–dependent zones of tonsil. These antibodies were used in dual-label immunohistochemistry in conjunction with commercial antibodies LN1 or LN2, which label B5-fixed paraffin-embedded B-cells[6,7] (Techniclone International, Santa Anna, Calif). In this procedure immunohistochemistry with the anti-T-cell antibody was completed through the development of the horseradish peroxidase chromogen. The sections were thoroughly washed; then either LN1 or LN2 was applied to the slide. The same immunohistochemical steps were used to visualize the binding of the LN-antibody, except that an alkaline phosphatase complex was used in the final step. By this technique cells binding UCHL1 or T2/48 were brown, whereas those binding LN1 or LN2 were pink. Serial sections were also stained with T2/48 or UCHL1 and the LN antibodies for investigation of the pattern of staining.

UCHL1 and T2/48 were further evaluated with 106 cases of human non-Hodgkin's lymphoma and 29 cases of Hodgkin's disease from archived materials at the University of Nebraska Medical Center. The non-Hodgkin's lymphomas had been previously and comprehensively characterized by immunohistochemistry on frozen tissue sections by means of monoclonal antibodies Leu-1 (CD5), Leu-3a/OKT4 (CD4), Leu-2a/OKT8 (CD8), Leu-4/OKT11 (CD3), and Leu-14 (CD22) (Leu antibodies: Becton-Dickinson, Sunnyvale, Calif; OKT antibodies: Ortho Diagnostics, Raritan, NJ). The frozen section immunophenotype of the non-Hodgkin's lymphomas was T-cell in 47 cases and B-cell in 59 cases. The non-Hodgkin's lymphomas were also classified histologically according to the International Working Formulation,[8] with the addition of a category for intermediate lymphocytic lymphoma.[9]

Results

Monoclonal Antibodies Not Marking Paraffin-Embedded Lymphocytes

Sixty-five of the 70 antibodies in the T panel did not mark lymphocytes in B5-fixed, paraffin-embedded human tonsil (Table 1).

Table 1—Monoclonal Antibodies Without Reactivity to Paraffin-Embedded Lymphocytes

D44	L25	100-3C6
K20	26.2	109-2D4
S152	2H8.3	CRIS7
XIX.8	9.6	K20
M241	7A9	Leu-3A
NA1/34	7H2.2	YTH26.5
30/3D6	8B25	YTH3.2
31/162	8F2.7	Anti-Leu-8
42/161	4B4	NA1/34
BL-TS-84	Leu-2A	T4
OR 101	BW135/80	TU13
John9-3	SCM2	TU68
1247	95-6-22	MG12
UCHT2	M-L412	NU-TPAN
PBS	M-T32	F937G2
VIT14	KS-6	Leu-2A
H25	KOLT-2	2A3
H366	UCHT1	4ELIC7
3Aid-14C2	OKT18A	HIT2
G3-7	CLB-9F4	HIT3
PHM3	MBG6	HI21
L24	T12/3PT12B8	

Monoclonal Antibodies Marking Paraffin-Embedded Lymphocytes in T-Dependent Areas

Five antibodies marked lymphocytes in T-dependent areas of the tonsil (Table 2). Weak staining of T-cell–dependent areas was observed with OKT17 and YTH80.103. Antibody UCHL1 had 3+ strong marking of interfollicular lymphocytes (Figure 1A); only scattered germinal center and follicular mantle zone cells were marked with this antibody, mirroring the normal distribution of T lymphocytes. Antibodies T2/48 and T2/53 had a similar pattern of staining, marking most interfollicular cells, follicular mantle zone cells, and scattered cells in the germinal center (Figure 1B). However, T2/53 stained fewer cells with weaker intensity than did T2/48.

Table 2—Antibodies Marking T-Cell Areas of Paraffin-Embedded Tonsil

Clone	Germinal center	Follicular mantle	Interfollicular
T2/48	1 + S	3 + S	3 + S
T2/53	1 + S	3 + S	3 + S
UCHL1	1 + W	1 + S	3 + S
OKT17	1 + W	1 + S	2 + W
YTH80.103	1 + S	1 + S	2 + W

0, negative; 1+, <10%; 2+, 10–75%; 3+, >75% cells labeled; W, weak; S, strong.

