Monoclonal Antibody Specific for Histone H1 Phosphorylated by Cyclin-Dependent Kinases: A Novel Immunohistochemical Probe of Proliferation and Neoplasia

David E. Burstein, Shimon Oami, Francine Dembitzer, Caryn Chu, Georgeta Cernaianu, Anatoly Leytin, Ecaterina Misilim, Subba Rao Jammula, James Strauchen, D. Stave Kohtz

Department of Pathology (SO, FD, CC, GC, AL, EM, SRJ) and Derald H. Ruttenberg Cancer Center (DEB, JS, DSK), Mount Sinai School of Medicine, New York, New York

Monoclonal antibody 12D11 (MAb 12D11) has been shown to bind histone H1 isolated from human placenta and other tissues but not histone H1 that has been digested with bacterial alkaline phosphatase. We show here that phosphorylation of phosphatase-treated histone H1 with cyclin dependent-kinase (CDK) restores binding by MAb 12D11. We conclude that MAb 12D11 selectively binds histone H1 that has been phosphorylated by CDKs, and we have investigated the use of MAb 12D11 as an immunohistochemical probe of CDK activity *in situ*. Previous immunofluorescence studies have revealed strong nuclear staining by MAb 12D11 in proliferating cultured cells and the absence of staining in terminally differentiated cells. Immunohistochemical staining of frozen and formalin-fixed, paraffin-embedded sections of benign tissues with MAb 12D11 was nuclear and confined to recognized foci of cell proliferation. In lymphoid germinal centers, MAb 12D11 preferentially stained large lymphoid cells with a relative lack of staining in small cleaved cells, contrasting with a lack of cell size discrimination observed with the monoclonal antibody proliferation probe, MIB-1. Tumor tissues displayed strong albeit heterogeneous staining of malignant cells by MAb 12D11, with little or no staining observed in surrounding nonneoplastic stromal cells. Differential staining by MAb 12D11 of invasive and *in situ* carcinoma suggest applications in prognostication. MAb 12D11 may also be useful in identification of tumors more likely to respond to therapeutic CDK inhibitors.

KEY WORDS: Cell cycle, Cyclin-dependent kinases, Histone H1, Immunohistochemistry, Phosphorylation-specific antibodies, Proliferation markers, Tumor markers.

Proliferation of both normal and neoplastic cells is marked by important changes in the expression and posttranslational modification of cell cycle and other regulatory proteins. Despite recent progress made in understanding the molecular basis of cell proliferation, the potential use of immunohistochemical staining of proliferation markers as a tool for tumor diagnosis and prognostication has been only partially explored. Presently, monoclonal antibodies (MAbs) MIB-1 and Ki-67, which detect a nuclear protein of unknown function, are used most commonly to identify actively proliferating normal and neoplastic cells in tissue sections (1). Other proliferation markers, including proliferating cell nuclear antigen (PCNA; for review, see (2)), topoisomerase II (3) and cyclins (4) also have been employed with some success. Each of these has specific disadvantages: The target of MAb MIB-1 is a protein of virtually unknown function, and does not appear to bind selectively in any specific phase of the cell cycle (1). PCNA has a long half-life, and may persist in cells which have become quiescent (5). Some cyclins are expressed at significant levels in nondividing cells (6–11). Other markers such as topoisomerase II appear promising, but evidence of their clinical utility awaits more extensive testing.

In a previous report, it was shown that MAb 12D11 specifically binds mammalian histone H1, and that binding of the antibody could be eliminated by digesting histone H1 with bacterial alka-

line phosphatase (12). Immunofluorescence microscopy of cultured cells revealed that MAb 12D11 stains the nuclei of proliferating primary human skeletal myoblasts, but antibody binding was eliminated upon terminal differentiation (12). In this report, we show that phosphorylation of phosphatase-digested human histone H1 with cyclin-dependent kinase (CDK), but not the other kinases that were tested, restores antigen binding to MAb 12D11. From these data, we have concluded that MAb 12D11 binds specifically to histone H1 that has been phosphorylated by active CDKs. Activation of CDKs is an essential event for progression through the cell cycle (13–17), and several studies have shown that histone H1 is phosphorylated significantly during the S and M phases of the cycle (18–20). Because histone H1 is an abundant protein present in the nuclei of virtually all vertebrate cells, we investigated the use of MAb 12D11 as a marker of cell proliferation in normal and neoplastic tissues.

