Proc. Natl. Acad. Sci. USA
Vol. 82, pp. 4803–4807, July 1985
Medical Sciences

Immunocytochemical staining of estrogen receptor in paraffin sections of human breast cancer by use of monoclonal antibody: Comparison with that in frozen sections

(heterogeneity of estrogen receptor)

AKIHIKO SHIMADA*, SATOSHI KIMURA*, KAORU ABE*†, KOICHI NAGASAKI*, ISAMU ADACHI*, KEN YAMAGUCHI*, MASAFUMI SUZUKI*, TAKASHI NAKAJIMA‡, AND LARRY S. MILLER§

*Endocrinology and ‡Pathology Divisions, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104, Japan; and §Cancer Research Laboratory, Diagnostics Division, Abbott Laboratories, North Chicago, IL 60064

Communicated by Elwood V. Jensen, March 18, 1985

ABSTRACT **Estrogen receptor (ER) in human breast cancer tissues was demonstrated in paraffin sections as well as in frozen sections by immunoperoxidase methods using monoclonal antibody (H222) against ER. The avidin-biotin-peroxidase complex method was used for the paraffin sections fixed in cold buffered formalin, and the peroxidase-antiperoxidase method was used for the fixed frozen sections. The results were compared with the ER content in the respective tumor tissue determined by dextran-coated charcoal assay. The specific staining for ER was located exclusively in the nuclei of cancer cells in both paraffin and frozen sections. Differences in the intensity and distribution of nuclear staining within a section were often observed, suggesting heterogeneity of the ER content of individual breast cancer cells. In 24 breast cancer tissues studied simultaneously by both paraffin and frozen section methods, 21 (88%) showed similar evaluation of the presence of ER. The results of immunocytochemical staining agreed with those of the dextran-coated charcoal assay in 89 (82%) of the 109 paraffin-sectioned tumor tissues and in 24 (86%) of the 28 frozen-sectioned tissues, indicating that ER can be demonstrated immunocytochemically by use of paraffin as well as frozen sections.**

It is generally established that human breast cancer, which is rich in estrogen receptor (ER), responds well to endocrine therapy (1) and that patients with ER-positive breast cancer have a more favorable clinical course and prognosis than those with ER-negative cancer (2–5). Therefore, determination of the ER content of breast cancer tissue is indispensable for selecting a regimen of treatment when there is a relapse or for predicting the prognosis. The methods of biochemical ER assay, however, are complicated and require radioactive materials. In addition, because of recent progress in diagnosing a smaller lesion, it is often impossible to obtain enough tissue (0.5 g or more) needed for the conventional radioligand assay. Furthermore, the biochemical ER assay cannot evaluate heterogeneity of ER content among the breast cancer cells, which could be a reason for the unresponsiveness to endocrine therapy of about 40% of patients with ER-positive breast cancer (6).

In order to circumvent these disadvantages of the current ER assay, many immunocytochemical methods using anti-estradiol antibody (7–11) and cytochemical methods using fluorescein- or peroxidase-labeled estradiol (12–15) have been proposed. However, sucrose gradient analysis revealed that the antibodies against estradiol do not necessarily detect the estradiol–ER complex (16). In addition, affinity of the estradiol-conjugate for ER was shown to be extremely low when compared with that of free estradiol (17–19). Although these published methods were aimed at visualizing the specific binding of estrogen to ER, the methods of tissue preparation used in these studies could not prevent the loss of ER through diffusion or the reduced ability of estrogen to bind to its receptor (20). Therefore, it is reasonable to conclude that the stains obtained by these immunocytochemical and cytochemical methods are not specific for ER (16–20).

Recently, monoclonal antibodies to human ER were developed by Greene *et al.* (21) and Miller *et al.* (22). By use of these monoclonal antibodies against ER, King and Greene first demonstrated ER in frozen tissue sections prepared from human breast cancer and other sources (23). These monoclonal antibodies are highly specific for ER and could serve as a more reliable probe for detecting ER in tissues. However, frozen sections have limitations in their use for detailed examination of tumor morphology and for retrospective studies.

