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Brief Communication

Novel Method for Estimating the Labeling Index in Clinical Specimens with the Use of Immunoperoxidase-labeled Antinucleoside Antibodies[1]

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SUMMARY

The labeling index determined by [^{3}H]thymidine autoradiography in cells from clinical specimens was compared with the percentage of the cells showing nuclear reactivity to immunoperoxidase-labeled antinucleoside antibodies. This nuclear immunoreactivity is specific for denatured or single-stranded DNA's and is detectable almost exclusively during DNA synthesis. Results with the two methods showed excellent agreement. The new method allowed rapid accurate assessment of S phase in the tumor cells from freshly isolated aspirated specimens as well as frozen sections, suggesting general applicability to estimation of the labeling index without autoradiography.

INTRODUCTION

The potential usefulness of knowledge of the percentage of tumor cells engaged in DNA synthesis (the labeling index) as a guide for radiotherapy and chemotherapy has been reviewed (8, 11, 14). The labeling index has been determined in human solid tumors *in vivo* with continuous infusions of [^{3}H]thymidine (13) or direct injection of the DNA precursor into solid tumors (12); unfortunately, these approaches are not generally applicable. Furthermore, even when tumor cells can be obtained from body fluids such as effusions or ascites, determination of the labeling index requires cell cultures, sterile precautions, and photographic development. These factors have doubtless interfered with more frequent determination of the labeling index in clinical studies.

To simplify determination of the labeling index, we have successfully used an indirect approach whose rationale is as follows. Cellular DNA synthesis and nuclear immunoreactivity to antinucleoside antibodies vary in parallel during S phase. These antibodies are specific for single-stranded nucleic acids (4, 6, 9, 10) which are detectable almost exclusively during DNA synthesis. Previous studies verified that nuclear immunoreactivity exhibited during S phase could be abolished by prior treatment with DNase but not RNase. Single-stranded RNA did not participate in this reaction under usual conditions, perhaps due to shielding by proteins (2). With an immunoperoxidase staining method and autoradiography made on cell preparations of identical clinical specimens, we were able to correlate nuclear immunoreactivity and incorporation of [^{3}H]thymidine as judged from autoradiography. Individual cells could be scored unambiguously by both techniques. The method was applicable to frozen sections as well as aspirated fluid specimens and aspirations from lymph nodes and solid tumors.

MATERIALS AND METHODS

Immunological Methods. The preparation of antinucleoside antibodies and fluorescent- and peroxidase-labeled antibody staining methods were described in previous publications (10, 15). Immunoperoxidase staining was performed with a 3-layer bridge technique: (*a*) rabbit anti-guanosine directly conjugated to peroxidase, according to the method of K. C. Hsu (15), 1:50 dilution, (*b*) sheep anti-rabbit globulin, 1:100 dilution, (*c*) rabbit anti-peroxidase, 1:100 dilution, (*d*) 20-min exposure to horseradish peroxidase (Sigma VI), 0.5 μg/ml, followed by (*e*) 10-min exposure to 0.003% peroxidase with diaminobenzidine as a color indicator. The rationale for using directly conjugated antinucleoside as the 1st layer as well as the bridge technique was to increase the intensity of positive staining. (Staining with anti-guanosine peroxidase alone was too weak.) In each case, immunological staining with antibody for 30 min was followed by a 10-min wash in phosphate-buffered saline (3). Promising results were obtained with rabbit anti-guanosine antibody followed by commercial goat anti-rabbit globulin conjugated with peroxidase (Miles-Yeda Kankakee, Ill.), in recent variant of the above technique.

Cytological Studies and Autoradiography. Freshly isolated clinical specimens from aspirates were labeled for 1 hr in Eagle's minimal essential medium with 10% fetal bovine

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serum with [³H]thymidine, 1 μCi (specific activity, 60 Ci/mmole). The cells were then sedimented (cytocentrifuge) onto microscope slides and fixed in acetone at −20° for 3 min. The slides were stained with anti-guanosine by the immunoperoxidase technique described above. The slides stained with immunoperoxidase were scored by 1 observer and then allowed to air dry; autoradiographs were obtained with the use of Kodak NTB2 emulsion and 4 to 6 hr of exposure. The autographs were then scored by a 2nd observer. Therefore, identical specimens were scored independently by both techniques. Furthermore, it was possible to score peroxidase staining beneath the plane of focus of the photographic emulsion, permitting examination of individual cells by the 2 techniques.

