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Relationship Between Radioligand Binding Assay, Immunoenzyme Assay and Immunohistochemical Assay for Estrogen Receptors in Human Breast Cancer and Association with Tumor Differentiation

JØRN ANDERSEN,* SØREN M. BENTZEN† and HANS S. POULSEN*

**Receptor Laboratory, The Danish Cancer Society, Department of Experimental Clinical Oncology, Radiumstationen, DK-8000 Aarhus C, Denmark and †Department of Radiophysics, Radiumstationen, DK-8000 Aarhus C, Denmark*

Abstract—*We have studied the merit of a new enzyme immunoassay (EIA) in relation to the results obtained with a conventional dextran-coated charcoal assay (DCC) of estrogen receptors (ER) in cytosols and nuclear extracts of human breast cancer tissue. The results of the two assays were related to cytosolic progesterone receptor content (PgR), semiquantified ER content in formalin-fixed paraffin embedded tissue specimens and tumor differentiation.*

The EIA was found stable at low cytosol protein concentrations (0.5 mg/ml). *The EIA and DCC assays were highly correlated both in cytosols* ($r = 0.92$, $n = 57$) *and nuclear extracts* ($r = 0.82$, $n = 25$), *but the EIA slightly overestimated the ER values in both ER fractions.*

A significant correlation between ER in nuclear (ER(N)) and cytosolic (ER(C)) fractions was established with both assays (DCC: $r = 0.90$, $n = 56$; *EIA*: $r = 0.83$, $n = 24$).

A qualitative relationship was established between PgR and ER fractions as determined with both assays, the best quantitative association was between PgR and ER(N(DCC)) ($r = 0.58$, $n = 34$, $P < 0.001$).

A significant qualitative and quantitative relationship was found between semiquantified ER content in formalin-fixed, paraffin-embedded tissue and ER(C(DCC)) ($r = 0.88$), *ER(N-(DCC))* ($r = 0.86$)), *ER(C(EIA))* ($r = 0.60$), *ER(N(CIA))* ($r = 0.64$) *and PgR* ($r = 0.65$).

Finally, we found tumor differentiation to be significantly associated with ER content as determined with all assays except for ER(N(EIA)).

We recommend the use of the DCC assay for routine analysis of ER until the clinical correlation of EIA results has been established.

INTRODUCTION

ER determination in human breast cancers is generally considered a valuable tool in the selection of patients for endocrine therapies. The response rate amounts to 50–60% in ER-positive tumors but only reaches 10% in ER-negative tumors [1, 2]. The predictive value is apparently raised by determining also the PgR and the ER levels in the nuclear pellets [3–5].

The DCC assay is based on 17-β-estradiol binding to cytosolic and/or nuclear tissue extracts. Though a routine application in ER determination, the DCC assay is beset with certain inherent limitations. First the assay usually does not measure ER already occupied by endogenous estrogen or ER in its early stages of synthesis not yet capable of binding hormone. Second, standardization is difficult and the charcoal has been shown to adsorb 10–20% of ER, making the assay less sensitive [6]. Third, avoidance of false negative results requires concentrated cytosols, which limits the use of the method [6, 7]. Thus if only small tumor samples are available, binding data cannot be obtained or must be less accurately determined [6]. Fourth, criticism has been raised against the estimation of ER estrogen binding in relation to non-specific estrogen binding and against the interpretation of binding data by the Schatchard transformation due to the limited experimental binding data available and the

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Correspondence and reprint requests to: Jørn Andersen, Receptor Laboratory, The Danish Cancer Society, Department of Experimental Clinical Oncology, Radiumstationen, DK-8000 Aarhus C, Denmark.

unknown concentration of free hormone in the equilibrium situation. Therefore, the estimation of the equilibrium constant, K_d, has been subject to profound criticism [8, 9].