In this study, methods were developed to demonstrate ER in cold formalin-fixed paraffin sections as well as in frozen sections of human breast cancer tissues by use of one of these monoclonal antibodies. The utility of immunocytochemical staining of ER was evaluated by comparing the results with the results of determining ER content by the dextran-coated charcoal (DCC) assay.

MATERIALS AND METHODS

Breast Cancer Tissues. One hundred and thirteen breast cancer tumors (104 primary and 9 metastatic tumor tissues) were obtained at surgery at the National Cancer Center Hospital and Keio University Hospital, Tokyo. The tumor specimen was cut into two pieces immediately after the resection. One piece was used for routine histological examination, and the other was stripped of adhering fat, quickly placed on ice, and further divided into several pieces for immunocytochemical staining and the DCC assay for ER. For immunocytochemical detection of ER, 109 tissues were processed by the paraffin section method, and 28 were processed by the fresh frozen section method. Thus, 24 tissues were examined as both paraffin and frozen sections. The piece of tumor for the DCC assay was frozen immediately and stored in liquid nitrogen until analyzed.

Monoclonal Antibody to ER. The monoclonal antibody (H222) used in this study was developed and characterized by one of us (L.S.M.) (22) and provided to investigations at the

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: ER, estrogen receptor; DCC, dextran-coated charcoal; ABC, avidin-biotin-peroxidase complex; DAB, 3,3′-diaminobenzidine tetrahydrochloride; PAP, peroxidase-antiperoxidase.
†To whom reprint requests should be addressed.

National Cancer Center of Japan. This monoclonal antibody was developed by injecting purified ER (21, 24) prepared from the MCF-7 human breast cancer cell line (25) into Lewis rats and hybridizing the spleen cells with mouse myeloma cells (Sp2/0-Ag14). This antibody has been shown to be ER-specific by several criteria, and cross-reactivity with other steroid receptors and cellular proteins was negligible (22).

Tissue Preparation and Immunocytochemical ER Staining for Paraffin Sections. The tumor pieces were fixed in buffered formalin (0.1 M sodium phosphate, pH 7.4/10% formalin) for 24 hr at 4°C. After being rinsed overnight at 4°C in 0.1 M sodium phosphate (pH 7.4), they were dehydrated with graded ethanol and embedded in paraffin. Paraffin sections (4 μm) were cut, deparaffinized with xylene, and rinsed thoroughly with absolute ethanol. Then they were soaked in absolute methanol containing 0.3% H_2O_2 for 30 min at room temperature to decrease the endogenous peroxidase activity. The sections were washed three times with 50 mM Tris·HCl, pH 7.6/137 mM NaCl (Tris/NaCl) and were incubated with normal rabbit serum (10% in Tris/NaCl) for 30 min at room temperature so as to reduce the nonspecific staining. Excess serum was removed by blotting, and the sections were incubated with the monoclonal antibody (H222, 10 μg/ml) or with normal rat IgG (10 μg/ml) for 30 min at 37°C. After being washed with Tris/NaCl, the sections were incubated with biotinylated rabbit antibody to rat IgG (15 μg/ml, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After being washed with Tris/NaCl, they were incubated with avidin-biotin-peroxidase complex (ABC) reagent (Vector Laboratories) for 30 min at room temperature in a moist chamber (26, 27). This was followed by another washing with Tris/NaCl and a subsequent reaction with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution (0.05 M ammonium acetate/citric acid, pH 5.5–6.0) containing 0.0075% H_2O_2 and 0.2 mg of DAB per ml) for color development (28) in the dark for 6 min. Finally, the sections were counterstained lightly with hematoxylin unless indicated otherwise.

