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The Stability of Estrogen and Progesterone Receptor Expression on Breast Carcinoma Cells Stored as PreservCyt Suspensions and as ThinPrep Slides

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BACKGROUND. Analysis of estrogen receptor (ER) and progesterone receptor (PR) status is an important ancillary test in the evaluation of positive breast fine-needle aspirates. This study compares the detection of ER and PR in breast carcinoma cells suspended in PreservCyt with that achieved with stored ThinPrep slides (TP).
METHODS. ER and PR positive mammary tumor cells (cell line ZR-75–1 spiked in PreservCyt by the American Type Culture Collection) were used to evaluate the stability of immunodetection of ER and PR under two conditions: 1) TP slides prepared immediately from PreservCyt and stored air-dried (stored TP) for up to 56 days, and 2) TP prepared from cells suspended in PreservCyt (newly prepared TP) on Days 1, 2, 5, 14, 21, 42, and 56. At each of the time periods, stored TP and newly prepared TP were analyzed for ER and PR using the same immunocytochemical staining protocol. The percentage of positive cells was calculated by counting 1000 cells/TP.
RESULTS. Positivity for ER and PR was demonstrated in both stored TP and newly prepared TP on Days 1, 2, 5, 14, 21, 42, and 56. Over the 56-day period, the number of ER positive cells ranged from 41% to 57% in stored TP and from 38% to 58% in newly prepared TP. The number of PR positive cells ranged from 31% to 41% in stored TP and from 26% to 37% in newly prepared TP. Mild, nonspecific cytoplasmic and nuclear staining occurred in all newly prepared TP (PR > ER).
CONCLUSIONS. ER and PR antigenicity was preserved in both stored TP and newly prepared TP of mammary tumor cells over a 56-day storage period. This demonstrates that ER and PR status can be evaluated in cytologic material from breast carcinoma using the ThinPrep technique. ***Cancer (Cancer Cytopathol)* 1998;84:355–60.** © *1998 American Cancer Society.*

KEYWORDS: breast carcinoma, fine-needle aspiration, estrogen receptor, progesterone receptor.

Fine-needle aspiration (FNA) is a cost-effective technique for diagnosing palpable and radiologically detected breast masses. Because definitive cytopathologic diagnoses of carcinoma are accurate, patients can be treated for primary, locally recurrent, or metastatic breast carcinoma based on FNA diagnoses alone. Ideally in such cases, ancillary studies that are required for selecting appropriate treatment, such as estrogen receptor (ER) and progesterone receptor (PR) analysis, are performed on cytopathologic samples if surgical excision is not required. Consequently, validated and standardized immunocytochemical staining protocols are needed for the type of cytopathologic preparation used.

ThinPrep (Cytyc Corporation, Boxborough, MA) is a Food and Drug Administration–approved method for preparing cytopathologic

specimens; it uses a liquid-based collection system to prepare thin-layer slides. In this system, aspirated cells are placed in buffered fixative (Cytolyt) rather than being smeared directly on glass slides. In the laboratory, the cell suspensions are centrifuged and resuspended in PreservCyt, and then prepared as thin-layer slides using the ThinPrep processor. Because the composition of Cytolyt and Preservcyt differs from other fixatives commonly used in pathology, such as 95% ethanol and 10% buffered formalin, the validity of using standard immunocytochemistry to detect ER and PR in aspirated breast specimens prepared with the Cytyc system requires study.

In this investigation, we used cell suspensions of an ER and PR–expressing mammary tumor cell line, ZR-75–1, to evaluate two protocols for processing breast FNAs prepared from the ThinPrep liquid-based collection system. Our aim was to investigate the preservation of ER and PR antigenicity in breast carcinoma cells suspended in PreservCyt, as well as in cells stored on ThinPrep slides, and to determine the length of time for which the suspended and/or stored material can still be utilized. Our results may be useful in developing and standardizing a protocol for performing ER and PR analysis on breast carcinomas diagnosed using FNAs prepared as thin-layer slides.

