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Rapid Communication

Antibody L26 Recognizes an Intracellular Epitope on the B-Cell-Associated CD20 Antigen

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Monoclonal antibody L26 is a highly selective marker of B cells and B-cell neoplasms in paraffin-embedded tissues, but it suffers from the drawback that the target molecule has not been identified. In this paper we provide evidence by two independent techniques that antibody L26 recognizes an intracellular epitope on the CD20 antigen (a pan B-cell marker). When this antigen was redistributed on the surface of unfixed viable B cells by incubation with monoclonal anti-CD20 followed by anti-mouse Ig, the diffuse cytoplasmic staining of L26 was abolished and replaced by coincident dotlike labeling for antibody L26 and the CD20 antigen. None of the other antibodies tested (covering 10 different B-cell-associated antigens) had this effect on the L26 staining pattern. Furthermore, COS-1 cells transfected with cDNA encoding the CD20 molecule gave positive staining with antibody L26 and with two other CD20 reagents, but not with antibodies to other pan B-cell markers (eg, CD19 and CD22). (Am J Pathol 136:1215–1222)

In recent years a series of monoclonal antibodies selectively reactive with human B lymphocytes have been produced (Table 1). Studies reported at the four International Workshops on Leucocyte Differentiation Antigens have allowed the molecules recognized by many of these antibodies to be identified and given a cluster of differentiation (CD) designation, and extensive literature has accumulated on their protein structure, on the genes that encode them and, in several cases, on their functional roles.

Although the B-cell-associated antigens listed in Table 1 have been used widely for the recognition of normal and neoplastic B cells in tissue sections,[1] their use in the context of routine histopathology has been restricted by the fact that they are denatured by routine tissue fixation and paraffin embedding. Pathologists who wish to detect B cells in routinely processed tissue are therefore obliged to choose from the few monoclonal reagents that react with this type of material. Unfortunately these reagents are often directed against unknown target molecules and/or against antigens that are not truly specific for B cells.

Empirical studies in a number of histopathology laboratories have shown that monoclonal antibody L26, first described by Ishii et al[2] in 1984, is the most selective marker among the currently available reagents for B cells in paraffin sections,[3–6] and in a recent review of published data, Norton and Isaacson[7] noted that it had been found to react with 99% and 93%, respectively, of low- and high-grade B-cell lymphomas, but with only 4% of high-grade T-cell neoplasms (all low-grade T-cell lymphomas having been negative). This level of cross-reactivity with non-B-cell neoplasms is lower than observed with other paraffin-reactive B-cell markers such as the LN and MB reagents,[7] and may be within the limits of experimental error.

Despite its wide use as a B-cell marker, the molecular target recognized by L26 has not been identified. The antibody was included in the last two International Workshops on Leucocyte Differentiation Antigens but it was categorized as an unclustered pan B-cell antibody. However, it was reported at the Third Workshop that the antigen recognized by antibody L26 is intracellular in location because strong staining of B cells is obtained in cell smears and tissue sections, but not when cells are labeled in suspension. This prompted us to wonder whether antibody L26 might react with an intracellular portion of one of the previously recognized B-cell-associated

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Table 1. *Human B-cell–Associated Antigens*

Antigen	Molecular weight	Comments	Antibodies used in this study	
			Reagent	Source
CD9	24 kd	Present on many non-B cells (eg, granulocytes, platelets)	BA-2	Hybritech, San Diego, CA
CD10 (CALLA)	100 kd	Only limited B-cell distribution. Present on many nonlymphoid cells	VIL-A1	Prof. W. Knapp, Vienna, Austria
CD19	90–95 kd	Pan B-cell marker	B4	Coulter Clone, Hialeah, FL
			HD37	Dakopatts, Glostrup, Denmark
CD20	32/37 kd	Pan B-cell antigen; appears later than CD19 in B-cell maturation	B1	Coulter Clone, Hialeah, FL
			93-1B3	Dr. R. Vilella, Barcelona, Spain
			Anti-Leu-16	Becton Dickinson, San Jose, CA
CD21 (CR2)	140 kd	Restricted B-cell marker; also on dendritic reticulum cells	B2	Coulter Clone, Hialeah, FL
CD22	140 kd	Pan B-cell marker; initially intracellular in location	Anti-Leu-14	Becton Dickinson, San Jose, CA
			4KB128	Author's Laboratory
CD23 (FcεRII)	45 kd	Present on only a minority of B cells, and also on some dendritic reticulum cells	Tü1	Biotest, Dreieich, FRG
CD24	42 kd	Present on many non-B cells	BA-1	Hybritech, San Diego, CA
CD37	40–45 kd	Weakly expressed on T cells and also found in macrophages	Y29/55	Dr. H. K. Forster, Hoffman-La Roche, Basel, Switzerland
FMC7 antigen	105 kd	Present on a subset of B cells	FMC7	Dr. H. Zola, Bedford Park, Australia
SmIg	Molecular weight dependent on Ig class	B-cell–specific marker	Polyclonal antisera	Kallestad Laboratories, Austin, TX

