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Monoclonal Antibody Technique for Detection of Estrogen Receptors in Human Breast Cancer: Greater Sensitivity and More Accurate Classification of Receptor Status Than the Dextran-coated Charcoal Method

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ABSTRACT

Estrogen receptor (ER) concentrations have been determined in 191 freshly prepared cytosols from breast cancer biopsies using both the monoclonal enzyme immunoassay (ER-EIA) and the dextran-coated charcoal (ER-DCC) methods in a single laboratory. The concentrations of the ER detected using the two methods are highly and significantly correlated (linear regression curve: ER-EIA = 15.5 + 0.82 ER-DCC; $r = 0.97$). Nevertheless, it may be most correct to interpret the data by resolving the correlation into two lines, one describing the fit for cytosols with low and intermediate concentrations (the first 75% of the distribution of ER values for all primary breast cancers: <217 fmol ER/mg cytosol protein) and one describing the fit for cytosols with the highest ER concentrations (*i.e.*, ≥217 fmol ER/mg cytosol protein).

Using a cutoff limit of 10 fmol/mg cytosol protein to distinguish between ER positive and ER negative biopsies, discrepancies in the classification of ER status were found in only 6% (12 of 191) of the cases using the two different methods. In all 12 cases, the ER concentrations as determined by both methods were in the lower range of receptor concentrations (0–53 fmol/mg cytosol protein). Of the 12 discrepancies, 10 biopsies were classified as ER negative using the ER-DCC method but ER positive using the ER-EIA method. Additional available data for these 10 patients indicate that the ER-EIA assay yielded the more biologically "correct" result. All 10 of these biopsies were either progesterone receptor positive or had nuclear ER.

By identifying the outliers of the linear regression curves (points exceeding the 80% confidence interval) of the logarithmically transformed ER concentrations, 9 of the 12 biopsies were identified. Thus, it is unlikely that the observed discrepancies are due to random events in most cases here. Since most of the few deviations observed appear to represent true differences in the sensitivities of the two methods, the ER-EIA method appears to be superior to the ER-DCC method in our hands.

The concentration of ER in 47 cytosols stored at −70°C for 3–6 yr was analyzed using the ER-EIA method, and results were compared to the concentration of ER found using the ER-DCC method on freshly prepared cytosols when the biopsies had been received at the laboratory. The linear regression curve of the correlation between ER concentrations determined using the two methods did not differ significantly from that found for the 191 freshly prepared cytosols.

INTRODUCTION

ER[3] determinations have been routinely performed at most major breast cancer centers since 1979 when, at a consensus development meeting held on "Steriod Receptors and Breast Cancer" in the National Institutes of Health, it was recommended that these analyses be performed for all primary breast cancer patients (1). The most commonly used technique for determination of ER to date is the DCC assay method, which was first described by Korenman and Dukes (2). Despite widespread efforts to standardize the ER-DCC assay method (3–8), assay results still vary, which is apparent both from results of these quality control trials as well as from the fact that the frequency of ER positivity among primary breast cancer patients is reported to vary between 38% (9) and 86% (10).

In both an American and a European trial, the monoclonal EIA that is designed for quantitation of ER in human breast cancer cytosols and that is commercially available (ER-EIA) has already been demonstrated to yield results that are significantly and highly correlated to those obtained using the DCC-assay method (11, 12). Since the ER-EIA method is based upon the recognition of the estrogen receptor molecule itself rather than receptor-ligand binding, it is independent of the presence of both endogenous hormones in the patients' tissue and proteins that nonspecifically bind estrogens. Furthermore, the assay method is both relatively simple and standardized. This method may therefore be more accurate and more easily reproducible than the DCC method. The purpose of the present investigation has been to evaluate whether the few deviations that are observed in assay results using the two methods (DCC *versus* EIA) are systematic rather than random. Furthermore, the question of whether the ER-EIA assay method can be used for cytosols that have been stored at −70°C for up to 6 yr has been investigated.

MATERIALS AND METHODS

Tissue. Freshly prepared cytosols from 191 breast cancer biopsies have been analyzed for ER concentrations using the DCC and the EIA assay methods. Both assays were performed on the same day for these cytosols.