MATERIALS AND METHODS

Preparation of Test Samples

Cells (approximately 5×10^6) from a well-characterized ER and PR positive mammary tumor cell line, ZR-75–1 (American Type Culture Collection [ATCC] CRL-1500), were used for the study. The ZR-75–1 cultures were derived from a malignant ascitic effusion in a woman age 63 years with metastatic ductal carcinoma of the breast. Receptor analysis using competitive binding assays at multiple concentrations of radiolabeled ligand have demonstrated that ZR-75–1 cells contain 29 fmol/mg cytoplasmic protein of ER and 43 fmol/mg cytoplasmic protein of PR.[1]

ZR-75–1 cells were spiked in 100 mL of PreservCyt provided to ATCC by Cytyc Corporation. The cell suspensions were equally divided into 5 vials containing approximately 1×10^6cells per 20-mL vial of PreservCyt at the company.

ER and PR immunocytochemical staining was performed on Days 1, 2, 5, 14, 21, 42, and 56 on thin-layer slides that were prepared using two different protocols. In one protocol, ThinPrep slides were prepared immediately from PreservCyt-suspended cells and stored air-dried at room temperature for up to 56 days. In the other protocol, newly prepared slides were made from the cell suspension on the designated days indicated above. The following describes in details the steps of both protocols.

ThinPrep slides were prepared from the PreservCyt-suspended cells using the ThinPrep Processor 2000. The ThinPrep slides were processed by placing a slide and a vial of PreservCyt on the ThinPrep 2000 processor. The instrument removes cells by suction onto a rotating cylinder capped with a polycarbonate filter. When a sensor detects a specified pressure increase resulting from blockage of the filter pores by cellular material, the suction is released and cells are transferred to a slide using slight positive pressure. The instrument is calibrated to remove a sufficient number of cells to produce a thin-layer slide. After this process, the slides were immediately immersed in 95% alcohol for 15 minutes, air-dried, and stored at room temperature (stored TP). The stored TP and remaining PreservCyt vials were transferred overnight to the George Washington University Medical Center, where, on Days 2, 5, 14, 21, 42, and 56, three ThinPrep slides were prepared from cells suspended in PreservCyt (newly prepared TP) then fixed in 95% alcohol for approximately 1 hour and air-dried.

Immunocytochemical Staining and Evaluation

Immunocytochemical staining for ER and PR with a negative control was performed on three stored TP and three prepared TP slides on Days 1, 2, 5, 14, 21, 42, and 56. The immunocytochemical preparations were evaluated by manually counting the number of positive nuclei in 1000 cells (SOT), and results were expressed as the percentage of positive cells. Staining intensity and background nonspecific staining were also subjectively evaluated. According to the criteria of Masood et al., tumors are considered hormone receptor positive when 20% or more of the cells are stained.[2] However, in our laboratory, samples in which 10% of the nuclei demonstrate specific staining are considered positive for ER or PR.

The staining protocol was also tested on breast carcinoma cells scraped from resected breast specimens that had been suspended in PreservCyt. Suspended cells from cases known to be ER and PR positive by immunohistochemistry performed on formalin fixed, paraffin embedded tissue sections were used to optimize the antibody dilution. A dilution run was also performed on the PreservCyt-suspended ZR-75–1 cell line. We found that the protocol that was optimal for ER and PR immunocytochemical staining was the same as the one used for formalin fixed, paraffin embedded sections in our laboratory. The protocol is summarized in Table 1.

TABLE 1
Immunocytochemical Protocol[a]

1. Buffer	5 min
2. Antigen Retrieval Citra (Biogenex)	Detailed below
3. Buffer	5 min
4. Primary antibody ER (Dako) 1:20, PR (Dako) 1:25	1 h at room temperature
5. Buffer	5 min
6. Link (Dako LSAB Kit)	10 min
7. Buffer	5 min
8. Streptavidin (Dako LSAB Kit)	10 min
9. Buffer	5 min
10. Chromogen-DAB (Sigma Tablets)	4 min
11. Buffer	5 min
12. Counterstain	

Antigen Retrieval Citra (Biogenex)

1. Heat 180 mL distilled water + 20 mL Antigen Retrieval Citra Concentrate in microwave for 3 min at 70% power.
2. Repeat Step 1.
3. Add slides to heated solution.
4. Heat slides in solution for 3 min.
5. Allow slides to stand in solution for 20 min.