Tissue Preparation and Immunocytochemical ER Staining for Frozen Sections. The tumor pieces were quickly frozen in an acetone/dry-ice bath. Cryostat sections (6 μm) were cut and fixed immediately in 0.01 M potassium phosphate, pH 7.4/145 mM NaCl (P_i/NaCl) containing 10% formalin for 15 min at 4°C. The slides were soaked in cold methanol at −20°C for 4 min and then in cold acetone at −20°C for 1 min. After being rinsed in P_i/NaCl, they were incubated with normal goat serum (2% in P_i/NaCl) for 15 min to reduce nonspecific staining. The tissue sections were then incubated successively with the monoclonal antibody (10 μg/ml) or with normal rat IgG (10 μg/ml), goat antibody to rat IgG (1:100), and rat peroxidase-antiperoxidase (PAP) (1:200) for 30 min each at room temperature in a moist chamber (29, 30). Each incubation was followed by a 5-min washing with P_i/NaCl. After a final rinsing in P_i/NaCl, the sections were incubated with DAB substrate solution for 6 min in the dark and counterstained with hematoxylin.

Evaluation of the Immunocytochemical Staining. Other than the faint nonspecific staining seen in the adjacent tissue section that had been incubated with normal rat IgG instead of the monoclonal antibody, the brown color produced by DAB in the tissue section that had been incubated with the monoclonal antibody was regarded as specific for ER. The average intensity of the specific staining was scored as negative, faint, moderate, or strong while scanning the tissue section. By use of a microscope grid, the percentage of positively stained tumor cells was also estimated in each tumor by scanning at least 1,000 cells in high power (×400) fields from multiple portions of each tumor.

DCC Assay for ER. The method of Rosen et al. and Menendez-Botet et al. (31, 32) was used with modifications. The modifications were as follows: The frozen tissues were pulverized in liquid nitrogen, and the powder was homogenized in a solution containing 10 mM Tris·HCl (pH 7.4), 1 mM EDTA, 3 mM sodium azide, 12 mM monothioglycerol, and 10% (vol/vol) glycerol. Aliquots of each cytosol were incubated overnight at 4°C with different concentrations—0.125, 0.25, 0.5, 1, and 2 nM of 17β-[2,4,6,7-^{3}H(N)]estradiol (112 Ci/mmol, New England Nuclear; 1 Ci = 37 GBq) with and without a 1,000-fold excess of 17β-estradiol (Sigma). Protein concentration was determined by the method of Lowry et al. (33). The number of binding sites and the dissociation constant of ER in each tumor were analyzed by the method of Scatchard (34) after subtracting nonspecific binding. The quantity of receptor $\geq$ 5 fmol/mg of cytosol protein with an inhibition of >60% by excess cold estradiol was taken as biochemically ER positive.

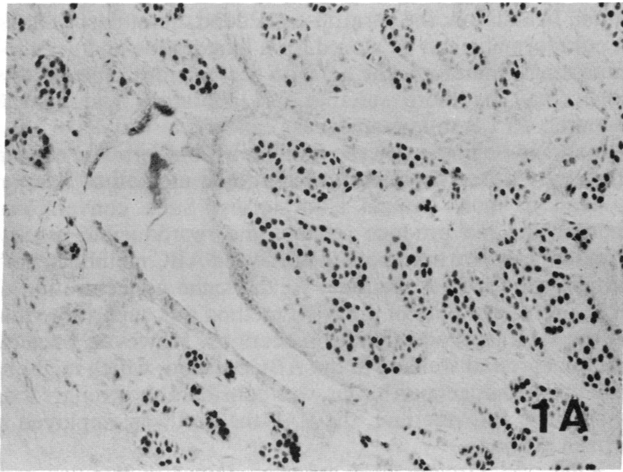
Statistics. Statistical significance of the difference between the two groups was determined by either the χ^2 test or Fisher's direct probability method.

RESULTS

Immunocytochemical Demonstration of ER in Breast Cancer Tissues. The specific ER immunoperoxidase staining was demonstrated exclusively in the nuclei in both paraffin (Fig. 1*A*) and frozen sections (Fig. 2). The ER-specific immunostaining covered the nucleus of the positive cell diffusely, but the intensity of the nuclear staining was not always uniform (Fig. 1*A*). Frequently, the distribution of positive nuclear staining varied considerably, depending on the area within a section (Fig. 3). The heterogeneity of the nuclear staining in distribution and intensity did not correspond to differences in tumor histology. However, the tumor cells with prominent nuclear atypia or pleomorphism tended to be negative for ER by immunocytochemistry.