Cytosolic ER was previously assumed to reside in the cytoplasm. Upon binding to estrogen it was thought to translocate to the nucleus and by binding to chromatin exerting the estrogenic effects. Recent studies with monoclonal antibodies have changed this, and all receptors are now assumed to be located in the nucleus. The fraction extractable under low salt conditions (cytosol) seems to be loosely bound and probably not filled with hormone, while the high-salt extractable ER (nuclear extract) seems to be bound to chromatin and its receptor site filled with endogenous estrogen [10–12].

The utilization of monoclonal antibodies has led to new methods of assaying ER in breast cancers by direct antigenic recognition of the receptor independent of the binding of labelled hormone to unoccupied receptors, thereby circumventing some of the difficulties mentioned above [13–16].

The aim of the present study was to compare data obtained with conventional DCC assays on low- and high-salt extracts with an immunoenzyme and an immunohistochemical ER assay using monoclonal ER antibodies.

MATERIALS AND METHODS

Tumor specimens

Routine steroid receptor analysis was performed on a total of 57 consecutive tumors excised by surgery. Tumor specimens were frozen immediately and stored at −80°C. Histopathological examination and histochemical ER analysis was performed on adjacent tumor specimens, fixed in 10% buffered formalin and paraffin embedded.

Preparation of cytosol and nuclear extracts

The tissue was minced with a pair of scissors, cooled in liquid nitrogen and homogenized using a Schwingmühle (Retch, F.R.G.). The tissue powder was suspended in TED buffer (0.01 M Tris, 1.0 mM EDTA, 1.0 mM dithiotreitol, pH 7.4) and centrifuged at 1000 ***g*** for 20 min. The pellet was resuspended twice in TED buffer and the supernatants pooled and centrifuged at 100,000 ***g*** for 1 h to obtain the low-salt extract (cytosol). The final pellet was further extracted by suspension in TED buffer containing 0.5 M NaSCN, pH 7.4. The suspension was shaken vigorously for 30 min and centrifuged at 150,000 ***g*** for 30 min to obtain the high-salt extract (nuclear extract). This technique exchanges approximately 90% of total cellular ER (Poulsen, unpublished results).

Dextran coated charcoal assay

The method has been described in detail elsewhere [6, 17]. Briefly, cytosolic ER analysis was performed by incubation with at least 10 different concentrations of 17-β-estradiol with or without a 100-fold excess of diethylstilbestrol. All incubations were performed in duplicate. Cytosolic PgR content was measured by inubation with at least 10 different concentrations of tritiated promegestone with or without a 100-fold excess of unlabeled hormone. Exchange DCC assay as described by Bresciani *et al.* [18] was used for nuclear ER measurements. Protein content was measured using Coomassie blue G250 dye (Biorad, F.R.G.), and the binding capacities are presented as fmol hormone bound per mg protein. ER and PgR positive is defined as $\geq$ 10 fmol ER/mg cytosol protein.

Estrogen receptor enzyme immunoassay (EIA)

The EIA is a solid phase enzyme immunoassay developed by Abbott Laboratories. Polystyrene beads covered by one monoclonal anti-ER antibody (D547) were incubated with the cytosols and appropriate controls. After washing the beads were incubated with a second monoclonal antibody (H222) conjugated with peroxidase enzyme to label the bound receptor. Finally incubation with hydrogen peroxide and *o*-phenylenediamide yielded a colored reaction product that could be measured in a spectrophotometer. With each run a standard curve was obtained by parallel incubation with dilutions of a known standard obtained from ER-positive MCF-7 cells. The standard range was 0–250 fmol/ml cytosol and unknown cytosols with a higher ER content were read as $\geq$ 250 fmol. To obtain a meaningful correlation between assays, data points with EIA results $\geq$ 250 fmol were excluded from regression analysis. ER positive was defined as $\geq$ 10 fmol ER/mg cytosol protein.