antigens listed in Table 1. An obvious candidate was the CD20 antigen (originally recognized by monoclonal antibody B1) because much of this molecule lies within the cell membrane and cytoplasm, with only a small part exposed on the cell surface (Figure 1). Furthermore, Ishii et al[2] reported that L26 immunoprecipitated two polypeptide chains with molecular weights of 30 and 33 kd, a finding very similar to that observed with known CD20 antibodies,[8] and Cartun et al[5] noted, when staining benign lymphoid tissue, the close similarity in labeling between L26 and antibody B1 (anti-CD20). The CD20 antigen, which shows no sequence homology to any other sequenced polypeptides, is a highly specific marker for B cells, although it appears later during B-cell differentiation than do CD19 and CD22.[1]

We have used two independent techniques to test this hypothesis. First, we performed cocapping experiments in which the CD20 molecules were redistributed on the surface of unfixed viable B cells by incubation in suspension at 37°C with a CD20 antibody followed by anti-mouse Ig. The cells were cytocentrifuged, fixed, and stained with antibody L26. If the intracellular epitope recognized by L26 is indeed on the CD20 molecule, one would expect that preincubation with a CD20 antibody such as B1 (but not with monoclonal antibodies to other surface antigens) would modify the intracellular staining pattern of L26 on B cells. In a second set of experiments, cells were transfected with cDNA clones that encode the CD20 molecule and then stained in cytocentrifuged preparations to determine if they had acquired cytoplasmic positivity for antibody L26. Because no other human genetic material is introduced into these transfected cells, positive staining with antibody L26 would constitute strong evidence that this reagent recognizes the CD20 molecule.

Materials and Methods

Cell Samples

Four human B-cell lines were studied in cocapping experiments, one (SMS-SB) of immature phenotype (surface Ig negative), and the other three (ROS-1, ROS-15, and ROS-17) of mature phenotype (expressing surface Ig).