Dual Label Immunohistochemistry

Antibodies T2/48, or UCHL1 were used together with LN1 and LN2 in a dual-label immunohistochemical technique. Germinal center and follicular mantle zone cells were both marked with LN2, while LN1 principally marked germinal center cells. In the same tissue section interfollicular cells were marked with UCHL1, but no cells appeared to be simultaneously marked with UCHL1 and LN1 or LN2 (Figure 2A and B). However, simultaneous marking of follicular mantle zone cells occurred with T2/48 and LN2 (Figure 2C).

Non-Hodgkin's Lymphoma

Overall, UCHL1 and T2/48 each marked 37 of 43 (86%) cases of B5-fixed T-cell lymphoma. The num-

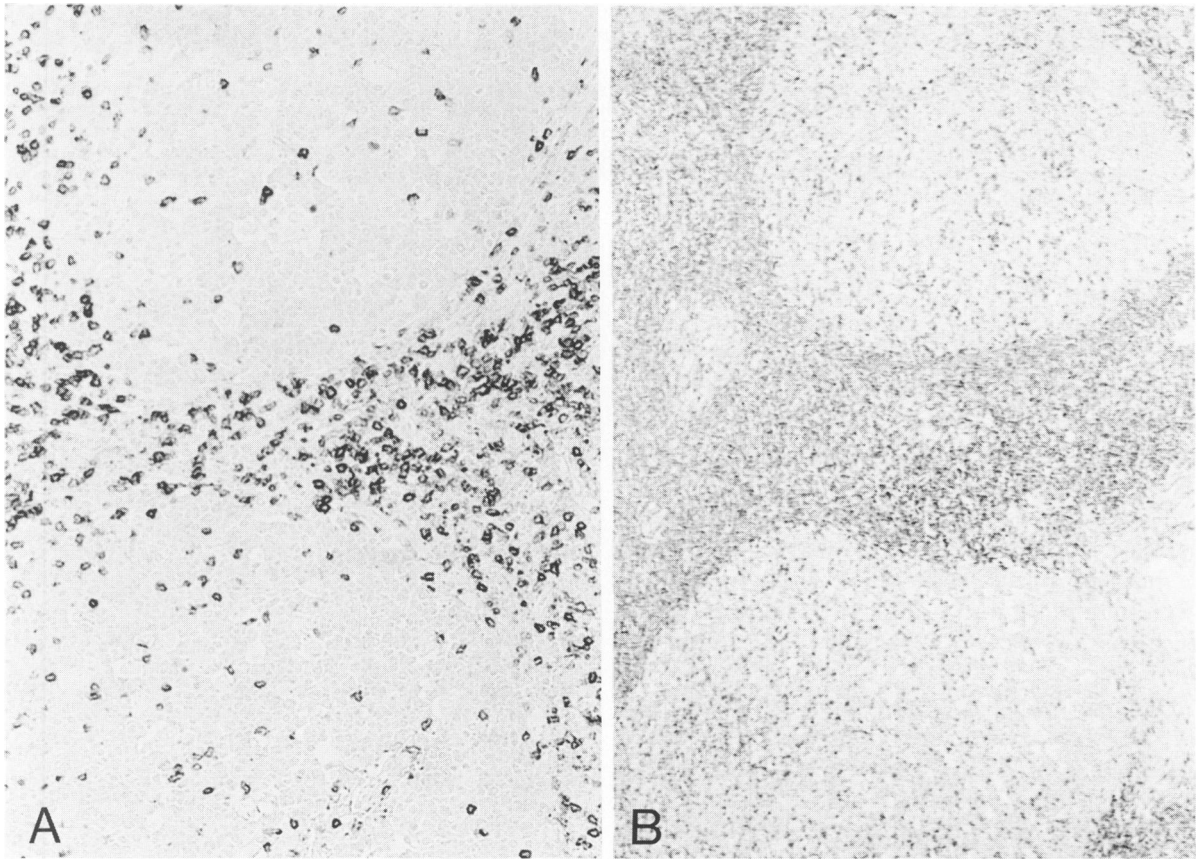

Figure 1A—B5-fixed human tonsil. Interfollicular cells are marked with UCHL1. Scattered germinal center cells also stain. (Immunoperoxidase–diaminobenzidine, original magnification, ×200) **B**—B5-fixed human tonsil. Interfollicular cells, follicular mantle zone cells, and scattered germinal center cells are marked with T2/48. (Immunoperoxidase–diaminobenzidine, original magnification, ×100)