FIGURE 1. Binding of MAb 12D11 to histone H1 is dependent on phosphorylation by cyclin-dependent kinase. Samples of human placental histone H1 were isolated by acid extraction and acetone precipitation, resolved by SDS-PAGE, and analyzed by Western blot with MAb 12D11. Lanes: M, markers: (top to bottom, Mr 180K, 116K, 84K, 58K, 48.5K, 36.5K, 26.5K). **Lane 1,** Five micrograms human placental histone H1. **Lane 2**, Five micrograms human placental histone H1, digested with bacterial alkaline phosphatase. **Lane 3**, Five micrograms human placental histone H1, treated first with bacterial alkaline phosphatase, then phosphorylated by cyclin-dependent kinase.
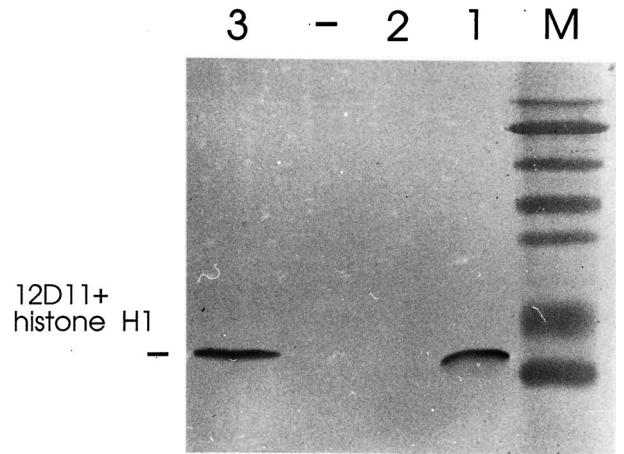
MATERIALS AND METHODS

Cell Culture and Reagents

Mouse hybridoma cells producing MAb 12D11 were cultured in DME medium containing 4.5 g/L of glucose and 10% fetal bovine serum. Hybridoma supernatants containing approximately 20 μg/mL of MAb 12D11 were produced by growing hybridoma cells to maximum density and removing the cells by centrifugation. Horseradish peroxidase–coupled goat anti-mouse IgM was from Boehringer Mannheim. Bacterial alkaline phosphatase was from Calbiochem. A multilink streptavidin-biotin immunoperoxidase staining kit was from BioGenex (catalog #QP900–9L.)

Kinase Reactions and Western Blotting

Histone H1 was purified from human placenta by acid extraction and acetone precipitation (11). Histone H1 was digested with 25 U of bacterial alkaline phosphatase (BAP) in 50 mM Tris (pH 8.3), 1 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ to remove serine and threonine phosphate groups. Histone H1 treated with BAP was isolated by acid extraction and acetone precipitation, then dissolved in water. To phosphorylate BAP-treated histone H1, 5 μg of substrate was incubated in 25 mM HEPES, 10 mM $MgCl_2$, 50 mM NaCl, 50 μM ATP, and kinase. Cyclic-AMP–dependent kinase (protein kinase A) catalytic subunit (Sigma), casein kinase (Sigma), or cyclin A/E-CDK2 purified from rat liver (gift of Tom Langan, University of Health Sciences Center, Denver) were incubated in 20 uL total reaction volume at 30° C for 20 minutes. Reactions and sham reactions lacking ATP were stopped by the addition of EDTA to 50 mM, and histones were isolated by acid extraction and acetone precipitation.