Frozen Sections. CH3/HeHa mice (30 g) bearing a transplantable mammary C3HBA adenocarcinoma were given i.p. injections of 100 μCi of [³H]thymidine (specific activity, greater than 60 Ci/mmole). Mice were sacrificed 1 to 3 hr after injection and, from the tumor, 4-μm frozen sections were made and fixed in acetone. Sections were stained with anti-guanosine antibodies by means of the immunoperoxidase technique. The slides were then overlayed with photographic emulsion and were developed for 3 days. For comparison, a Krebs II ascites tumor (growing s.c.) in Swiss mice was also studied.

RESULTS

Tumor cell specimens from 6 untreated cancer patients were aspirated from lymph nodes or ascites fluid. Cells were labeled with [³H]thymidine in short-term cultures and then were stained for antinucleoside immunoperoxidase reactivity. Tests on 3 of the patients were repeated within a week; in 2 patients, the repeat tests were made after the subjects had just begun chemotherapy or radiotherapy. Results with the 2 techniques agreed and were nearly identical to initial values. Details are given in Table 1. The results were plotted for each cell preparation, as shown in Chart 1.

The *dotted line* through the origin indicates results anticipated if the 2 tests correlated perfectly, *i.e.*, slope = 1.00. A linear regression line (not shown) was fitted to the data and was found statistically to coincide with the ideal curve. The data indicated a slope of 0.96 with 95% confidence limits of 0.82 to 1.1. The correlation coefficient was 0.978.

Frozen sections were made of mammary adenocarcinomas from mice previously given injections of [³H]thymidine. The sections were stained and scored by both methods, as described above. Results shown (*solid line*) include a determination made on a Krebs II ascites tumor growing s.c. Excellent correlation of the labeling index with immunoreactivity was found with determinations made from frozen sections. It is not yet known if the slight apparent deviation from the ideal curve is significant.

Labeling index variation in the mammary adenocarcinoma samples tested might be accounted for by the differences in tumor size or other factors beyond the scope of this report. With Krebs II ascites tumor cells growing in a solid form, there was much necrotic debris; no cells were

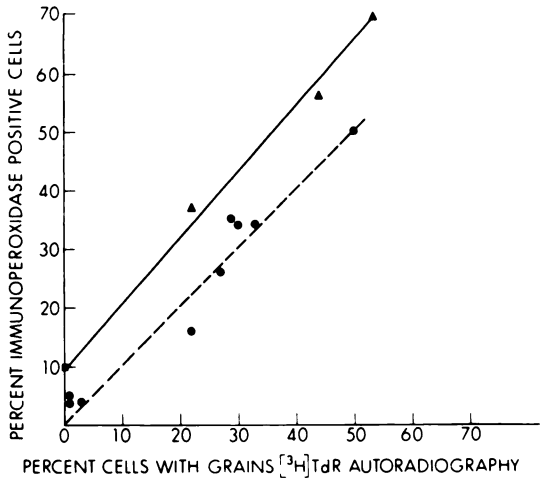

Chart 1. Correlation of [³H]thymidine ([³H]TdR) incorporation and nuclear immunoperoxidase reactivity. ●, clinical aspirates; ▲, C3H mouse mammary adenocarcinoma; ■, solid form of Krebs II ascites tumor.

Table 1

Comparison of [³H]thymidine labeling index and immunoreactivity to antiguanosine antibodies by immunoperoxidase technique in clinical specimens

At least 200 cells were scored to determine nuclear immunoreactivity and 500 cells were scored for determination of the labeling index by autoradiography.