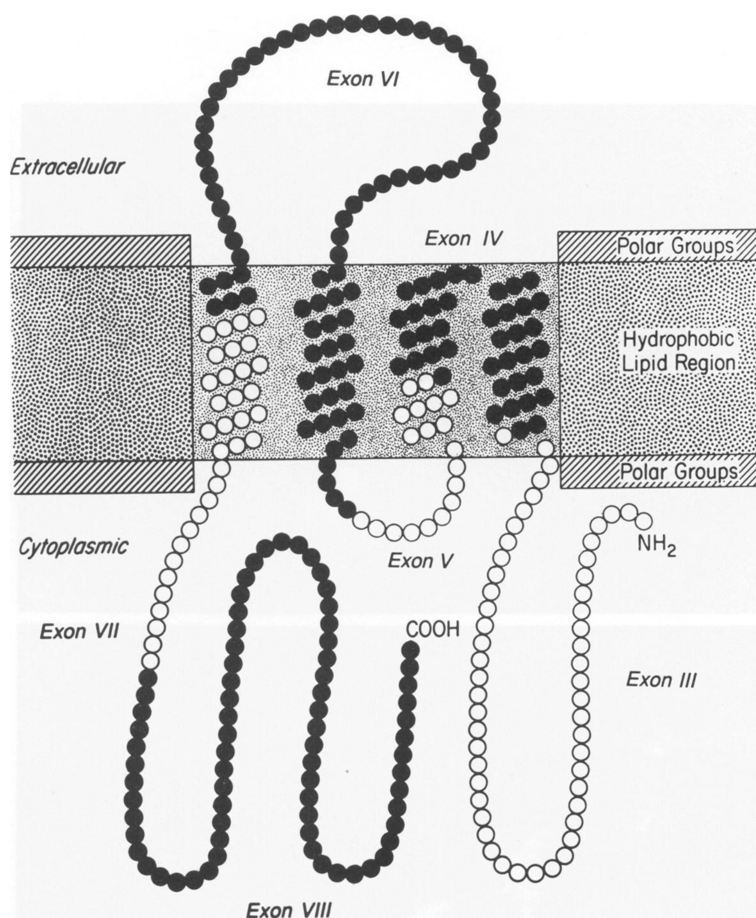

Figure 1. *Schematic diagram of the human B-cell-associated CD20 antigen, showing that only a small portion of the molecule (15%) is exposed on the cell surface, the remainder found either within the cell membrane (35%) or within the cell cytoplasm (50%). Reproduced, with permission, from Tedder et al.*[10]

cDNA Clones

A 1.0-kb cDNA clone (CD20.6) encoding the CD20 antigen was obtained from Dr. I. Stamenkovic[9] and a second 2.1-kb CD20 cDNA clone (pB1-21A-29) was provided by Dr. T. Tedder.[10]

Transfection of Cell Lines

COS cells were transfected using the procedure described elsewhere.[11] DNA (0.6 ml dissolved at 1.7 μg/ml in TRIS-buffered saline (TBS) containing 0.5 mg/ml DEAE Dextran) was added to a flask of COS-1 cells. They were incubated at room temperature for 30 minutes, washed in TBS, and incubated for 5 hours at 37°C in tissue culture medium containing 0.1 mmol/l (millimolar) chloroquine diphosphate. Cells were then washed and treated with TBS containing 10% DMSO for 2 minutes before washing again in TBS followed by normal tissue culture medium. Cells were cultured for a further 24 hours and then harvested using 0.05% trypsin/0.02% EDTA, spun down gently, resuspended in tissue culture medium, and restored to their original flasks. After 3 more days in culture, cells were harvested using 0.02% EDTA and cytospin preparations made. These were air dried and stored unfixed at −20°C until use.

Antibodies

The specificity and sources of antibodies used in this study are listed in Table 1. Rhodamine (TRITC)-conjugated goat anti-mouse Ig was obtained from Nordic Immunological Laboratories (Tilburg, The Netherlands) and anti-CD3 and rabbit anti-mouse Ig from Dakopatts (Glostrup, Denmark). Alkaline phosphatase: antialkaline phosphatase (APAAP) complexes were prepared in one of the authors' laboratories. Antibody L26 was obtained from Dakopatts and conjugated with biotin by a conventional procedure.[12] Fluorescein isothiocynate (FITC)-conjugated streptavidin was obtained from Chemicon (Temecula, CA). All reagents were used at optimal dilutions as determined in preliminary dilution experiments.

Immunocytochemical Staining

Cocapping Experiments

Cells from each of the four B-cell lines were suspended at a concentration of 2×10^6 cells per milliliter