ER: estrogen receptor; PR: progesterone receptor.
a The same protocol is applied to formalin fixed, paraffin embedded sections in our laboratory.

TABLE 2
Results of ER Staining

	Day						
	1	2	5	14	21	42	56
Type of TP	% of positive cells[a]						
Stored	45	46	49	41	57	53	53
Newly Prepared	50	38	40	41	58	45	51

ER: estrogen receptor; TP: ThinPrep slides.
a Percentage is based on counting 1000 cells.

TABLE 3
Results of PR Staining

	Day						
	1	2	5	14	21	42	56
Type of TP	% of positive cells[a]						
Stored	32	33	31	39	36	41	33
Newly Prepared	30	32	32	26	37	32	36

PR: progesterone receptor; TP: ThinPrep slides.
a Percentage is based on counting 1000 cells.

RESULTS

The immunocytochemical staining results of the ZR-75–1 cells for ER and PR are summarized in Tables 2 and 3. ER and PR antigenicity was preserved in both stored TP and newly prepared TP of mammary tumor cells over a 56-day storage period. Specific nuclear staining was identified for ER and PR on stored TP and newly prepared TP for all time periods. Positive staining was comparable in intensity in both stored TP and newly prepared TP. However, loss of cytologic detail resulting in a hazy appearance of the nucleus was consistently noted in stored TP slides. This artifact was attributed to air-drying artifact and inadequate length of fixation time of the cells in 95% alcohol following preparation of the slides. Mild, nonspecific cytoplasmic and nuclear staining was recognized in all negative control slides made according to the newly prepared TP protocol. This nonspecific staining was more noticeable in the PR-stained slides than in the ER-stained slides.

DISCUSSION

FNA is frequently used to diagnose breast carcinoma. In most situations, the cytopathologic diagnosis is followed by surgical resection of the tumor. In these cases, ER and PR analysis is usually deferred until a tissue specimen is available. However, there are an increasing number of situations in which ER and PR determinations performed on cytopathologic material would be useful in patient management, i.e., for patients who require preoperative chemotherapy, in cases of metastatic or recurrent breast carcinoma, and in cases of metastatic carcinoma in which a breast primary is considered in the differential diagnosis.

Several techniques for demonstrating ER and PR expression in cytologic specimens have been reported to produce a good correlation between staining on cytologic and histologic specimens and agreement with biochemical assays.[3–8] In our laboratory, ER and PR immunostains are performed on conventional smears using the same steps of fixation and processing that are used for frozen sections. Therefore, special handling at the time of the FNA and good coordination with the staff of the immunohistochemistry laboratory to ensure immediate smearing, fixing, and storing of the specimen are necessary. Fixation involves the use of formaldehyde, cold methanol, and cold acetone, which are not readily available in cytology laboratories or in clinicians' offices.[3,4,7] In addition, the slides must be stored at −10 to −20 degrees centigrade in a sucrose solution to preserve antigenicity. Furthermore, distribution of the diagnostic malignant cells may vary between smears, and the slides specifically prepared for receptor studies may contain insufficient cellular material for ER and PR analysis.[3]

Marchetti et al. described a method in which cells suspended in isotonic cell culture medium were concentrated on a small surface area by cytocentrifugation.[4] This technique was intended to preserve morphologic detail and limit reagent costs. Hudock et al.