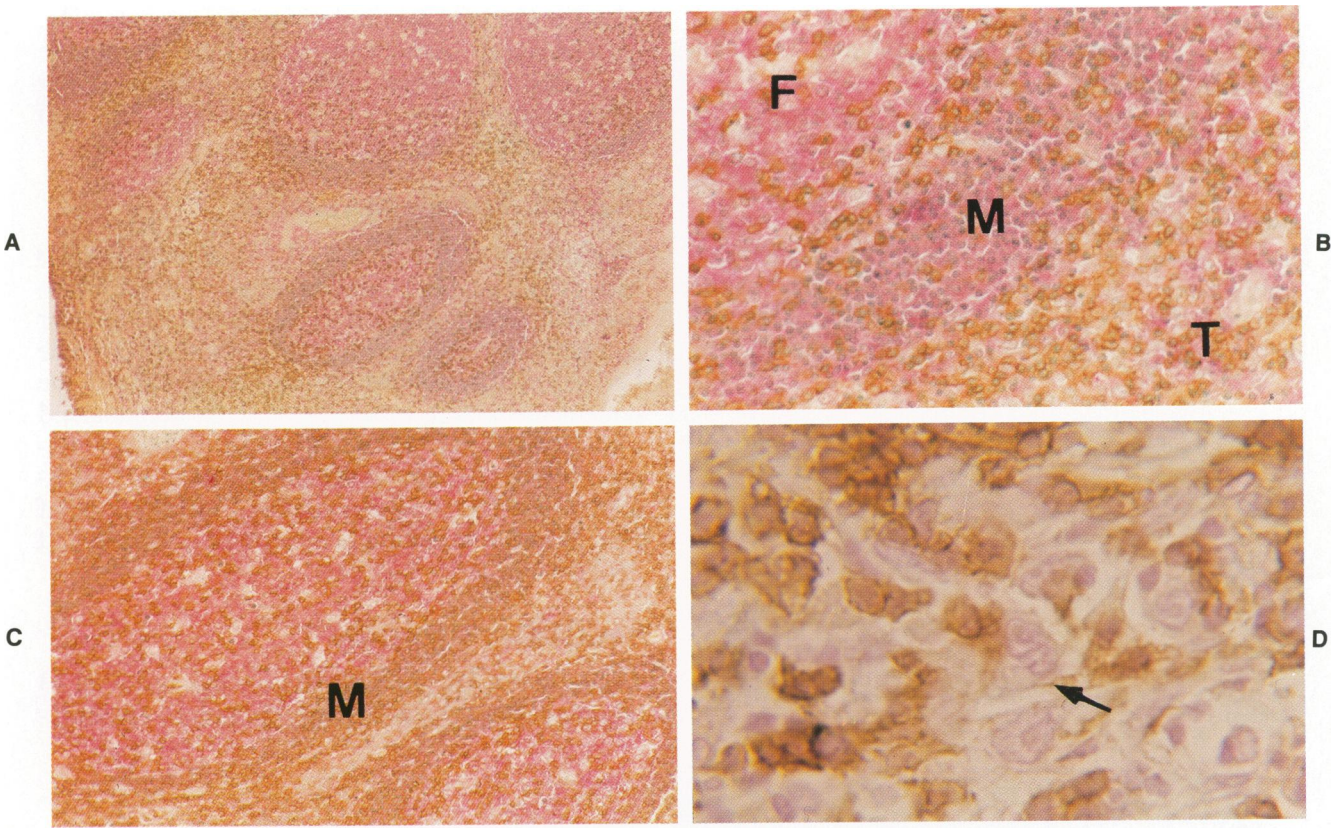

Figure 2A—B5-fixed human tonsil. Germinal center cells and follicular mantle zone cells are marked with LN2 (*pink*, alkaline phosphatase). Interfollicular cells and scattered cells are marked with UCHL1 (*brown*, diaminobenzidine) (Original magnification, ×40) **B**—Same as **A**. *F*, follicle; *M*, mantle-zone; *T*, T-cell–dependent area. (Original magnification, ×400) **C**—B5-fixed human tonsil. Interfollicular cells, follicular mantle zone cells (*M*), and scattered cells in the germinal center are marked with T2/48 (*brown*, diaminobenzidine). B lymphocytes are marked with LN2 (*pink*, alkaline phosphatase). (Original magnification, ×200) **D**—Hodgkin's disease. No staining is present in Reed–Sternberg cells (*arrow*). However, many adjacent small lymphocytes are marked with UCHL1. (Immunoperoxidase-diaminobenzidine, original magnification, ×400)

ber of cases marked with each antibody varied according to histologic subtype and the type of fixation (Table 3). Nearly all peripheral T-cell lymphomas with large-cell or mixed-cell histologic features were marked with these antibodies (Figure 3). Failure to stain was most frequent in lymphoblastic lymphoma. Only 50% of the formalin-fixed T-cell lymphomas were marked with UCHL1 and T2/48. In the formalin-fixed cases the number of cells that stained and the intensity of staining were usually less than in B5-fixed tissue.