Samples were resolved by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred electrophoretically to nitrocellulose filters. The blots were fixed with 10% acetic acid/25% isopropanol, washed several times with water, and blocked with 5% nonfat dry milk in 25 mM Tris (pH 7.4) and 150 mM NaCl (TBS). The blots were incubated with MAb 12D11 (full-strength hybridoma supernatant supplemented with 5% nonfat dry milk and 0.5% Tween 20) for 18 hours at 4° C. The blots were washed twice in TBS supplemented with 0.5% Tween 20 (TBS-Tween), then incubated for 2 hours (4° C) with horseradish peroxidase-coupled goat anti-mouse IgM in TBS-Tween supplemented with 1% bovine serum albumin. The blots were washed three times with TBS-Tween, then developed with 1 mg/mL 4-chloro-1-naphthol (BioRad) in TBS supplemented with 20% methanol and 0.015% hydrogen peroxide.

Frozen Sections

Frozen sections were stained using a two-step immunoperoxidase staining protocol. Sections on polylysine-treated slides were fixed in 2.5% formalin for 10 to 40 minutes, washed three times with phosphate buffered saline (PBS), incubated with MAb 12D11 hybridoma supernatant diluted 1:1–3 in PBS supplemented with 0.75 M NaCl, 5% horse serum (HS), and 5% nonfat dry milk. The sections were incubated with primary antibody for 2 hours

at 37° C, then washed four times with PBS supplemented with 0.75 M NaCl. The sections were incubated with horseradish peroxidase-labeled goat anti-mouse IgM second antibody in PBS supplemented with 1% bovine serum albumin for 1.5 hours at 37° C. The sections were then washed four times with PBS, then developed in PBS with diaminobenzidine (1 mg/mL) and hydrogen peroxide (0.015%).

Formalin-Fixed, Paraffin-Embedded Sections

Sections (5 μm) were prepared from archival, formalin-fixed, paraffin-embedded surgical pathology specimens. Sections were prepared using an antigen-retrieval method (21), followed by streptavidin-biotin staining. Paraffin was removed from sections in xylene followed by graded alcohols. Endogenous peroxidase activity was blocked with 3% H_2O_2 for 15 minutes, and sections were washed with water. Antigen retrieval was performed as follows: sections were heated to 90° C for 15 minutes in 0.01 M citric acid buffer, pH 6.0, then cooled to room temperature for 20 minutes. Sections were washed with PBS, then incubated with MAb12D11 culture supernatant, 1:20 dilution, in PBS supplemented with 0.75 M NaCl, 5% casein, and 5% horse serum for either 1 hour at 37° C or overnight at ambient temperature. Sections were washed four times in PBS, then incubated with a multilink biotinylated anti-immunoglobulin (BioGenex) for 20 minutes at ambient temperature. The sections were washed four times with PBS, then incubated with streptavidin-coupled horseradish peroxidase (BioGenex) for 20 minutes at ambient temperature. The sections were then washed four times, developed with diaminobenzidine (see above) for 20 minutes, then washed in water for 5 minutes. Some slides were counterstained with hematoxylin, and all slides were then dehydrated through graded alcohols and xylene and coverslipped.

For MIB-1, standard antigen retrieval and staining methods were used: slides were exposed to 0.11 M sodium citrate, pH 6.0, at 100° C for 5 minutes, cooled for 20 minutes, and washed with PBS. Sections were then incubated overnight at ambient temperature with MIB-1 diluted 1:10–1:20 in PBS supplemented with 2.5% nonfat dry milk, 5% goat serum, and 0.2% bovine serum albumin. Slides were washed in PBS, then processed for immunodetection with a streptavidin-biotin immunoperoxidase kit and diaminobenzidine, as above.

Benign proliferative tissues studied included colon, small intestine, and tonsils. Tumors studied included adenocarcinomas of mammary, endometrial, ovarian and colonic origin, head and neck squamous carcinomas, and non-Hodgkin's lymphomas.

RESULTS

MAb 12D11 Specifically Binds Histone H1 that Has Been Phosphorylated by Cyclin-Dependent Kinases

Human histone H1 was isolated from placenta and analyzed by Western blot with MAb 12D11. Consistent with previous results (12), MAb 12D11, and IgM, bound the upper, slower migrating band in the mammalian histone H1 doublet (Fig. 1, Lane 1). Also consistent with previous observations (12), digestion of histone H1 with bacterial alkaline phosphatase (BAP) ablated binding by MAb 12D11 (Fig. 1, Lane 2). When BAP-digested histone H1 was phosphorylated with a mixture of cyclin A/CDK2 and cyclin E/CDK2 isolated from regenerating rat liver, binding to MAb 12D11 was recovered (Fig. 1, Lane 3). In contrast, phosphorylation of BAP-digested histone H1 by either protein kinase A or casein kinase I did not restore binding to MAb 12D11 (data not shown). Together, these results show that MAb 12D11 does not bind unphosphorylated histone H1 and specifically binds histone H1 that has been phosphorylated by cyclin-dependent kinases.