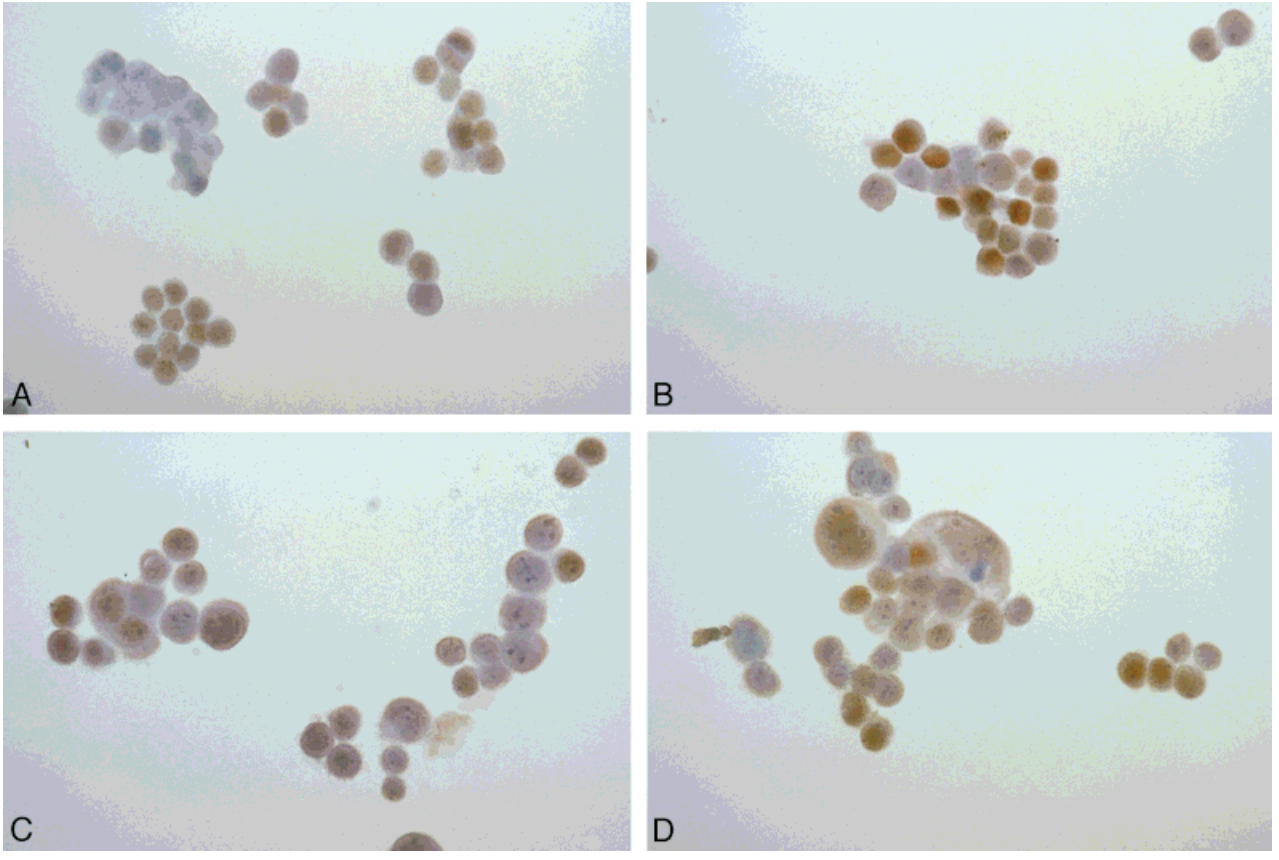

FIGURE 1. Day 1: Estrogen receptor stain on a stored ThinPrep slide (A) and progesterone receptor stain on a stored ThinPrep slide (B) show specific nuclear staining. A mild loss of cytologic detail is apparent. Estrogen receptor stain on a newly prepared ThinPrep slide (C) and progesterone stain on a newly prepared ThinPrep slide (D) show similar nuclear staining; however, a slight nonspecific staining, mostly cytoplasmic, is also noted.

studied the effects of several fixatives (including Shandon cytospin collection fluid, ethanol, and formalin) used with various preparations (such as smears and cell blocks) on the performance of ER and PR stains.[8] They reported that the smears fixed in Shandon cytospin collection fluid for ER had the largest number of positive tumor nuclei and the greatest staining intensity, whereas cell blocks fixed in formaldehyde were preferable for PR studies.