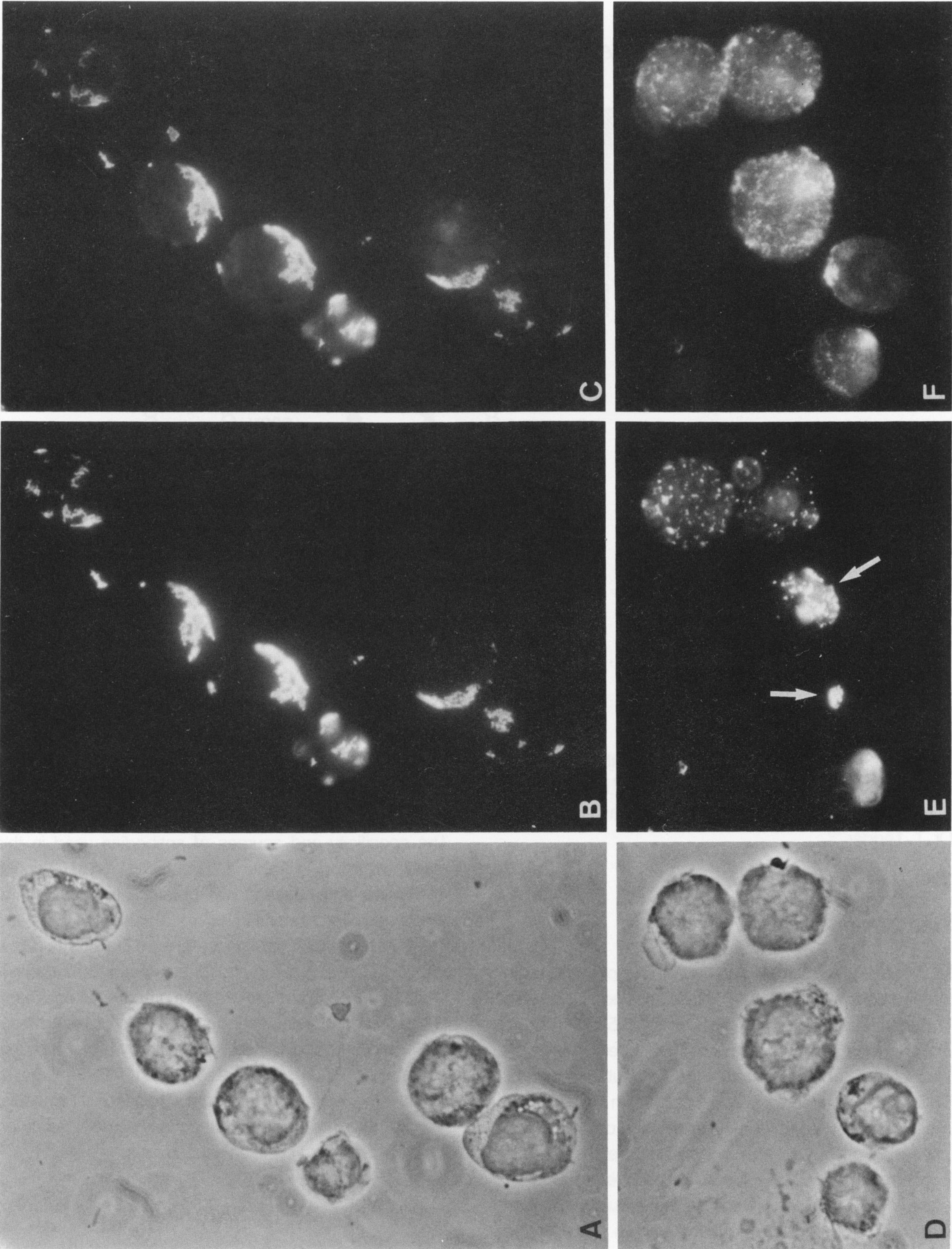
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Figure 2. *Cocapping experiments using antibody L26 in conjunction with anti-CD20 and anti-CD19, on the B-cell line ROS-17.* **A**: *Phase-contrast morphology after capping with anti-CD20 (B1).* **B**: *The same cells showing that CD20 antigen is present in a capped dotlike distribution, often localized to one pole of the cell (TRITC).* **C**: *L26 staining (FITC) is completely identical in distribution to the CD20 antigen, and diffuse cytoplasmic staining for L26 is abolished (see* **F**); **D**: *Phase-contrast morphology of cells after capping with anti-CD19 (B4).* **E**: *The same cells showing dotlike staining of CD19 antigen (TRITC). Two cells in which staining is largely restricted to a cap at one pole of the cell are arrowed.* **F**: *L26 staining (FITC) in the same cells shows that diffuse cytoplasmic labeling persists and is clearly different in appearance from the CD19 staining.*

and 50 μl aliquots were incubated with 50 μl of antibody at 37°C for 30 minutes while being gently agitated. Cells were washed twice in 2 ml of pH 7.8 phosphate-buffered saline containing 0.5% bovine serum albumin (PBS/BSA). The cell pellet was then resuspended in 50 μl PBS/BSA and incubated for 90 minutes at 37°C with 50 μl TRITC-conjugated goat anti-mouse Ig. Cells were washed again (as above), resuspended in 100 μl PBS/BSA, and two cytocentrifuge preparations were made using 50 μl of cell suspension per preparation. Slides were air dried for 15 minutes, fixed in acetone at 4°C for 10 minutes, and washed in PBS (pH7.8) for 15 minutes at room temperature (RT). After excess buffer was removed, the cytocentrifuged cells were first incubated with normal mouse serum (NMS; dilution 1:100) for 30 minutes at RT to block free antigen-binding sites of the goat anti-mouse-Ig antiserum, followed by washing in PBS for 15 minutes at RT. Excess buffer was removed and the cytocentrifuged cells were then incubated with biotin-labeled L26 for 30 minutes at RT. After a wash in PBS for 15 minutes, excess buffer was again removed and cells were incubated with FITC-streptavidin for 30 minutes (RT). Slides were then washed in PBS for 15 minutes, dried with paper tissue, and mounted in glycerol/PBS (9:1) pH 8.6 containing 1 mg/ml paraphenylenediamine. Preparations were evaluated using a Zeiss Standard 16 microscope with a IV FL epi-illumination condensor and phase-contrast facilities.[13]