Although no cytoplasmic staining was observed in the frozen sections, it was seen in a few paraffin sections. However, it was very faint when compared with the nuclear staining, and similar faint cytoplasmic staining was also seen in slides of paraffin sections incubated with normal rat IgG (control). Moreover, no nuclear staining was observed in the negative controls of either paraffin (Fig. 1*B*) or frozen sections (data not shown). The faint staining seen occasionally in the connective tissue, necrotic tissue, leukocytes and erythrocytes was also regarded as nonspecific, because it also was observed in the negative control sections. Thus, only the nuclear staining was considered specific for ER. The tissue sections were considered immunocytochemically as ER positive if they contained any cells showing nuclear staining regardless of the staining intensity. By this criterion for the immunocytochemical staining for ER, 43 of the 109 tissues (39%) examined by the paraffin section method and 14 of the 28 tissues (50%) examined by the frozen section method were evaluated as ER positive, but the difference was not statistically significant. Among the positive tissues, the percentage of immunocytochemically ER-positive cells ranged from 5% to 72% in paraffin sections and from 21% to 81% in frozen sections.

Comparison of Immunocytochemical Staining in Paraffin and Frozen Sections. The results of the immunocytochemical staining of ER in both paraffin and frozen sections are shown in Table 1. The nuclear staining specific for ER was positive in 12 of the 24 tumors in the paraffin sections and in 13 tumors in the frozen sections. The results of the staining in the two types of sections agreed in 21 of the 24 tumors. There was a good correlation between paraffin and frozen sections both in terms of the intensity of staining and the percentage of positively stained cells. In each tumor, the ER positivity



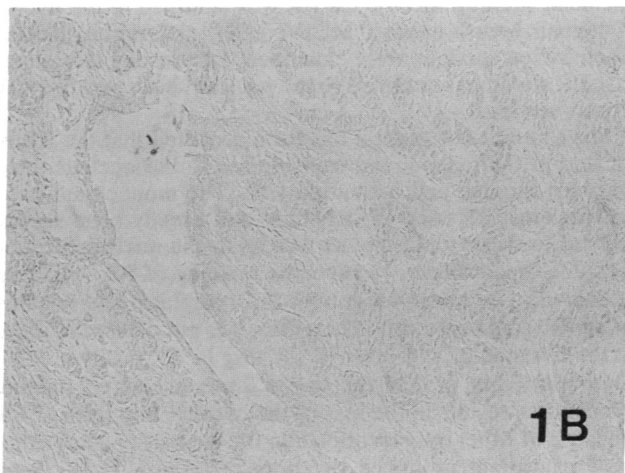


FIG. 1. Immunocytochemical staining of an ER-positive breast cancer in paraffin sections. This tumor was taken from a 62-year-old woman. The ER content estimated by DCC assay was 57.6 fmol/mg of cytosol protein. Adjacent sections were treated either with 10 μg of monoclonal antibody IgG, H222 (*A*) per ml or with 10 μg of normal rat IgG per ml as the negative control (*B*) and were stained as described in *Materials and Methods* without counterstain. Note that the specific staining for ER is located exclusively in the nuclei of cancer cells and that the nuclear staining showed various intensities (*A*). (Original magnification = ×165.)

evaluated by the immunocytochemical method also correlated well with that determined by the DCC assay. However, the intensity of staining and the percentage of ER-positive cells were not always proportional to the ER content estimated by the DCC assay.