UCHL1 and T2/48 marked 1 of 56 (1.8%) and 11 of 56 (19.6%) B5-fixed B-cell lymphomas, respectively (Table 4). The one B-cell lymphoma that marked with UCHL1 had diffuse large-cell histologic features (Figure 4A). Several different histologic subtypes of B-cell lymphoma were marked with T2/48, including all 4 cases of intermediate lymphocytic lymphoma (Figure 4B).

Table 3—T-Cell Lymphomas Marked With UCHL1 and T2/48

	UCHL1		T2/48	
Histology	B5	Formalin*	B5	Formalin
Diffuse small cleaved	2/2	1/2	2/2	2/2
Diffuse mixed	9/10	4/8	9/10	4/8
Diffuse large cell	2/2	ND‡	2/2	ND
Immunoblastic clear	6/7	0/2	7/7	0/2
Immunoblastic polymorphic	7/7	3/3	7/7	3/3
Immunoblastic epithelioid	3/3	0/2	3/3	1/3
Immunoblastic NOS†	3/3	2/2	3/3	1/2
Lymphoblastic	3/6	1/4	1/6	1/4
Mycosis fungoides	2/3	1/1	2/3	1/1
Total	37/43	12/24	36/43	13/25
Percent	86.0	50.0	86.0	52.0

*Ten percent neutral buffered formalin.
†NOS, not otherwise specified.
‡ND, not done.

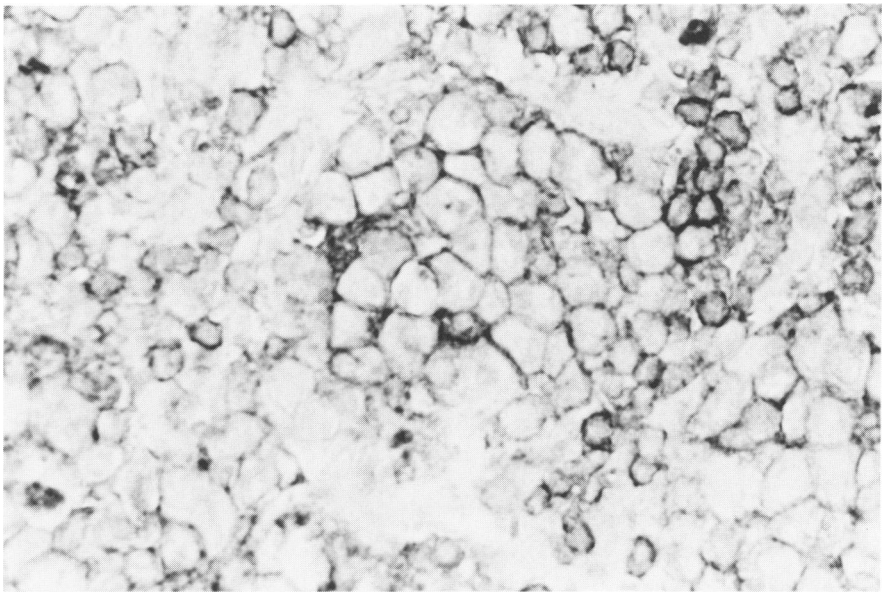

Figure 3—T-cell lymphoma. Neoplastic cells have strong cell-surface membrane staining with UCHL1. (Immunoperoxidase-diaminobenzidine, original magnification, ×400)