To investigate whether analysis of frozen (−70°C) cytosols stored for a number of yrs yields viable results using the ER-EIA method, ER positive cytosols (range of ER concentration from 15 to 1148 fmol/mg cytosol protein) kept for 3–6 yr were used. The results of the ER-EIA assay were compared to the results of the ER-DCC assay originally obtained using the freshly prepared cytosol (*i.e.*, 3–6 yr ago).

DCC Assay. In all cases, the DCC steroid binding assay method was used in accord with the recommendations put forth by the EORTC (13) with the minor modifications that are described elsewhere (14).

ER-EIA Assay. The ER-EIA assay method was performed as recommended by the manufacturers (Abbott Laboratories) on the cytosols used for the DCC assay but were further diluted 2- to 6-fold. Thus, they contained a final protein concentration of approximately 0.2–1.5 mg/ml and a maximum concentration of monothioglycerol of 5 mM. This cytosol protein concentration is lower than that recommended by the manufacturers, but it facilitates assessment of samples with high ER concentrations. If despite this dilution, the ER concentration in the cytosol used for the ER-EIA assay was so high as to exceed the limits of the reference standard curve (which occurred in 5 of 114 cases), the final reaction solution was diluted 2-fold with 1 N sulfuric acid mixed, the absorbance was recorded, and the value of the diluted reactant was used to calculate the ER concentration from the reference standard curve.

Analysis of ERn. The ER-EIA assay method as described earlier (15)

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[3] The abbreviations used are: ER, estrogen receptor; DCC, dextran-coated charcoal; EIA, enzyme immunoassay; ERn, nuclear estrogen receptor.

was used on 0.6 M KCl-Tris extracts of the crude nuclear pellet to determine ERn concentrations.

Protein Analysis. Protein determinations were performed using the Bio-Rad method (Coomassie brilliant blue) with Kabi Diagnostica human serum albumin protein standard as a reference. Receptor concentrations determined using the DCC method are expressed in relation to protein concentration of the DCC-treated cytosol, while those determined using the EIA method are expressed in relation to the protein concentration in the diluted cytosol used for the assay.

A concentration of 5 fmol ER/mg cytosol protein is accepted here as being a reliable level of detection of receptors.

Statistical Methods. Conventional statistical tests have been applied to the data. The particular test used in the individual circumstance is indicated in the text or in the relevant table or legend. A value of $P < 0.05$ was accepted as being significant.

RESULTS

Study of Freshly Prepared Cytosols. Parallel analyses of 84 biopsies (range of ER concentration, 0–2022 fmol/mg cytosol protein) were performed at two different cytosol protein concentrations using the ER-EIA method. For the series analyzed at high cytosol protein concentrations (0.5–1.5 mg/ml) 10 of the 84 samples exceeded the highest value of the standard curve (>500 fmol ER/ml cytosol). In contrast, all 84 samples could be directly assessed in the series with the low cytosol protein concentrations (0.2–0.5 mg protein/ml). ER concentrations for the 74 samples assayable at both cytosol protein concentrations were equivalent [linear regression equation: ER (dilute cytosol) = −2.3 + 1.00 ER (concentrated cytosol); $r = 0.99$]. Thus, for the remainder of the investigation, assays were performed using a 6-fold dilution of the cytosol preparation that is routinely used for the DCC assay.

There is a highly significant ($P < 0.0001$) linear correlation between assay results using the DCC and the EIA assay method to determine cytosolic ER in a total of 191 biopsies analyzed (Fig. 1A). The intercept of the line at the y-axis (15.5) is significantly different from 0 ($P < 0.0002$), and while greater values for ER are determined in the lower range of receptor concentrations (<86 fmol/mg) using the ER-EIA method, lower values are found for biopsies in the higher range of ER concentrations (>86 fmol/mg). Because tumors with the higher concentrations of ER tend to yield higher results using the DCC compared to the EIA method, two regression curves may more accurately describe the data than a single curve. To investigate this, linear regression analysis was performed on biopsies with cytosol values within the first three quartiles *versus* the last quartile of the distribution of ER concentrations in primary breast cancers from a large series of tumors ($n = 3728$; median value = 62; quartiles = 12 and 217 fmol ER/mg cytosol protein). Each of the resultant regression curves is highly significant (both $P < 0.0001$) (Fig. 1 B and C) while the slopes of the regression curves differ significantly ($P < 0.01$).