Immunohistochemical ER assay

The method has been described in detailed elsewhere and is using formalin-fixed paraffin-embedded tissue [14]. It has a slightly lower sensitivity than assays using fresh or frozen tissue, compared with which the frequency of ER positives is diminished by 10%. These false negatives all have an ER content < 100 fmol ER/mg protein. The lower sensitivity is caused by fixation and tissue processing [14, 15], the effects of which can be partly reversed by enzyme treatment of the specimen before immunostaining, probably by exposing antigenic sites disclosed by the fixation [15, 16]. Briefly, the formalin-fixed paraffin-embedded biopsies were cut in 6 μm sections, deparaffinized and rehydrated. After incubation with trypsin to digest excess aldehyde linkages and addition of 10% normal goat serum to block non-specific staining,

monoclonal ER antibody (H222, Abbott) was added for 16 h at 4°C. The ER–anti-ER binding is visualized with biotinylated goat anti-rat and avidin–biotin–peroxidase complex followed by 9-ethyl-aminocarbazole/hydrogen peroxide as chromogen. Positive staining was localized to the nucleus of epithelial cells, and the staining intensity was arbitrarily graded 0–100–200–300 with 300 representing the most intensive staining. The number of stained nuclei and the epithelial fraction was quantified using a microscopic grid. A total score was obtained by multiplying the three variables (score = staining intensity × % stained nuclei × % malignant epithelium). Controls were established by replacing the monoclonal antibody with the corresponding non-immune immunoglobulin to rule out non-specific staining. The antibody used has been shown to be specific for ER by immunoblotting, by its binding within normal tissues and by inhibition of staining by preadsorption of the antibody with excess ER.

Histology

Histopathologic examination was performed on hematoxylin–eosin stained sections adjacent to those used for histochemical and DCC/EIA assays. The tumors were histologically graded (I: highly differentiated, II: intermediately differentiated, III: poorly differentiated) based on the criteria of Bloom and Richardson [19] for classification of ductal carcinomas according to tendency to tubular formation, nuclear pleomorphism and number of mitoses/hyperchromasia.

Statistical analysis

Quantitative variables were correlated by linear regression, and slope, intercept, their standard error, and the correlation coefficients were estimated from a least squares fit. From these estimates a 95% confidence interval for the regression parameters was calculated. Correlation coefficients were compared as suggested by Hald [20]. Wilcoxon's rank sum test was applied to test for different centerings of underlying distributions in any two independent groups. Fisher's exact probability test was applied to test for variations in event frequencies among tumor groups.

RESULTS

Influence of cytosol protein content on EIA values

Figure 1 shows the effect of cytosol protein concentrations on ER determination with the EIA assay. Recordings at both high and low ER levels proved almost constant at protein concentrations ≥ 0.5 mg protein/ml cytosol.

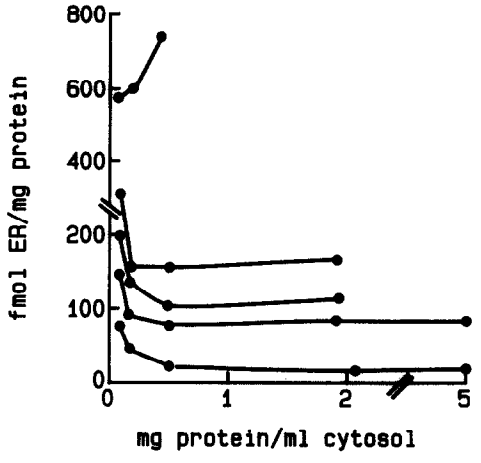

Fig. 1. Effect of cytosol protein concentration on ER determination with the EIA assay. Five different cytosols diluted with TED buffer.