Hodgkin's Disease

Twenty-nine and 13 cases of Hodgkin's disease were examined with UCHL1 and T2/48, respectively. No Reed–Sternberg or atypical mononuclear cells had membrane or cytoplasmic staining with either antibody (Figure 2D). However, many of the background small lymphocytes demonstrated cell surface membrane staining.

Nonlymphoid Tissues

No staining of normal tissue from the liver, kidney, pancreas, lung, colon, thyroid, skin, muscle, breast, prostate, or uterus occurred with either UCHL1 or T2/48. Similarly, a variety of formalin-fixed neoplasms also failed to mark with these antibodies (Table 5). UCHL1 and T2/48 both marked normal thymocytes in the cortex and medulla of B5-fixed thymus gland and thymocytes of two lymphocytic thymomas. A slightly greater number of cortical cells were marked with T2/48 than with UCHL1. No staining of Hassall's bodies was seen.

Table 4—B5-Fixed B-Cell Lymphomas Marked With UCHL1 or T2/48

Histology	UCHL1	T2/48
Small lymphocytic	0/5	1/5
Follicular small cleaved cell	0/1	2/2
Follicular mixed cell	0/9	1/9
Follicular large cell	0/9	1/9
Diffuse intermediate lymphocytic	0/4	4/4
Diffuse cleaved cell	0/2	0/2
Diffuse mixed	0/2	1/2
Diffuse large cell	1/18	1/18
Immunoblastic	0/5	0/5
Small noncleaved	0/1	ND*
Total	1/56	11/56
Percent	1.8	19.6

ND, not done.

Discussion

Because most lymphocyte cell-surface antigens do not survive formaldehyde fixation and paraffin embedding, immunophenotyping has usually been performed on fresh or frozen tissue. Immunologic studies of paraffin-embedded tissue have largely been limited to the demonstration of cytoplasmic immunoglobulin in plasmacytoid B cells and plasma cells.[10] The commonly used murine monoclonal antibodies from CD3, CD4, and CD8 have had little or no ability to mark T lymphocytes in paraffin sections.[11] This has hampered experimental studies of lymphoproliferative, autoimmune, and inflammatory disorders.

There are several reasons the immunophenotyping of paraffin-embedded lymphocytes is desirable. Most laboratories have archives of paraffin blocks that permit study of uncommon diseases and when studied retrospectively provide for long-term patient follow-up. The cellular morphology is much better in paraffin sections than in frozen sections, thus permitting morphologic analysis of the relationship between cells. Finally, despite the best intentions of investigators, many important tissues are inadvertently placed in fixative, and the opportunity to study frozen tissue section is lost.