Using the commonly used cutoff limit of 10 fmol receptor/mg cytosol protein to distinguish between ER positive and ER negative biopsies, the distribution of 191 biopsies as ER positive or negative using the two assay methods is shown in Table 1. Only 12 of 191 discrepancies are found; complete data for these 12 biopsies are given in Table 2. While the 12 patients shown in Table 2 have relatively low concentrations of cytosol ER as measured by both the DCC and the EIA methods, all but one of the biopsies are receptor positive for either progesterone receptor and/or ERn.

In order to evaluate whether these discrepancies in classification of receptor positivity/negativity are due to "true" differ-

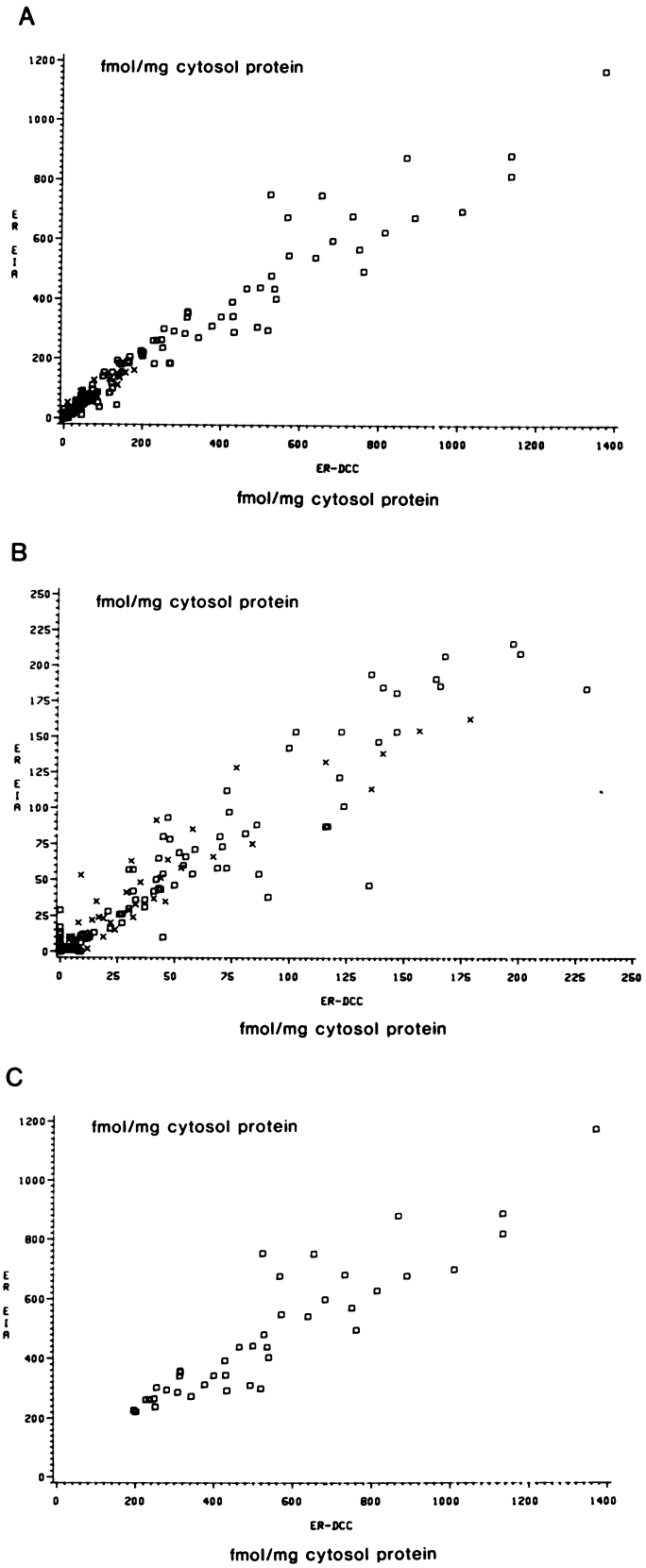

Fig. 1. Correlation between ER concentrations (fmol ER/mg cytosol protein) determined on the same day using the DCC (*ER-DCC*) and EIA (*ER-EIA*) assay methods on 191 freshly prepared cytosols from breast cancer biopsies. *A*, overall analysis; linear regression equation: ER-EIA = 15.5 + 0.82 ER-DCC; $r = 0.97$. *B*, quartiles 1–3 of the distribution of ER concentrations; $n = 150$, linear regression equation: ER-EIA = 7.2 + 0.92 ER-DCC; $r = 0.93$. *C*, quartile 4 of the distribution of ER concentration; $n = 41$, linear regression equation: ER-EIA = 84.7 + 0.72 ER-DCC; $r = 0.92$. Patients <50 yr (×); patients ≥50 yr (□).