Transfected Cells

Before staining, cytocentrifuge preparations of the transfected COS-1 cells were brought to room temperature and fixed in neat acetone for 10 minutes. Slides were allowed to air dry and stained using the APAAP immunoalkaline phosphatase technique,[14] with one repetition of the anti-mouse Ig and APAAP complex incubation stages to enhance the intensity of the reaction. At the completion of the reaction, cytospin preparations were counterstained with hematoxylin and mounted for microscopy.

Results

"Co-capping" Experiments

All four B-cell lines SMS-SB, ROS-1, ROS-15, and ROS-17 expressed the CD20 membrane antigen as determined by labeling in suspension with antibody B1. They also exhibited the typical cytoplasmic staining pattern when labeled with L26 in cytocentrifuge preparations. The immunophenotype of the B-cell lines is given in further detail in Table 2. In the cocapping experiments (Table 2) the cytoplasmic L26 staining pattern in the four B-cell lines was completely abolished in CD20-capped cells and replaced by a dotlike staining pattern that coincided completely in its distribution with that of CD20 (Figure 2). In contrast, preincubation with other antibodies against B-cell–associated antigens did not abolish the cytoplasmic L26 staining (Figure 2). It is interesting that limited dotlike reactivity with L26 was seen after previous incubation with antibody FMC7, and also with antibodies to the CD37 antigen or surface membrane immunoglobulin, without disappearance of the typical cytoplasmic L26 staining (Table 2).

Table 2. *Results of Cocapping Experiments: Double Immunofluorescence Staining for Surface Markers and Cytoplasmic L26 on Four B-cell Lines*

Antibodies	Surface marker positivity of cell lines				Co-capping of surface marker and L26 staining on cell lines			
	SMS-SB	ROS-17	ROS-15	ROS-1	SMS-SB	ROS-17	ROS-15	ROS-1
Control (NMS)	−	−	−	−	−	−	−	−
CD9 (BA-2)	+	−	+	+	−	−	−	−
CD10 (VIL-A1)	−	+	+	+	−	−	−	−
CD19 (B4)	+	−	+	+	−	−	−	−
CD20 (B1)	+	+	+	+	+*	+*	−	+*
CD20 (Leu-16)	+	+	+	+	+*	+*	+*	+*
CD21 (B2)	−	+	+	−	−	−	+*	−
CD22 (Leu-14)	+	+	+	+	−	−	−	−
CD23 (Tü1)	−	−	+	−	−	−	−	−
CD24 (BA-1)	+	+	−	+	−	−	−	−
CD37 (Y29/55)	−	+	+	+	−	+/−†	−	+/−†
FMC7	−	−	+	+	−	−	−	+/−†
Anti-SmIg	−	+	+	+	−	+/−†	−	+/−†

* The typical cytoplasmic staining of L26 was completely replaced by a dotlike staining, which was identical to the dotlike staining of CD20.
† The typical cytoplasmic staining of L26 remained, but there was partial codistribution of L26 staining with the cell-surface marker.

Table 3. *Reactivity of COS-1 Cells After Transfection with cDNA Encoding the CD20 Antigen* *

Specificity	Antibody	Reaction on transfected COS cells†
CD20	93-1B3	Scattered strongly stained cells
CD20	B1	Scattered weakly stained cells
?	L26	Scattered strongly stained cells
CD19	HD37	No cells stained
CD22	4KB128	No cells stained
CD3	3D4	No cells stained

* Staining was performed on cytospin preparations using the APAAP technique (see Materials and Methods and Figure 2).

† Reaction pattern of COS-1 cells after transfection with the CD20.6 cDNA clone[10] or the pB1-21A-29 cDNA clone.[10]

Labeling of Transfected Cells

When the COS cell line was transfected with either of the two cDNA clones encoding CD20 (see Materials and Methods) and stained with a panel of monoclonal antibodies, both antibody L26 and two control CD20 antibodies labeled scattered cells (Table 3 and Figure 3). In contrast, control antibodies to unrelated antigens provided no labeling of transfected cells.