Patient	Diagnosis	Cell source	Status	%+ peroxidase	%+ autography
474	Ovarian cancer	Ascites	Untreated	5	1
373	Lymphoepithelioma of the pharynx	Nodular mass, neck	Untreated	50	50
376	Cancer of the lung	Aspirate of neck node	Untreated	16	22
476	Adenocarcinoma of the stomach	Ascites	Untreated	34	33
476	Adenocarcinoma of the stomach	Ascites	3 days after start of chemotherapy	26	27
479	Adenocarcinoma of the cecum	Ascites	Untreated	4	3
479	Adenocarcinoma of the cecum	Ascites	Untreated	4	1
378	Adenocarcinoma of the rectum	Aspiration of inguinal node	Untreated	34	30
378	Adenocarcinoma of the rectum	Ascites	After 1000 rads	35	29

labeled by autoradiography, and only 9% immunopositive cells were found.

Fig. 1 shows the typical appearance of the labeled preparations which were used to study the mammary adenocarcinoma using frozen sections.

DISCUSSION

The generally excellent correlation between results obtained by the 2 methods, as also applied to solid tumors, has not been previously described. Our own previous attempts to use fluorescein-labeled antinucleoside antibodies were interfered with by variable amounts of autofluorescence encountered in certain clinical specimens. Endogenous peroxidase activity was not encountered in clinical or mouse mammary tumor cells (Fig. 1*e*). The advantage of the immunoperoxidase technique is that it can be scored by visible optics, the stain is permanent, and the staining procedure can be carried out with commercially available material in 4 hr. Although human tumor cell specimens from body fluids are readily accessible for study by both techniques, the applicability of the immunoperoxidase technique to frozen sections may make this approach an especially valuable one, opening the way to determining the labeling index in routine clinical specimens.

We do not yet know if a low background of immunoreactive cells which do not incorporate [^{3}H]thymidine will be a common finding. Clearly, more study will be needed with diverse clinical material before this test can be generally used as a substitute for [^{3}H]thymidine incorporation as an index of DNA synthesis.

It is possible that the method may be equally applicable to studies of tumors from patients undergoing radiotherapy or chemotherapy. However, previous studies in HeLa cells suggest that certain discrepancies may be anticipated (1). For example, in HeLa cells, radiation-induced G_2 arrest entailed immunoreactivity without [^{3}H]thymidine incorporation for several hr. The usual correlation resumed after the cells escaped from G_2 arrest.

Antinucleoside antibodies against halogenated pyrimidines have recently been proposed as the basis of a means to estimate the labeling index in cells studied after short-term cultures (7), an approach which also may eliminate the need for autoradiography based on [^{3}H]thymidine incorporation.

Finally, it is worth noting that thymidine kinase-dependent incorporation of [^{3}H]thymidine or pyrimidine analogs may not be a totally reliable indication of DNA synthesis, employing a scavenger pathway. In certain clinical situations, [^{3}H]thymidine incorporation did not provide an authentic indication of DNA synthesis (5). Immunoreactivity to antinucleoside antibodies, presumably due to DNA strand separation during synthesis, might actually be more appropriate in such situations.