Immunostaining of Benign Tissues

Frozen and routinely processed formalin-fixed, paraffin-embedded sections of benign tissue from colon, small intestine, and tonsils were stained with MAb 12D11. In colon (Fig. 2, A–B) and small intestine (data not shown), staining was confined to nuclei of cells at the base of glandular epithelium, the site of epithelial mitogenesis (22). The pattern of staining of colon crypt epithelial nuclei with MAb 12D11 was qualitatively similar, but quantitatively lower than immunostaining by MIB-1/Ki-67 (Fig. 2, A–B).

Prominent nuclear staining with MAb 12D11 was noted in lymphoid germinal centers in colonic Peyer's patches (data not shown) and in germinal centers in reactive tonsillar/adenoidal lymphoid tissue (Fig. 2C). MAb 12D11 differed from MIB-1 staining in benign lymphoid tissue (Fig. 2D) in several respects: 1) The number of MAb 12D11-positive cells within germinal centers was consistently lower than the number of MIB-1-positive cells (Fig. 2, C–F). 2) A peripheral zonal staining pattern was often more readily apparent in MAb 12D11-stained germinal centers than it was with MIB-1-staining (Fig. 2, C–D), corresponding well to the zone of mitogenesis (23). 3) Large activated lymphocytes were preferentially stained by Mab 12D11, with relative sparing or weak, equivocal staining of small cleaved lymphocytes. In contrast, MIB-1 stained both cell types (Fig. 2, E–F). This suggests that MAb 12D11 staining more effectively discriminates sub-
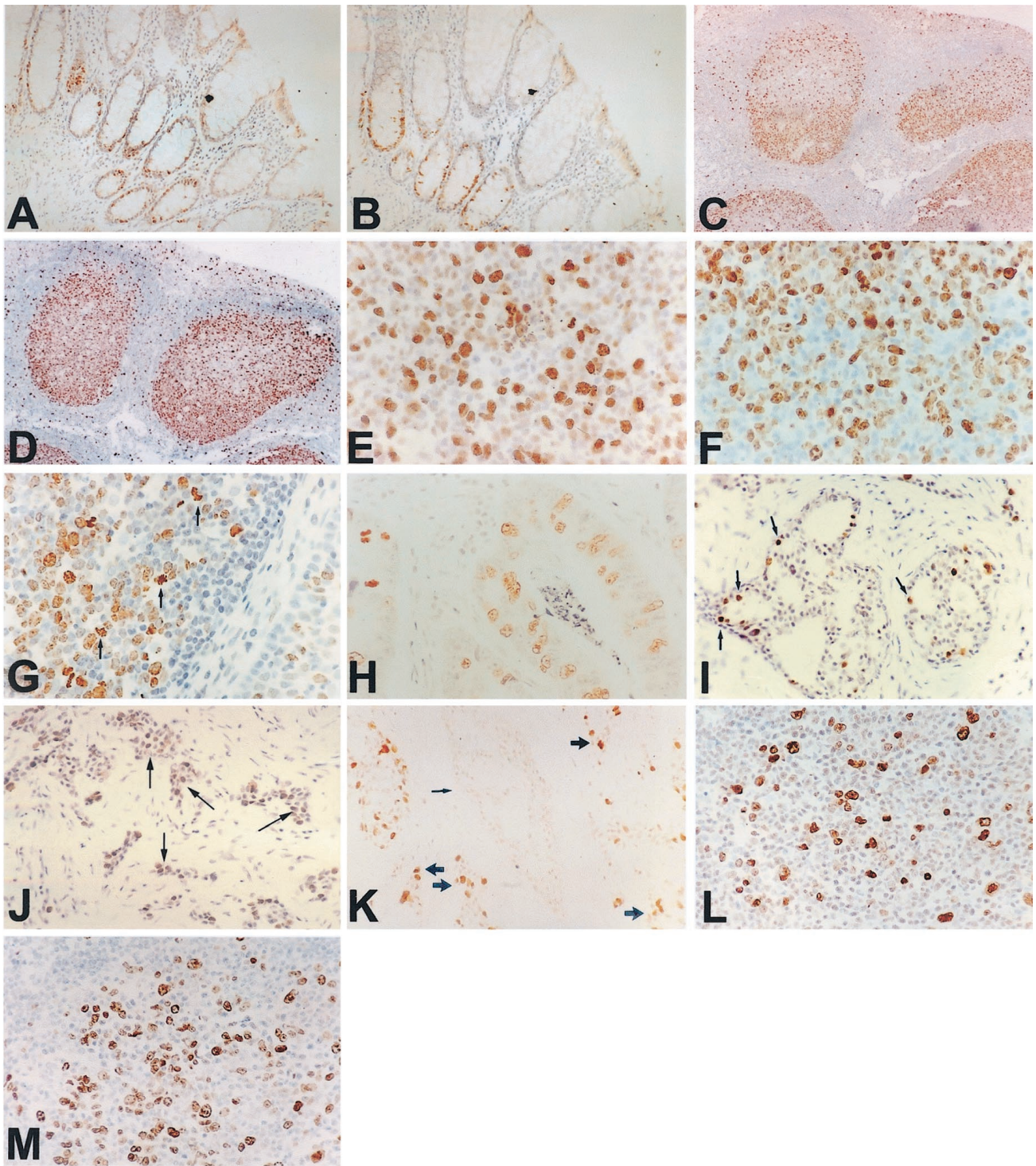