Table 1 *Classification of ER status using the DCC and EIA methods*

Classification of ER status using both the ER-EIA and the ER-DCC assay methods is shown for the 191 freshly prepared cytosols. Samples with ≥10 fmol/mg cytosol protein are classified as being ER positive. A significant association was found ($X^2 = 136.87$, $P < 0.0001$) and the concordance was 94% (179 of 191).

	ER-EIA method		
ER-DCC method	*ER positive*	*ER negative*	*Total*
ER positive	133	2	135
ER negative	10	46	56
Total	143	48	191

Table 2 *Data for 12 patients with discordant classification of receptor positivity using the two assay methods*

The first 10 patients are classified as ER positive using the ER-EIA method but ER negative using the ER-DCC method, while the opposite is the case for the remaining 2 patients in the table.

Patient	ER-EIA fmol/mg cytosol protein	ER-DCC fmol/mg cytosol protein	ERn fmol/mg nuclear protein	PGR[a] fmol/mg cytosol protein	Age (yr)
ER-DCC < ER-EIA					
1	53[b]	9	129	586	48
2	29[b]	0	370	140	76
3	20[b]	8	54	[d]	46
4	17[b]	0	[d]	12	73
5	13[b]	0	298	3	69
6	13[b]	0	[d]	18	56
7	12[b]	0	126	304	50
8	11	9	19	0	77
9	10	5	47	29	37
10	10	4	28	0	42
ER-DCC > ER-EIA					
11	9[c]	12	[d]	16	53
12	2[b]	12	[d]	0	25

[a] PGR, progesterone receptor.
[b] Biopsies for which points on the logarithmically transformed plot of ER concentrations determined using each of the two methods exceed the 80% confidence limit. Only the 160 biopsies that had an ER concentration >5 fmol/mg cytosol protein using *at least one* of the assay methods were considered.
[c] Biopsies for which points on the logarithmically transformed plot of ER concentrations determined using each of the two methods exceed the 80% confidence limit. Only the 142 biopsies that had ER concentrations ≥5 fmol/mg cytosol protein using *both* of the assay methods were considered.
[d] Information is missing.

ences in assay results or to random events, all biopsies having a measurable ER concentration (≥5 fmol ER/mg cytosol protein) using *at least one* of the assay methods were included in a linear regression analysis of the logarithmically transformed values for ER concentrations. Of the 160 biopsies fulfilling this criterion, 20 points exceeded the 80% confidence interval of the line of regression. Seven of the 20 biopsies are among those shown in Table 2 that have a discordant classification of receptor status. The same type of analysis was consequently applied to the data for those biopsies with detectable ER values (*i.e.*, ≥5 fmol/mg cytosol protein) using *both* assay methods in order to evaluate whether there may be a specific trend in under- or overestimation of ER concentrations of ER positive biopsies using the two assay methods. Of the 191 biopsies, 142 fulfilled this criterion. Six of the biopsies identified in the first analysis were also identified here as having values exceeding the 80% confidence interval, and 14 additional biopsies were identified. Two of these 14 biopsies appear among those shown in Table 2. In both analyses, the outliers characteristically had lower ER concentrations than the corresponding group as a whole (ER concentration of outliers *versus* total group = 10 *versus* 69 fmol/mg for the 160 biopsies with measurable ER using at least one of the assay methods, and 46 *versus* 87 fmol/mg for the 142 biopsies with measurable ER using both assay methods).

Study of Frozen Cytosols. Of the 47 cytosols kept for 3–6 yr at −70°C before performing the ER-EIA assay, 23 cytosols had been prepared in assay buffer without and 24 cytosols with 10 mM sodium molybdate in the assay buffer. Since the linear regression equations for both sets of samples did not differ significantly (Table 3), they were pooled to evaluate the correlation between the originally obtained result using the DCC method on freshly prepared cytosols with the result determined years later using the EIA method on the frozen cytosols. The linear regression equation found for these 47 stored cytosols does not differ significantly from that found for the 191 fresh cytosols in the first part of this investigation.