Comparison of Immunocytochemical Staining with ER Content Determined by DCC Assay. In the paraffin sections as shown in Table 2, 49 of the 109 tumor tissues showed an ER content ≥ 5 fmol/mg of cytosol protein in the DCC assay, and 36 of these ER-positive tissues (73%) were immunocytochemically positive for ER. While the ER content in 10 of the 49 ER-positive tissues was relatively low (5–15 fmol/mg of cytosol protein), 7 of these 10 tissues were evaluated immunocytochemically as ER positive, suggesting that low content of ER by the DCC assay does not necessarily lower the sensitivity of the immunocytochemical evaluation. Also, of the 60 tumor tissues negative for ER by the DCC assay, 53 (88%) had no nuclear staining in the paraffin sections. Thus, the overall agreement with the DCC assay was 82% (89/109) for the paraffin sections, the association being significant ($P < 0.001$). Also, in the frozen sections (Table 2), 12 of the 28 tumors were ER positive by the DCC assay, and 11 of these

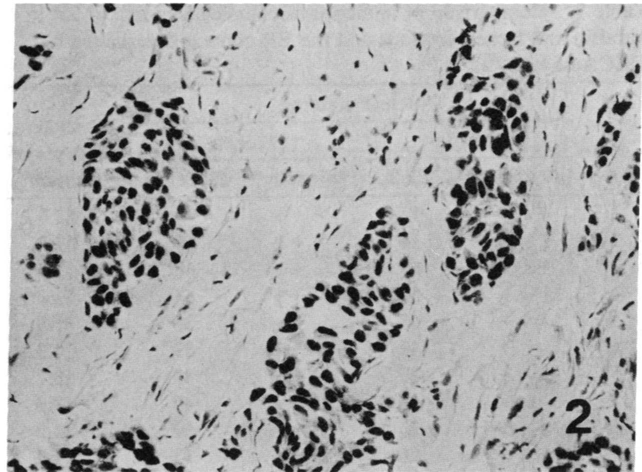


FIG. 2. Immunocytochemical staining of an ER-positive breast cancer in a frozen section. This tumor was taken from a 55-year-old woman. The ER content estimated by the DCC assay was 14.2 fmol/mg of cytosol protein. Specific staining for ER is located exclusively in the nuclei of cancer cells. This section was counterstained lightly with hematoxylin. (Original magnification = ×165.)

12 (92%) were immunocytochemically positive for ER. Of the 16 tissues that were ER negative by the DCC assay, 13 (81%) had no nuclear staining in the frozen sections. Therefore, the overall agreement was 86% (24/28) for the frozen sections ($P < 0.001$).

DISCUSSION

The immunocytochemical staining for ER in the cold formalin-fixed paraffin sections showed good correlations with that in the frozen sections. Furthermore, the immunocytochemical staining in both paraffin and frozen sections correlated well with the ER positivity determined by the DCC assay. These data indicate that ER protein is demonstrable in paraffin as well as frozen sections by immunocytochemistry using the monoclonal antibody, H222. Thirteen of the 49 paraffin-embedded tumors, positive for ER by the DCC assay, were negative on immunostaining (Table 2). It is

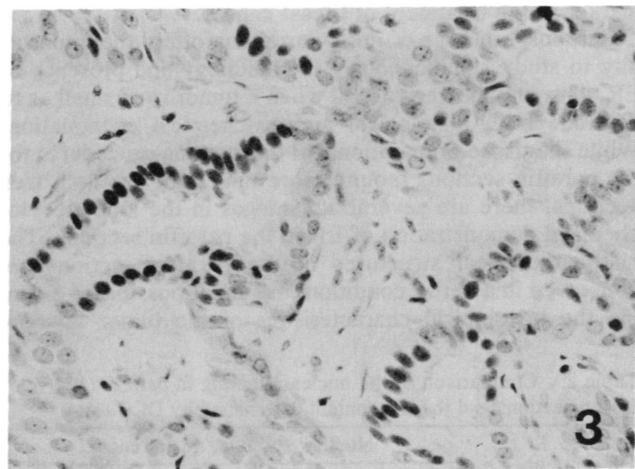


FIG. 3. Heterogeneity in the nuclear staining of an ER-positive breast cancer. This tumor was taken from a 68-year-old woman, and its ER content estimated by the DCC assay was 10.1 fmol/mg of cytosol protein. The immunocytochemical staining was carried out on paraffin sections with light counterstain. The ER-positive cells are present just as a mosaic among the ER-negative cells. (Original magnification = ×330.)