ACKNOWLEDGMENTS

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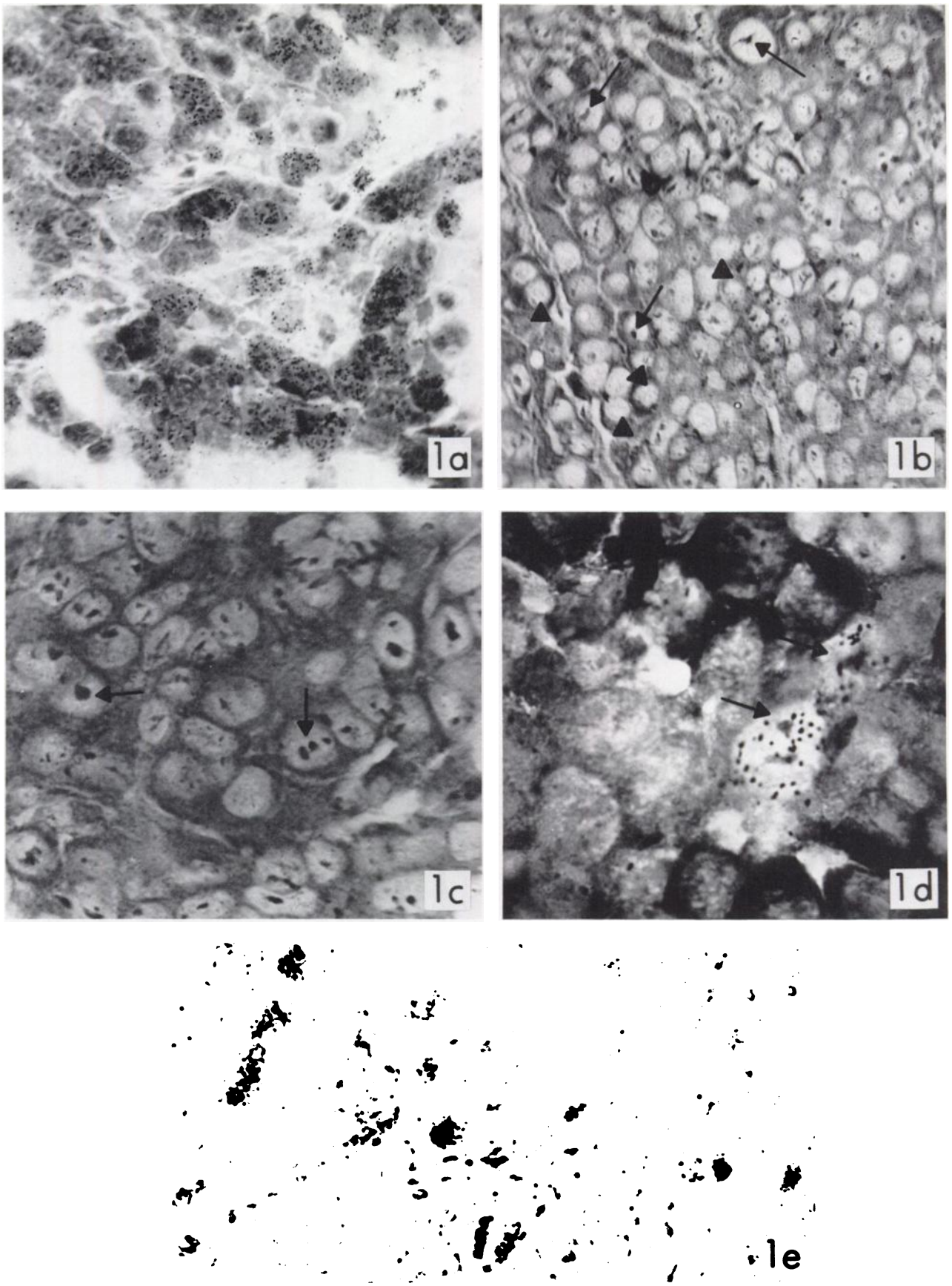
Fig. 1. [^{3}H]Thymidine-labeled C3H mouse mammary adenocarcinoma frozen sections: *a*, autoradiograph of Giemsa-stained section. Note the prominent nuclei with overlying grains. × 450. *b*, immunoperoxidase stain; *arrows*, nuclei containing dark amorphous immunopositive nuclear bodies; *arrowheads*, negative nuclei. × 450. *c*, immunoperoxidase stain. × 1000. *Arrows*, positive nuclei. *d*, immunoperoxidase-stained cells with overlying radiograph. *Arrows*, 2 doubly labeled cells, surrounded by several cells which are negative by both techniques. No direct intracellular morphological correlation between the stains and grains was observed. Dark regions at *top* and *bottom* represent heavy nonspecific background staining. × 1080. *e*, autoradiograph of cells stained only with antiperoxidase rabbit globulin, followed consecutively by peroxidase, diaminobenzidine, and hydrogen peroxide. Note absence of endogenous peroxidase activity. Nuclear immunoreactivity was not detected with this antiperoxidase rabbit globulin which was not specific for nucleosides. × 450.



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