DISCUSSION

Data from the Danish Breast Cancer Cooperative Group project indicates that there are two basically different criteria with regard to ER determinations that are important for the optimal clinical management of breast cancer patients. First, distinction between the presence and absence of receptors in the tissue appears to be the critical factor for the prognostic value of ER determinations in primary breast cancer patients not treated with systemic adjuvant therapy (16, 17). Studies from several centers have demonstrated that ER-positive patients have longer recurrence-free survivals (16–19), while other studies have not found such a correlation (9, 20–23). Notably, the frequency of ER positivity is high (70–76%) in the former but low (38–58%) in the latter group of studies.

Second, and in contrast to the above, quantitative estimations of ER appear to be most valuable in the prediction of the outcome of either adjuvant endocrine therapy in the primary disease and/or response to palliative endocrine therapy in the advanced disease. In both cases, the greatest benefit of endocrine therapy is observed among patients with high ER concentrations (17, 24).

Thus, for optimal management of the disease, it is critical to (*a*) accurately distinguish between ER-negative and ER-positive patients and (*b*) quantitate the amount of ER present in the tumor tissue.

The overall correlation between the results using the DCC and the EIA assay methods has already been demonstrated to be excellent, which is confirmed here. However, the data presented here reemphasize a fact already recognized for the ER-DCC assay: the concentrations of ER that are most difficult to reliably determine are those that are low (4). The notable features in the present investigation are that (*a*) the EIA assay is more sensitive than the DCC assay and (*b*) the few discrepancies found using the two assay methods appear to reflect true differences in the ability of the techniques to detect ER. Of the 12 biopsies with discrepant classifications of receptor status using the two methods, 9 exceeded the 80% confidence level of

Table 3 *Correlations between ER-EIA and ER-DCC assay results using frozen cytosols*

Correlations between results obtained on 47 frozen cytosols using the ER-EIA and the ER-DCC assay methods are shown. The correlations between assay results are evaluated for cytosols overall as well as separately stored in buffer either with or without 10 mM sodium molybdate.

Cytosol	*n*	ER-EIA (median)	ER-DCC (median)	Linear regression equation	*r*
Without molybdate	23	90	90	ER-EIA = 22.2 + 0.89 ER-DCC	0.78
With molybdate	24	272	271	ER-EIA = 82.7 + 0.70 ER-DCC	0.90
Overall	47	168	155	ER-EIA = 49.7 + 0.76 ER-DCC	0.89

the overall correlation of results using the two methods. Thus, it is unlikely that the observed discrepancies are due to random events for these 9 patients. Furthermore, in view of the remaining available data, it would appear that in 7 of the 9 cases, the EIA assay method yielded the most biologically reasonable result since ERn and/or progesterone receptor were detected in the biopsies.

It may be worthwhile to note that the results obtained using the EIA method are converted to fmol ER using a standard curve based upon ER from the MCF-7 cell line. Thus, any comparison between the DCC and the EIA method ultimately compares the results of the experimentor using his/her own DCC assay method to those of the reference laboratory(s) used by the manufacturers in establishing the concentration of ER in the MCF-7 standard provided with the kit.

The number of ER molecules determined using the ER-EIA and the ER-DCC methods is found to be approximately the same here for the majority of patients (ER <217 fmol/mg cytosol protein) but significantly lower values are found using the ER-EIA method for patients with high (ER ≥217 fmol/mg cytosol protein) ER concentrations. The reasons for this difference are subject to speculation. Perhaps they are based upon a difference in the ability of the two methods to determine very high concentrations of ER. Alternatively, tissue from patients with unusually high ER concentrations may also contain high(er) concentrations of proteins other than the specific ER that bind estradiol but are not recognized by the monoclonal technique. In this conjunction it might be worthwhile to note that all of the patients with ER concentrations exceeding 217 fmol/mg in the present investigation were postmenopausal.

Cytosols that have been kept at −70°C for a number of years can be reanalyzed using the ER-EIA method to obtain valid results. Because of the greater reproducibility of the ER-EIA assay method, perhaps such analyses could form a better basis for intercenter comparisons than results using the ER-DCC method.

The introduction of the monoclonal ER-EIA assay method appears to represent a major advance in the diagnostic tools available for treatment of breast cancer patients. Not only is the assay more easily reproducible than the DCC assay method (11, 12) but, and perhaps most importantly, it more accurately identifies those patients without ER in their tumor tissues.

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