Discussion

The majority of diagnostic histopathology laboratories that receive lymphoma biopsies attempt to categorize these tumors as being of B- or T-cell origin, either by carrying out immunohistologic staining themselves or by referring material to another laboratory. This investigation is frequently hampered by the lack of fresh frozen tissue, and thus there has been much interest in the identification of monoclonal antibodies that will detect B- or T-cell–associated antigens in routinely processed pathologic material. However the list of B- and T-cell reactive antibodies that are suitable for use on routinely processed formalin-fixed, paraffin-embedded material is restricted (Table 4). Furthermore, these reagents suffer from the drawback that the molecular targets that they recognize have not been identified (eg, MB2) and/or that they are of only limited specificity, eg, CD43 antibodies react not only with T cells but also with cells of B-cell and myeloid lineage.

These drawbacks are of practical relevance because the reaction of an antibody cannot be related to data ob-

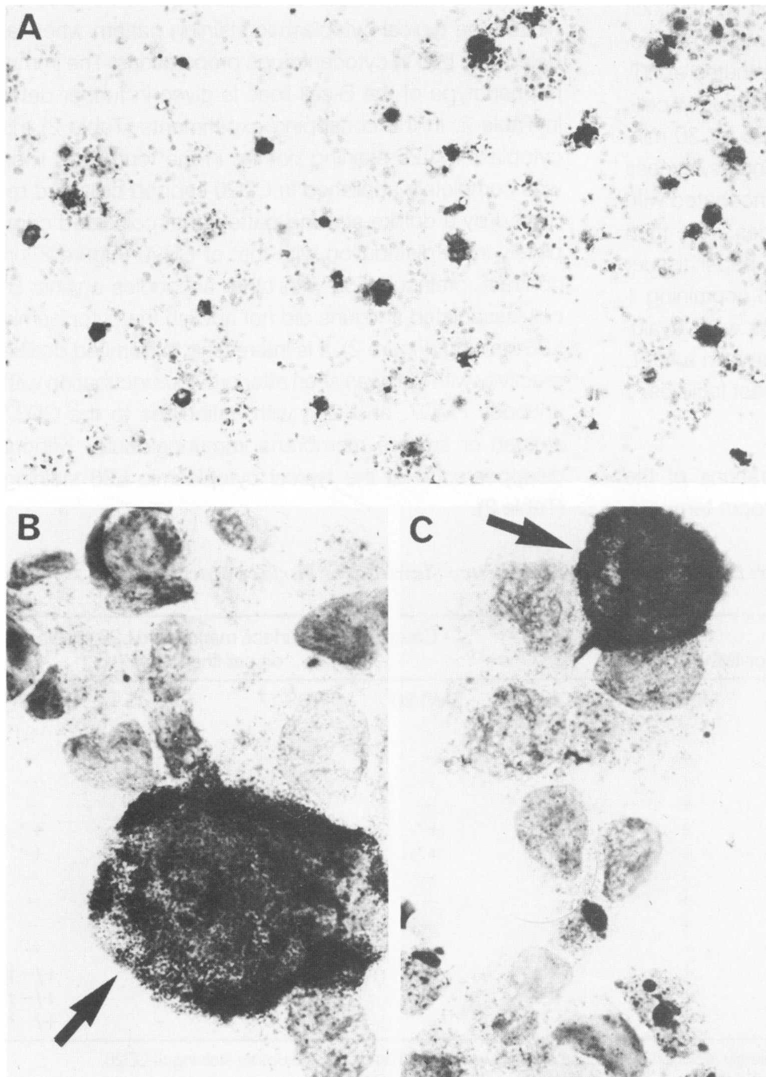

Figure 3. *Immunocytochemical labeling of COS monkey kidney cells after transfection with a cDNA clone encoding the CD20 antigen.* **A**: *Antibody L26 stains scattered cells with strong cytoplasmic reactivity.* **B and C**: *Higher power views of the same cells showing the essentially identical labeling obtained with the anti-CD20 antibody 93-1B3. Antibody B1 gave a similar but weaker reaction (see Table 3). APAAP staining technique.*