FIGURE 2. MAb 12D11 and MIB-1 immunoperoxidase staining. **A**, Benign colonic epithelium. MAb 12D11, formalin-fixed, paraffin-embedded. **B**, Benign colonic epithelium, parallel section. MIB-1. **C, E**, Benign adenoidal/tonsillar tissue, MAb 12D11, formalin-fixed, paraffin-embedded. **C**, Low power; **E**, high power. **D, F**, Benign adenoidal/tonsillar tissue, MIB-1, parallel section. **D**, Low power; **F**, high power. **G**, Benign adenoidal/tonsillar tissue, MAb 12D11: fragmented nuclei, high power (**see arrows**). **H,** Colonic adenocarcinoma, MAb 12D11, formalin-fixed, paraffin-embedded, high power. **I**, Mammary carcinoma. MAb 12D11, frozen section, formalin-fixed, counterstained with hematoxylin. **Arrows,** Scattered positively stained nuclei in intraductal carcinoma, cribriform type, mostly at periphery of duct. **J,** Mammary carcinoma, same specimen as (**I**), area of invasive carcinoma, MAb 12D11. Note greater percentage of positively stained cells than in intraductal component in (**I**) (**see arrows**). **K**, Mammary carcinoma, same specimen as (**I**) and (**J**), no counterstain. MAb 12D11. Note intraductal area (**thin arrow**) and invasive nests (**thick arrows**). **L,** Follicular lymphoma, mixed small cleaved and large cell type. MAb 12D11, formalin-fixed, paraffin-embedded. **M**, Follicular lymphoma, mixed small cleaved and large cell type, parallel section. MIB-1.

sets of activated germinal center lymphocytes than does staining with MIB-1. In parafollicular regions, both MAb 12D11 and MIB-1 selectively stained scattered large immunoblast-like cells. Germinal centers from other benign lymphoid populations, including reactive lymph nodes and Hashimoto's thyroiditis, showed similar findings (data not shown). Staining of fragmented nuclei by MAb 12D11 was noted in some germinal centers Fig. 2G), possibly represents with cells undergoing apoptosis, a common event in germinal centers (24). In contrast, nuclear fragments in necrotic regions of tumors did not stain with MAb 12D11 (data not shown).