Liquid-based cytology permits the collection of the FNA sample in a fixative suitable for both cytopathologic diagnosis and ancillary studies. Because the cells obtained in multiple passes may be collected in one vial, a single collection should be sufficient for diagnosis and ancillary testing in most cases. The ThinPrep processor mixing action permits the preparation of multiple slides of a relatively consistent appearance; consequently, slides prepared for immunostaining from malignant aspirates can be presumed to contain diagnostic cells. Our data indicate that the portion of the specimen that is not used for cytopathologic diagnosis may be stored and utilized for ancillary studies, such as ER and PR staining, at a later time.

The suitability of PreservCyt-suspended material for immunocytochemical analysis has been demonstrated in several studies.[9,10] Leung et al. tested commonly used antibodies on ThinPrep slides and established that, except for some lymphoid markers, various antigens can be demonstrated by immunocytochemistry.[9] In addition, when compared with the conventional smears, the preparations showed a more even staining of cells with no entrapment of immunoreagents in thick cell aggregates. In addition, the slides had a cleaner background, which resulted in an easier interpretation than could be achieved with smears. Dabbs et al. also tested commonly used antibodies, including ER and PR, on direct smears and ThinPrep slides prepared from 41 resected specimens.[10] The ThinPrep slides showed equal or greater intensity and distribution of proper staining than direct smears. False-negative results were identified in three direct smears and two ThinPrep slides, whereas a single ThinPrep slide was determined to show false-