Table 1. Comparison of immunocytochemical staining of ER in paraffin and frozen sections and the ER content determined by DCC assay

Case no.	Nuclear staining: Paraffin Intensity*	Paraffin % Cells†	Frozen Intensity*	Frozen % Cells†	ER content, fmol/mg of protein
1	+++	72	+++	79	452.6
2	++	34	++	65	67.3
3	+++	66	+++	68	57.6
4	++	64	++	64	53.7
5	++	66	++	36	31.5
6	+	41	++	53	20.3
7	++	20	++	45	18.7
8	−	0	++	49	17.3
9	+++	23	+++	69	14.2
10	+++	60	+++	81	11.1
11	++	63	++	66	9.2
12	++	44	++	54	<5
13	−	0	++	21	<5
14	+	15	−	0	<5
15	−	0	−	0	<5
16	−	0	−	0	<5
17	−	0	−	0	<5
18	−	0	−	0	<5
19	−	0	−	0	<5
20	−	0	−	0	<5
21	−	0	−	0	<5
22	−	0	−	0	<5
23	−	0	−	0	<5
24	−	0	−	0	<5

*+++, Strong; ++, moderate; +, faint; −, negative.
†Percentage of positive-staining cells.

unlikely that these false negatives would have been positive by immunostaining of frozen sections since there was such excellent correlation of the immunostaining results when both paraffin and frozen sections were examined in the same tumor (Table 1). In contrast, there were 7 positive tumors by immunostaining of paraffin sections that were negative by the DCC assay. These examples of "false positive" and "false negative" results will require evaluation of patient outcomes and the results of endocrine therapy to establish the relative value of immunostaining versus the DCC assay as a useful marker in the treatment of breast cancer.

Certainly, the use of immunostaining offers the opportunity to study simultaneously the histology and presence of ER. This may be very useful when a tumor is so small as to preclude both histological and biochemical examination. While the tissue preparation and the staining procedures for the paraffin sections require more time than for the frozen sections, there are several advantages in the immunocytochemical demonstration of ER in the paraffin sections. The histopathological structures of the paraffin sections are conserved in a better condition, making it possible to examine the histological characteristics of the tumor in more detail. In addition, the paraffin-embedded tumor tissues fixed in cold formalin can be stored for a longer time and used for restaining. Therefore, the paraffin sections described in this paper may be more suitable for laboratory and clinical research on ER in human breast cancer.

Table 2. Comparison of the nuclear staining in paraffin and frozen sections and the ER content determined by DCC assay

ER content, fmol/mg of protein	Nuclear staining, no. of cases: Paraffin sections Positive	Paraffin sections Negative	Frozen sections Positive	Frozen sections Negative
<5 (Negative)	7	53	3	13
≥5 (Positive)	36	13	11	1

It should be noted that the fixation in cold formalin used in this study differs from the conventional method of fixation utilized in most clinical laboratories. Such conventional fixation did not produce reliable and reproducible results. Preliminarily, we tried both the PAP and ABC method in both paraffin and frozen sections. At the same concentration of H222, the sensitivity of the ABC method was superior to that of the PAP method in paraffin sections. However, because the background staining in the ABC method, which might be due to the endogenous biotin, was considerably stronger than that in the PAP method, the PAP method was employed in frozen sections.

King and Greene (23) reported that the immunocytochemical staining specific for ER was confined to the nuclei of human breast cancer, MCF-7 cells, and rabbit uterus, when frozen sections were examined. Our results also show that ER staining is confined to the nuclei in both paraffin and frozen sections.