Table 4. *Antibodies for Staining B and T Cells in Routinely Processed Tissue*

Antibody*	Target molecule	Comments
B-cell Markers		
L26	CD20	High degree of specificity for B cells
4KB5, MB1, MT2	CD45R	Also label some T cells
LN1	CDw75	Also labels follicle centers, B cells, and erythrocytes.
LN-2, MB3	HLA class II (CD74)	Also labels macrophages and nonlymphoid cells
MB2	Unknown	Also labels macrophages, epithelia, and endothelium
T-cell Markers		
MT1, Leu-22, DF-T1	CD43 (Leucosialin)	Also label myeloid cells and some B cells
UCHL1	CD45RO	Also labels some B cells, macrophages, and myeloid cells

* Reference to the origin of the antibodies and to their staining reactions can be found in two reviews by Norton and Isaacson.[7,15]

tained with other reagents when the target molecule is unknown; in contrast, if an antibody reacts with a well-recognized leukocyte-associated antigen, there is often a substantial body of published data, based on the use of a number of different antibodies to the target antigen and on staining fresh tissue and cell samples, to help in interpretation of the results. Furthermore, ignorance of the molecular specificity of antibodies may lead laboratories inadvertently to use two or more reagents of the same specificity under the impression that their reactions are independent. An example is provided by two recent reports published in 1989[3,16] that described a 'novel' paraffin-reactive anti–T-cell antibody (Leu-22) but that did not indicate (because the information was not available at the time) that this antibody is directed against CD43 (leucosialin) and is thus identical in specificity to antibody MT1, a T-cell–associated marker that was described in several publications in the past 3 years.[17]

The fact that most of the antibodies in Table 4 are of only limited specificity for B or T cells also introduces problems that are of clinical relevance, because, in several instances, negative reactions do not exclude the cell lineage of a neoplasm nor do positive reactions provide reliable proof of its origin. For example, the reactions of CD45RA antibodies such as 4KB5 are ambiguous because these reagents will react with most, but not all, B-cell tumors, while some T-cell neoplasms give positive reactions. The T-cell marker UCHL1, also against a variant of the leukocyte common antigen (CD45RO), and CD43 antibodies offer further examples of this type of problem.

For these reasons, antibodies that not only react with routinely fixed tissue material but that are also directed against well-defined B- or T-cell specific molecules, represent a great improvement on existing reagents. In two recent reports from one of the authors' laboratories we have described how this may be achieved in the case of T cells by using antibodies selective for the CD3 molecule, a marker that is essentially specific for T cells.[18,19] The present report, indicating that antibody L26 is against the CD20 molecule, represents a similar advance in the context of B-cell markers. The two techniques we have used to prove the specificity of this reagent are independent of each other and hence their combined application provides strong evidence that L26 is a CD20 reagent. Cocapping experiments provide an elegant and powerful technique for demonstrating that epitopes recognized by two different antibodies are either on the same cell-surface molecule or on two physically associated molecules. The cocapping obtained with the CD20 antibody B1 and antibody L26 is highly suggestive that the latter reagent recognizes an intracellular epitope on the CD20 molecule. It was interesting that limited cocapping of the target molecule L26 by three other antibodies was observed in these experiments, which may conceivably indicate a physical association between CD20 and the molecules recognized by these other antibodies. In this context, it is important that anti-surface immunoglobulin was one of the antibodies that induced partial cocapping because there is some indirect evidence that immunoglobulin and CD20 antigen may be physically associated in the B-cell membrane.[20]

The technique of testing monoclonal antibodies of unknown specificity against cell lines transfected with cDNA clones encoding leukocyte-associated molecules provided valuable specificity data for antibodies in the Third Workshop on Leucocyte Differentiation Antigen, eg, those in the CD14 group, and was used much more extensively 2.5 years later in the Fourth Workshop because many more genes encoding CD antigens had been isolated in the meantime. The positive reaction of an antibody with transfected cells provides strong evidence that it is directed against the product of the transfected gene, although it may be noted that lack of staining is less informative because aberrant glycosylation by transfected cells may cause false-negative results. Antibody L26 was included in the Fourth Workshop, but it was not recognized as being a CD20 antibody by screening of transfectants, presumably because this analysis was performed by staining cells in suspension, a technique that would not detect an intracellular epitope. In our experiments both CD20 transfectants expressed the cytoplasmic L26 epitope.

In this paper we report that monoclonal antibody L26 detects an intracellular epitope of the pan B-cell antigen

CD20. In consequence, histologists using this reagent to characterize lymphomas can henceforth report lymphomas that react with antibody L26 as 'CD20 positive' and, given the extensive evidence for the B-cell specificity of this molecule, also add that the neoplasm is therefore almost certainly of B-cell origin.

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