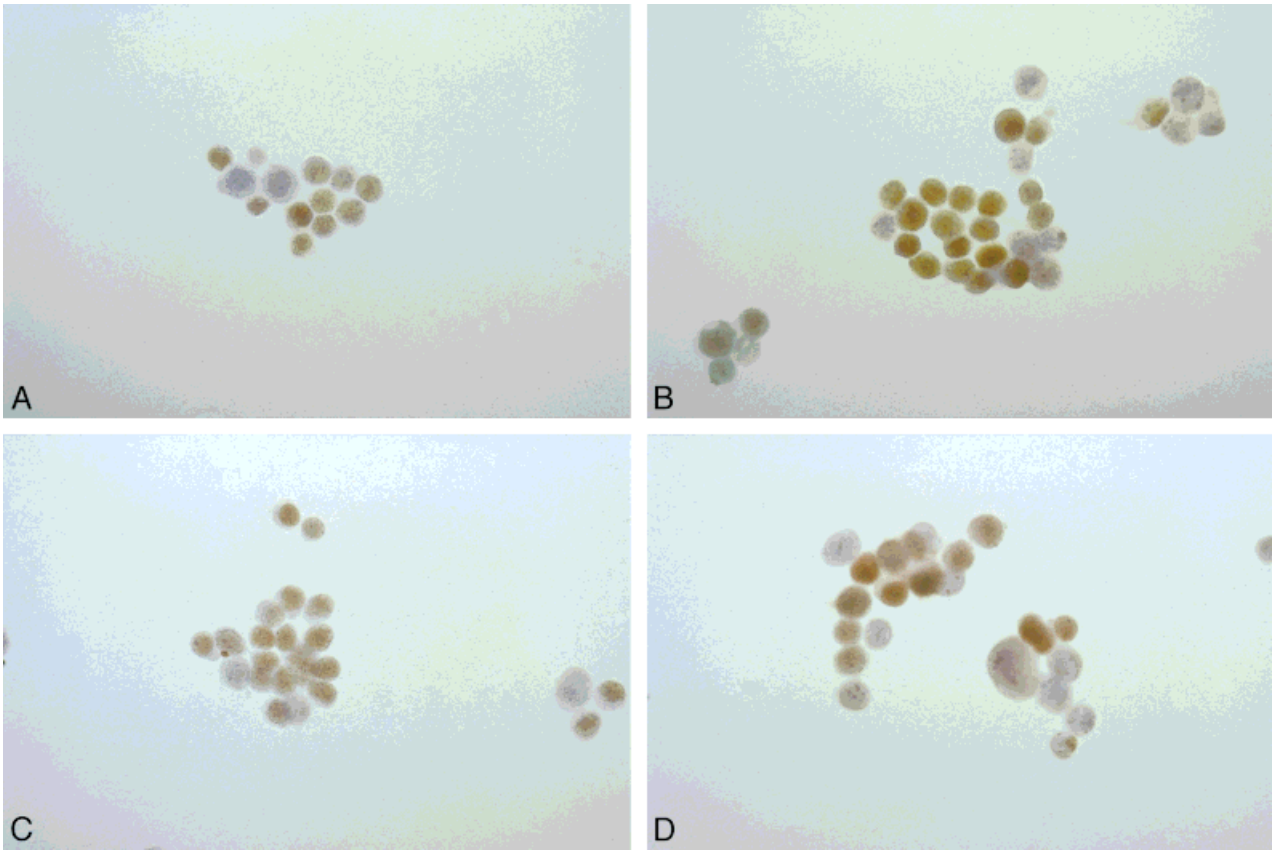

FIGURE 2. Day 21: Similar staining intensity and specificity is present for estrogen receptor stain on a stored ThinPrep slide (A), progesterone receptor stain on a stored ThinPrep slide (B), estrogen receptor stain on a newly prepared ThinPrep slide (C), and progesterone receptor stain on a newly prepared ThinPrep slide (D).

positive staining. Other reported advantages of the ThinPrep method include the ability to use higher antibody dilutions and prepare a large number of slides for immunocytochemical panels.[10,11] However, the decrease in the cost of the reagents may be counteracted by the cost of the ThinPrep processor and its consumables, especially in laboratories handling a smaller number of specimens.

This study demonstrates that specific nuclear staining for ER and PR was achieved in both stored TP and newly prepared TP after 1, 2, 5, 14, 21, 42, and 56 days of storage. Over the 56-day period, the number of ER and PR positive cells remained relatively constant and was similar with the two types of preparations. Mild, nonspecific cytoplasmic and nuclear staining occurred in all newly prepared TP and was more pronounced in the PR stains than in the ER stains. Loss of cytologic detail resulting in a hazy appearance of the nucleus was present on all stored TP and was attributed to air-drying artifact. Subsequently, we established that this artifact can be avoided by fixing the ThinPrep slides for a longer period of time before air-drying them. In our hands, extending the fixation of the ThinPrep slides for 1 hour instead of 15 minutes in 95% alcohol prior to air-drying has proven effective in overcoming this technical problem without altering the ER and PR results.

Based on our results, we conclude that ER and PR status can be evaluated in FNA material from breast carcinomas by using the ThinPrep technique and by applying our tested immunostaining protocol. Qualitative ER and PR analysis can be done at any time during the 3-week preservation period recommended by the manufacturer, and, as demonstrated, up to 56 days after collection. This will provide flexibility in performing ER and PR analysis long after the cytologic diagnosis of carcinoma is rendered, when such studies are deemed necessary for patient management.

In summary, the ThinPrep technology offers valid advantages in the performance of ER and PR analysis. Sample collection and storage is simple and permits the collection of the FNA sample for both cytopathologic diagnosis and ancillary studies. The technology permits the preparation of multiple slides of a rela-

tively consistent appearance. The distribution of malignant cells is relatively homogenous in all prepared slides, and the portion of specimens not used for cytopathologic diagnosis may be stored and utilized for ER and PR analysis up to 56 days after collection. Others studies,[9] as well as our own experience, have also shown that the ThinPrep immunostained slides have a clean background and even staining with no entrapment of immunoreagents in thick cell aggregates. However, we are not advocating that laboratories switch to using ThinPrep technology because it is better than the conventional techniques for cytologic preparations or ER and PR analysis. Moreover, it is also important to emphasize that there is an increased cost when this technology is used.

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