For a number of years it has been accepted that the ER is located in the cytosol and translocates to the nucleus after estrogen binding and activation (35). The monoclonal antibody against ER used in this study has already been shown to bind to the cytoplasmic as well as to the nuclear form of ER (22). Accordingly, the specific staining of ER would be expected to be observed in both the cytoplasm and nucleus of an ER-containing cell. Therefore, it is not clear why there is the absence of cytoplasmic staining in ER-positive cells. One possibility is that the tissue fixation and/or staining procedures results in the loss of the cytoplasmic form of ER because of either translocation into the nucleus or some other type of artifact produced by these processes. It has been reported that when unfixed frozen sections are used, considerable amounts of ER are released into the supernatant (20). However, in the case of paraffin sections, the cytosolic ER may not be as easily washed out because the tumor tissues are already fixed in buffered formalin.

An alternative possibility, proposed recently by King and Greene (23), is that ER might be present exclusively in the nuclei of ER-positive cells. There are several reports that support this concept and suggest that the cytoplasmic form of ER represents ER released from the nucleus during extraction (36, 37). This is an interesting interpretation, but further studies are needed to conclude that ER is present only in the nucleus.

It has been reported that the ER content varies from site to site even in the same breast cancer (38, 39). Similarly, in the present study we often noticed that ER-positive tumors showed patchwork or mosaic staining patterns, and that the intensity of the nuclear staining of ER-positive cells varied from cell to cell. These findings may be one of the reasons for the discrepancy between the results of the immunocytochemical staining and those of the DCC assay. Therefore, when a tumor is large, it would be of interest to compare the results of the ER-immunostaining of multiple portions from the same tumor with that of the DCC assay.

The immunocytochemical assay is the most suitable method for examining the heterogeneity of ER in breast cancers. The heterogeneity of the ER stain may be attributable to the mosaic of cells with different ER contents, the phase of the cell cycle, and/or the heterogeneity of the ER molecule itself. A detailed study on ER heterogeneity may provide additional information regarding the clinical unresponsiveness seen in about 40% of patients with ER-positive breast cancer.

We thank Prof. Elwood V. Jensen for his advice concerning this study. We are also grateful to Prof. Osahiko Abe and Dr. Kouji Enomoto, Department of Surgery, Keio University School of Medicine, Drs. Hiroshi Yamamoto and Takeshi Nanasawa, National Cancer Center Hospital, Tokyo, for their continuous support and advice. This work was supported in part by Grants-in-Aid for Cancer Research (57-8, 57S, 58-2, 58-12), a Research Grant for Intractable Diseases, and Grant-in-Aid from the Ministry of Health and Welfare for Comprehensive 10-Year Strategy for Cancer Control, Grants-in-Aid for Cancer Research from the Ministry of Education and Science, Japan.