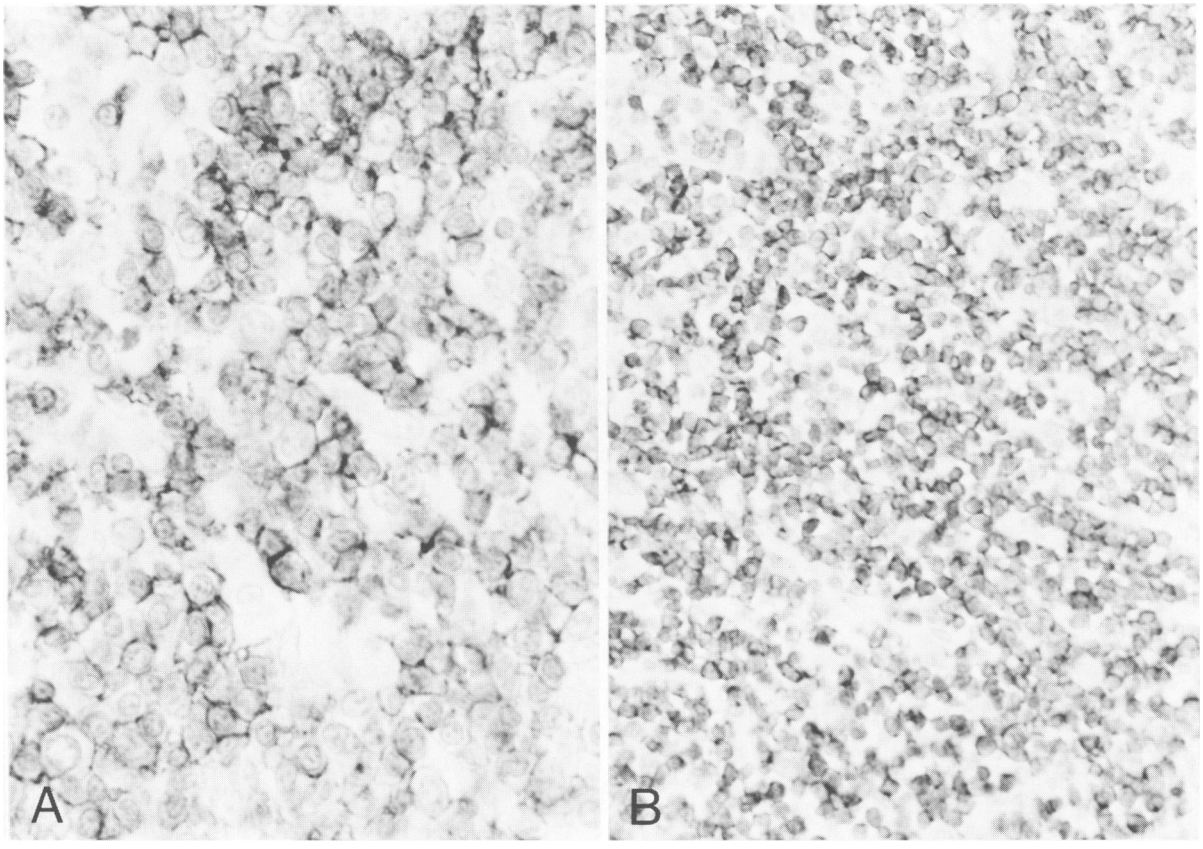

Figure 4A — Paraffin-embedded diffuse large-cell lymphoma marked with UCHL1. In parallel frozen tissue tumor cells were Leu-14$^+$. (Immunoperoxidase-diaminobenzidine, original magnification, ×400) **B**—All cases of intermediate lymphocytic lymphoma were marked with T2/48. (Immunoperoxidase-diaminobenzidine, original magnification, ×400)

Table 5—Nonlymphoid Tumors Not Marked With UCHL1 or T2/48

Adenocarcinoma
Colon
Breast
Ovary
Prostate
Lung
Thyroid
Squamous cell carcinoma
Uterine cervix
Lung
Skin
Neurogenous
Neurofibroma
Neuroblastoma
Melanoma
Anaplastic astrocytoma
Mesenchymal
Malignant fibrous histiocytoma
Alveolar rhabdomyosarcoma
Extraskeletal myxoid chondrosarcoma
Ewing's sarcoma
Others
Megakaryocytic leukemia
Medullary carcinoma of thyroid

One of the first lymphoid cell-surface antigens to be identified in paraffin tissue section was leukocyte common antigen.[12] This antigen is a 200-kd glycoprotein (T-200) expressed on most leukocytes derived from the bone marrow. Some antibodies against the T-200 antigen mark lymphocytes in paraffin-embedded tissue.[13-15] These antibodies have been useful to the surgical pathologist for identifying "undifferentiated" neoplasms.[15,16] Further expanding the ability of experimental and surgical pathologists to study paraffin-embedded lymphocytes have been the monoclonal antibodies LN1, LN2, and LN3, which mark paraffin-embedded B lymphocytes with varying degrees of specificity.[6,7] In different studies a combination of LN1 and LN2 has proven satisfactory in the identification of over 90% of B-cell lymphomas.[7,17]

In our studies the majority of antibodies from the T-cell panel of the Third International Workshop (Oxford, 1986) did not mark paraffin-embedded lymphocytes. However, three antibodies intensely labeled

the cell surface of lymphocytes in the T-cell–dependent areas of the tonsil. UCHL1 strongly stained interfollicular cells and a scattering of cells in the germinal center and follicular mantle zone, mirroring the normal distribution of T cells.[18] T2/48 and T2/53 marked interfollicular cells and follicular mantle zone cells with uniform strong staining. Weak staining of some cells in T-cell–dependent areas occurred with the antibodies OKT17 and YTH80.103.