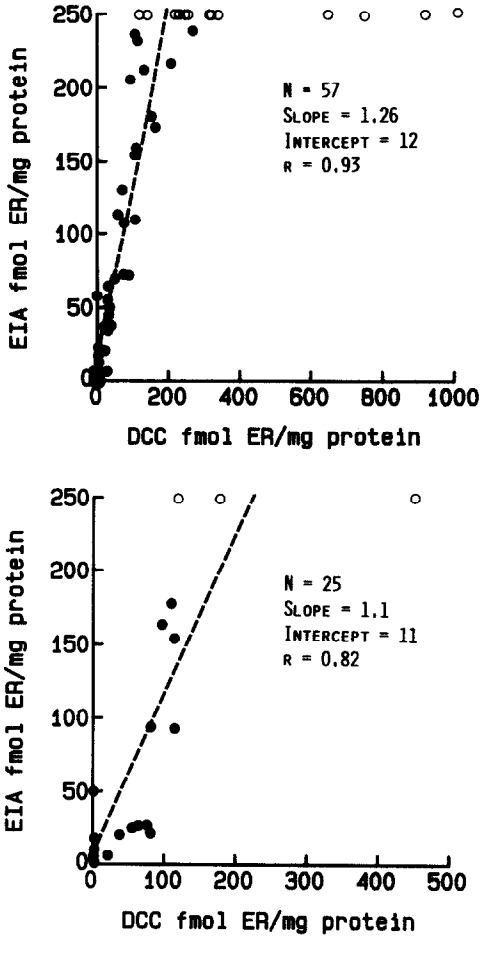

Fig. 2. Comparison of the number of binding sites determined by DCC and EIA assays in breast cancer cytosols (top) and nuclear extracts (bottom). ○ Points (top: 13, bottom: 3) not included in graph calculation (for details, see Materials and Methods).

DCC vs. EIA

Cytosolic ER was assayed with both techniques in all 57 tumors. The results are shown in Fig. 2. The correlation coefficient was 0.93 ($P < 0.001$), the slope of the regression line 1.26 ± 0.09 (one standard error of the estimate), and the y-intercept was found at 12 fmol. Four cytosols with an ER

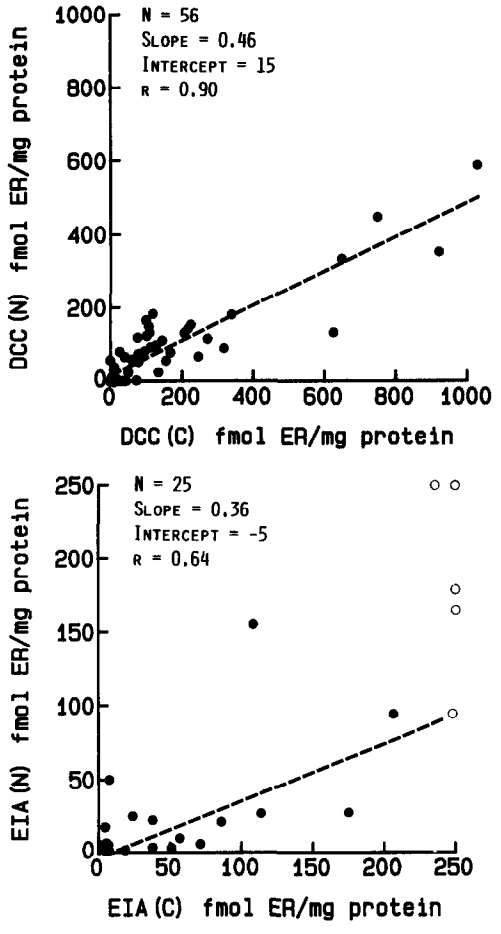

Fig. 3. Relationship between cytosolic (C) and nuclear (N) ER fractions as determined with the DCC assay (n = 56) *(top) and EIA assay* (n = 25) *(bottom).* ○ *Points* (n = 5) *not included in graph calculation (for details, see Material and Methods).*

content of 17, 20, 23 and 55 fmol, respectively, were found positive by EIA but negative by DCC. Thus, the EIA assay tends to overestimate the ER content relative to the DCC assay.

The DCC and EIA assay were compared in 25 nuclear extracts. The data are presented in Fig. 2. There was a close agreement between the two assays (correlation coefficient: 0.82 ($P < 0.001$), slope of regression line 1.1 ± 0.1). Three nuclear extracts with ER contents of 10, 18 and 50 fmol, respectively, were found positive by EIA but negative by DCC. The reverse was found in one extract with an ER content of 19 fmol.