1. Jensen, E. V. (1981) *Cancer* **47**, 2319–2326.
2. Cooke, T., George, D., Shields, R., Maynard, P. & Griffiths, K. (1979) *Lancet* **i**, 995–997.
3. Knight, W. A., Livingston, R. B., Gregory, E. J. & McGuire, W. L. (1977) *Cancer Res.* **37**, 4669–4671.
4. Maynard, P. V., Blamey, R. W., Elson, C. W., Haybittle, J. L. & Griffiths, K. (1978) *Cancer Res.* **38**, 4292–4295.
5. Rich, M. A., Furmanski, P. & Brooks, S. C. (1978) *Cancer Res.* **38**, 4296–4298.
6. McGuire, W. L. (1975) *Cancer* **36**, 638–644.
7. Ghosh, L., Ghosh, B. C. & Gupta, T. K. D. (1978) *J. Surg. Oncol.* **10**, 221–224.
8. Kurzon, R. M. & Sternberger, L. A. (1978) *J. Histochem. Cytochem.* **26**, 803–808.
9. Nenci, I., Beccati, M. D., Piffanelli, A. & Lanza, G. (1976) *J. Steroid Biochem.* **7**, 505–510.
10. Pertschuck, L. P. (1976) *Res. Commun. Chem. Pathol. Pharmacol.* **14**, 771–774.
11. Taylor, C. R., Cooper, C. L., Kurman, R. J., Goebelsmann, U. & Markland, F. S. (1981) *Cancer* **47**, 2634–2640.
12. Dandliker, W. B., Brawn, R. J., Hsu, M. L., Brawn, P. N., Levin, J., Meyers, C. Y. & Kolb, V. M. (1978) *Cancer Res.* **38**, 4212–4224.
13. Lee, S. H. (1978) *Am. J. Clin. Pathol.* **70**, 197–203.
14. Pertschuck, L. P., Gaetjens, E., Carter, A. C., Brigati, D. J., Kim, D. S. & Fealey, T. E. (1979) *Am. J. Clin. Pathol.* **71**, 504–508.
15. Walker, R. A., Cove, D. H. & Howell, A. (1980) *Lancet* **i**, 171–173.
16. Mercer, W. D., Edward, D. P., Chamness, G. C. & McGuire, W. L. (1981) *Cancer Res.* **41**, 4644–4652.
17. Chamness, G. C., Mercer, W. D. & McGuire, W. L. (1980) *J. Histochem. Cytochem.* **28**, 792–798.
18. Parl, F. F., Wetherall, N. T., Halter, S., Schuffman, S. & Mitchell, W. M. (1984) *Cancer Res.* **44**, 415–421.
19. Penny, G. C. & Hawkins, R. A. (1982) *Br. J. Cancer* **45**, 237–246.
20. Underwood, J. C. E., Sher, E., Reed, M., Eisman, J. A. & Martin, T. J. (1982) *J. Clin. Pathol.* **35**, 401–406.
21. Greene, G. L., Nolan, C., Engler, J. P. & Jensen, E. V. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5115–5119.
22. Miller, L. S., Tribby, I. I. E., Miles, M. R., Tomita, J. T. & Nolan, C. (1982) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **41**, 520 (abstr.).
23. King, W. J. & Greene, G. L. (1984) *Nature (London)* **307**, 745–747.
24. Greene, G. L. & Jensen, E. V. (1982) *J. Steroid Biochem.* **16**, 353–359.
25. Soule, H. D., Vaszquez, J., Long, A., Albert, S. & Brennan, M. J. (1973) *J. Natl. Cancer Inst.* **51**, 1409–1416.
26. Hsu, S. M., Raine, L. & Fanger, H. (1981) *Am. J. Clin. Pathol.* **75**, 734–738.
27. Hsu, S. M., Raine, L. & Fanger, H. (1981) *J. Histochem. Cytochem.* **29**, 577–580.
28. Vacca, L. L., Abrahams, S. J. & Naftchi, N. E. A. (1980) *J. Histochem. Cytochem.* **28**, 297–307.
29. Sternberger, L. A., Hardy, P. H., Cuculis, J. J. & Meyer, H. G. (1970) *J. Histochem. Cytochem.* **18**, 315–333.
30. Miller, L. S. & Konrath, J. G. (1984) *J. Steroid Biochem.* **20**, 1633 (abstr.).
31. Rosen, P. P., Menendez-Botet, C. J., Nisselbaum, J. S., Urban, J. A., Miké, V., Fracchia, A. & Schwartz, M. K. (1975) *Cancer Res.* **35**, 3187–3194.
32. Menendez-Botet, C. J., Nisselbaum, J. S., Fleisher, M., Rosen, P. P., Fracchia, A., Robbins, G., Urban, J. A. & Schwartz, M. K. (1976) *Clin. Chem. (Winston-Salem, NC)* **22**, 1366–1371.
33. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
34. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672.
35. Jensen, E. V. & DeSombre, E. R. (1973) *Science* **182**, 126–134.
36. Martin, P. M. & Sheridan, P. J. (1982) *J. Steroid Biochem.* **16**, 215–229.
37. Welshones, W. V., Lieberman, M. E. & Gorski, J. (1984) *Nature (London)* **307**, 747–749.
38. Poulsen, H. S. (1981) *Eur. J. Cancer* **17**, 495–501.
39. Straus, M. J., Moran, R., Muller, R. E. & Wotiz, H. H. (1982) *Oncology* **39**, 197–200.