Because of the strong staining of T-cell areas by UCHL1 and T2/48, these antibodies were reliable for all histologic types of T-cell lymphoma, with the exception of lymphoblastic lymphoma. Only one (1.8%) B-cell lymphoma was marked with UCHL1. T2/48, however, marked 19.6% of the B-cell lymphomas. The marking of all intermediate lymphocytic lymphomas by T2/48 is similar to Leu-1, a pan-T-cell murine monoclonal antibody that also recognizes follicular mantle zone cells and lymphomas derived from those cells.

UCHL1 was prepared by S. Smith in the laboratory of P. Beaverly.[19] It is a murine IgG 2a monoclonal antibody, with a molecular weight of 180–185 kd. Studies of the reactivity of UCHL1 with normal and neoplastic tissues by Norton et al[20] concur with our finding that UCHL1 is a reliable marker for T cells. However, several important differences between the two studies should be noted. None of the B-cell lymphomas reported in their series had membrane staining with UCHL1, whereas we found positive staining in 1 case. Although Norton et al favored neutral buffered formalin over mercurial-based fixatives, we found B5 superior to buffered formalin. Specifically, seven of our B5-fixed T-cell lymphomas had membrane staining with UCHL1 that was not present in corresponding formalin-fixed tissue. Only one T-cell lymphoma was marked in formaldehyde-fixed tissue and not in B5-fixed tissue. The discrepancies between the two studies are probably due to differences in the preparation of fixatives, tissue processing, and/or titer of antibody employed in the immunohistochemical procedure. In view of these differing observations, a prudent approach may be to examine both B5 and buffered formalin-fixed tissue when this antibody is used for investigative purposes. Finally, we did not find the cytoplasm of Reed–Sternberg or other normal cells to stain with UCHL1, as reported by Norton et al.[20]

T2/48 and T2/53 were prepared by E. Monostori (Szeged, Hungary) for investigation of the early events of T-cell activation. Although the functional properties of these two antibodies are as yet unknown, our studies suggest some similarity to Leu-1 and TU1. Leu-1 and TU1 react with T cells and with cells residing in the follicular mantle zone and the light zone of the germinal center.[21] Leu-1 will also mark intermediate lymphocytic lymphoma, which is a B-cell lymphoma derived from cells of the follicular mantle zone.[22] T2/48 and Leu-1 differ in that the latter will mark cells of small lymphocyte lymphoma, a feature we did not uniformly observe with T2/48. Nevertheless, the capacity of T2/48 to mark normal and neoplastic mantle zone lymphocytes suggests that the antibody may recognize a B-cell differentiation antigen.

Although UCHL1 and T2/48 are not completely characterized, preliminary data reported at the Third International Workshop suggests that both antibodies may cluster with antibodies to the leukocyte common antigen (CD45). The leukocyte common antigen is a 200-kd glycoprotein. Multiple epitopes are present on the leukocyte common antigen, some of which may be preferentially detected on T or B cells. The heavy glycosylation of the leukocyte common antigen may account for its ability to withstand routine fixation and tissue processing. Further studies of the biochemistry of the leukocyte common antigen and the specificity of UCHL1 and T2/48 for the antigen are clearly needed.

Our studies indicate that T2/48 and UCHL1 mark normal and neoplastic T lymphocytes in paraffin tissue section. UCHL1 appears more specific for T cells, in that T2/48 marks a subset of normal B cells in the human tonsil. These antibodies offer experimental and surgical pathologists exciting opportunities to study immunoproliferative disorders, immune-mediated diseases, and aspects of lymphocyte differentation in paraffin section.

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