Relationship between ER(C) and E(N) fractions

Nuclear receptors were studied as a function of cytosolic receptors with both assays. The relationship between nuclear and cytosolic ER fractions in the DCC assays is shown in Fig. 3. There was a highly significant correlation between the two fractions [correlation coefficient: 0.9 ($P < 0.001$), slope of the regression line 0.46 ± 0.03]. Nine tumors were ER(C) positive but ER(N) negative, two tumors were ER(C) negative but ER(N) positive.

Table 1. Relationship between cytosolic (C) and nuclear (N) estrogen receptor (ER) as determined with DCC and EIA assays and cytosolic progresterone receptor (PgR) assay

	DCC(C)		DCC(N)		EIA(C)		EIA(N)	
	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
PgR								
Pos.	16	1	18	0	18	0	7	0
Neg.	5	11	2	14	5	11	3	7
P-value*	< 0.001		< 0.001		< 0.001		0.01	

*Fisher's exact probability test for identical proportions of ER positive tumors in the groups of PGR positive and negative tumors.

Table 2. Correlation coefficients between staining features and cytosolic estrogen receptor (DCC assay)

	Immunohistochemical staining feature				
	*I**	*N*†	*C*‡	*I* × *N*	*I* × *N* × *C*
Correlation coefficient	0.70	0.68	0.19	0.81	0.88

**I* = Staining intensity.
†*N* = Number of stained cells.
‡*C* = Cellularity of specimen. 56 tumors.

The corresponding relationship between ER fractions in the EIA assay is shown in Fig. 3. A correlation coefficient of 0.64 ($P < 0.01$) was obtained, the slope of the regression line was 0.36 ± 0.15. The wide confidence limits were due to the small number of tumors ($n = 20$) left for analysis.

Relationship between PgR assay and ER assays

Table 1 states the association between ER and PgR. A qualitative (positive/negative) relationship to ER(C) and ER(N) was found in both assays. Significant linear correlations were demonstrated between PgR and ER(C(DCC)) ($r = 0.44$, $P = 0.01$), ER(N(DCC)) ($r = 0.58$, $P < 0.001$) and ER(C(EIA)) ($r = 0.48$, $P = 0.01$), but not to ER(N(EIA)) ($r = 0.20$, $P > 0.05$). There was no difference at the 5% significance level between the individual groups, probably due to the small number of tumors analyzed.

Relationship between ER(C) and ER(N) and immunohistochemical ER assay

The immunohistochemical staining was located to the nucleus in all cases. The score system was originally designed to optimize the semiquantitative ER content relative to ER(C(DCC)) results, as the combination of staining intensity, number of stained cells and fraction ER containing epithelium gives an average of the ER contant throughout the specimen. This is reflected in Table 2 where the correlation to

Table 3. Quantitative (A) and qualitative (B) relationship between immunohistochemical (histoch.) estrogen receptor (ER) content and cytosolic (C) and nuclear (N) ER content as determined with DCC and EIA assays

A	DCC(C)	DCC(N)	EIA(C)	EIA(N)	PgR
Correlation coefficient	0.88	0.86	0.60	0.64	0.65
Number	56	56	55	24	31
P-value*	<0.001	<0.001	<0.001	<0.001	<0.001

B		DCC(C)		DCC(N)		EIA(C)		EIA(N)		PgR	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
Histoch.	Pos.	38	0	31	7	37	7	12	3	16	4
	Neg.	2	16	3	15	3	15	3	6	1	10
P-value†		<0.001		<0.001		<0.001		<0.01		<0.001	

*Correlation coefficients 0.88 and 0.86 significantly different from 0.60, 0.64 and 0.65 at 95% level.

†Fisher's exact probability test for identical proportions of ER-positive tumors in the groups of histochemically ER-positive and negative tumors.