Immunostaining of Malignant Cells

Adenocarcinomas of mammary, endometrial, ovarian and colonic origin, head and neck squamous carcinomas, and non-Hodgkin's lymphomas were stained with MAb 12D11. Staining was more prominent in the neoplastic cells of tumors, with nonstaining or focal weak staining of nonneoplastic tumor stromal cells, tumor vessel endothelium, and tumor-infiltrating leukocytes (Fig. 2H). The percentage of MAb 12D11-positive nuclei varied between tumors of the same histopathologic type, suggesting heterogeneity in the degree of CDK-mediated histone H1 phosphorylation within specific histopathologic categories. Heterogeneity was also observed between different regions of the same tumor, probably associated with cell cycle phase and/or differences in the rates of cell division. In one case of cribriform-type intraductal carcinoma, staining was confined to occasional cells, most at the periphery of the duct. However, adjacent nests of infiltrating carcinoma cells showed a considerably higher percentage of positively staining nuclei than the noninvasive component of the tumor (Fig. 2, I–K). This suggests that MAb 12D11 may distinguish invasive malignant cells from preinvasive neoplasia in certain tumors. However, the basis for this distinction may be differences in proliferation rates between *in situ* and invasive tumor components. Nuclear debris in necrotic areas of comedo carcinoma was nonstaining with MAb 12D11 (data not shown), contrasting with positive staining of fragmented nuclei in benign cells in lymphoid germinal centers that may represent cells undergoing apoptosis.

Several aspects of MAb 12D11 staining of non-Hodgkin's lymphomas were consistent with the patterns observed in benign lymphoid tissue. Preferential staining of large rather than small lymphoid cells by MAb 12D11 was observed in lymphoid malignancies as well. In several cases of follicular lymphoma, for example, MAb 12D11 preferentially stained large lymphoid cells in con-

trast to MIB-1 that stained both large and small cells (Fig. 2, L–M). As in benign tissue, the percentage of lymphoma cell nuclei stained by MAb 12D11 was consistently lower than the percentage stained with MIB-1. In small lymphocytic lymphoma, MAb 12D11 staining was confined to the larger lymphoid cells that are present in loose aggregates (pseudofollicular proliferation centers) and are believed to represent the proliferative component of this neoplasm (25). In contrast, the predominant population of small cells, which is believed to accumulate but not divide, was nonstaining (data not shown).

DISCUSSION

The role of MIB-1 and other markers such as PCNA in predicting tumor behavior has been the subject of numerous studies. Unlike the target proteins of other proliferation probes such as MIB-1, which are expressed in proliferative states but are absent in quiescent states, histone H1 is ubiquitously expressed, independent of the state of cell division. However, phosphorylation of histone H1 at CDK sites clearly does correlates with cell proliferation (12). We present evidence that MAb 12D11, which detects CDK-phosphorylated histone H1, is a novel immunohistochemical probe of proliferation. The biochemical and biological basis of MAb 12D11 as a probe of proliferation and/or prognosis rests upon its ability to detect histone H1 only when phosphorylated by CDKs.

The immunohistochemical staining properties of MAb 12D11 are entirely consistent with the known expression and activation properties of the cyclin-dependent kinases (CDKs). CDKs are a set of related kinases that govern the highly regulated sequence of cellular events which underlie entry into and progression through the cell cycle (for reviews, see Refs. 13–17). Cyclins are the regulatory subunits of the cyclin-dependent kinases, and are expressed differentially during the cell cycle. The G_1 cyclins include the D-type cyclins and cyclin E, which form active complexes with CDK4 and 6 and CDK2, respectively (26–28). CDK4 and 6 bound to D-type cyclins do not have histone H1 kinase activity, whereas CDK2 bound to cyclin E has potent histone H1 kinase activity (19, 20, 29, 30). D cyclins are expressed throughout G_1, whereas cyclin E (and hence, histone H1 activity) appears only in late G_1. In late G_1 and S phase, cyclin A binds and activates CDK2, while in G_2 and M cyclin A and B-type cyclins bind and activate CDK1 and 2; all of these cyclin–CDK complexes phosphorylate histone H1. Hence, it is not surprising that MAb 12D11 stains a lower percentage of cells than MIB-1/Ki-67 in nonneo-

plastic proliferative tissues since 12D11 should react with cells from late G_1 through G_2-M, whereas MIB-1 stains throughout the entire cell cycle. In a quantitative study comparing 12D11 and MIB-1 staining in gynecologic tract lesions, the mean proportion of positively stained cells was also higher in MIB-1– compared with 12D11-stained sections in normal squamous epithelium and in all grades of squamous intraepithelial lesions; the relationship of the 12D11 labeling index to the MIB-1 index was statistically highly significant (manuscript submitted).

A number of small molecules with CDK-inhibitory activity have been isolated, some of which are being tested as anti-cancer agents (31–34). Two of these, flavopiridol and 7-hydroxy-staurosporine, show promise in early clinical trials (Senderowicz MD, National Cancer Institute, personal communication). In preliminary studies, we have found loss of MAb 12D11 reactivity upon treatment with the CDK inhibitor flavopiridol in a carcinoma from a patient with a strong clinical response to the drug (unpublished data). Furthermore, the correlation of prognosis with specific aberrations in CDK pathways in certain human tumors suggests a case for MAb 12D11 as a potentially useful prognostic marker. For example, breast carcinomas that aberrantly overexpress cyclin E have been shown to have a worse prognosis (35, 36). Because cyclin E activates CDK2, which possesses potent histone H1 kinase activity, this subset of breast cancers might be expected to demonstrate extensive MAb 12D11 staining. In contrast, breast carcinomas that overexpress cyclin D1 (which activates CDKs 4 and 6, which lack significant histone H1 kinase activity) have been shown in two studies to have an improved prognosis (35, 37). These tumors would be expected to have less extensive MAb 12D11 staining than would cyclin E-overexpressing tumors.

Consistent with a possible role as a prognostic marker was the staining by MAb 12D11 of a case of cribriform intraductal mammary carcinoma and adjacent invasive carcinoma. Increased staining by MAb 12D11 in nests of infiltrating mammary carcinoma compared with adjacent cribriform intraductal carcinoma suggests that increased histone H1 phosphorylation may correlate with progression from *in situ* to invasive carcinoma in some tumor types. This may reflect an enhanced proliferation rate in invasive compared with *in situ* carcinoma, but alternatively could result from aberrations in expression of cyclins, CDK inhibitors, and other regulatory factors associated with invasive but not *in situ* carcinomas. Further studies are in progress to test this hypothesis.

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Book Review

Sun T: Flow Cytometric Analysis of Hematologic Neoplasms. A Color Atlas and Text, 2nd ed, 296 pp, Philadelphia, Lippincott Williams & Wilkins, 2002 ($169.00).

This is the second completely revised and updated edition of Dr. Sun's book of flow cytometry (FCM) applied to the diagnostic study of lymphomas and leukemias. It consists of two distinct parts: an introductory part comprising chapters on the technical aspects of FCM and other diagnostic techniques used in the work-up of hematopathologic diseases, as well the new classifications; and the second part, titled *Clinical Application*, consisting of 38 case studies. The book shows that flow cytometry has become an invaluable part of the laboratory work-up of lymph node and bone marrow biopsies. At the same time the book clearly shows that FCM is not 'an independent diagnostic tool,' repeatedly emphasizing that FMC data should be interpreted only in the context of other laboratory and clinical data.

The case-study approach to teaching FMC is a very efficient way of demonstrating the clinical value of this technique. The cases are well chosen and instructive and almost all illustrate some valuable diagnostic points. The data for each case include flow histograms and countergrams, corresponding color figures of hematologic or pathologic findings, and, whenever indicated, immunohistochemical, molecular, or cytogenetic findings. Readers looking for quick review will appreciate the summary tables of salient features for each entity under discussion. References for each case are up to date.

The book will be used mostly by fellows and residents in pathology and hematology, but I would also recommend it to anybody else trying to learn FMC hematopathology. The book could be used as a reference by diagnostic hematopathologists as well.

Marin Nola
University of Nebraska Medical Center
Omaha, Nebraska