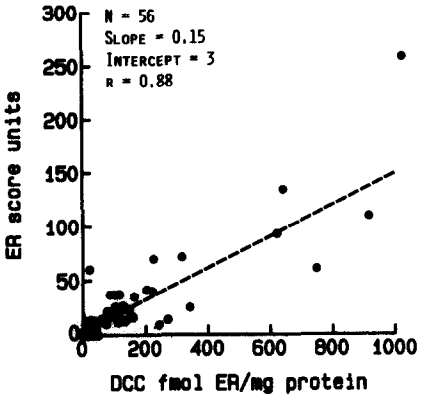

Fig. 4. Comparison between histochemical ER score and DCC results in cytosols (n = 56).

ER(C(DCC)) is shown to increase as the individual score factors are combined. The qualitative and quantitative relationships between immunohistochemical results and cytosolic and nuclear receptors are shown in Table 3 and the detailed relation between immunohistochemistry and ER(C(DCC)) in Fig. 4. The best correlations were found to cytosolic and nuclear ER as assayed with DCC and a lesser but still significant correlation to ER(C(EIA)), ER(N(EIA)) and PgR.

Receptor status and pathological parameters

Only infiltrating ductal carcinomas were graded ($n = 55$). Six were grade I, 38 grade II and 11 grade III. The malignancy grades were related to receptor content as determined by the different techniques. The results are shown in Table 4, where tumor receptor content is ranked within the histologic grades and compared by Wilcoxon's rank test. The histologic grade and receptor content were significantly correlated in ER(C(DCC)), ER(C(EIA)), ER(N(DCC), and ER(immunohistoch.), and the difference was mainly between grade I or II tumors and grade III tumors. The lack of correlation to ER(N(EIA)) and PgR might be explained by the relatively low number of tumors analyzed in these categories.

DISCUSSION

To avoid underestimation of estrogen receptors, a cytosol protein concentration > 1.5–2 mg/ml is recommended in the DCC assay [6, 7]. ER is readily recorded at protein concentrations as low as 0.25 mg/ml by the EIA assay (Fig. 1), allowing reliable ER determination in even small tumors [21]. The difference between the DCC and the EIA assays could be explained by a comparatively greater instability of the ligand binding site or the increased ER adsorption by the dextran-coated charcoal at low protein concentrations.

Comparison of DCC and EIA assays in cytosols demonstrated a linear relationship. ER data were slightly overestimated with the EIA assay as compared with the DCC assay [slope of regression line, β: 1.26 ($1.08 < \beta < 1.44$)] and four of 57 tumors with a relatively low ER content were classified ER-positive by EIA but negative by DCC. These findings have been reproduced in other studies comparing DCC and EIA assays [22, 23].

The reasons for the overestimation of ER with

Table 4. Relationship between histologic grade and tumor progesterone receptor content (PgR), histochemical ER content (histoch.), and cytosolic (C) and nuclear (N) ER content determined with DCC and EIA assays

		DCC(C)	DCC(N)	EIA(C)	EIA(N)	Histoch.	PgR
Grade	I vs.II	>0.1*	>0.1	>0.1	>0.1	>0.1	>0.1
	I vs. III	<0.01	>0.1	<0.01	>0.1	>0.05	>0.1
	II vs. III	<0.01	<0.05	<0.01	>0.1	<0.01	>0.1
Number		55	55	54	24	53	27

*P-values for the hypothesis of identically centered ER distribution in the various histologic grades was tested by Wilcoxon's rank-sum test.

the EIA assay are not entirely clear. The EIA standard curve is plotted using dilution of lyophilized MCF-7 breast cancer cell cytosols where the ER content was originally determined by a DCC assay. A slope > 1 indicates preservation of a larger number of antigenic than ligand binding sites in the unknown breast tumor cytosols, a difference that might be ascribed to the procedure adopted for extraction of the cytosols. Another possibility is that DCC underestimates ER because a part of the receptor sites are filled with endogenous estrogen, while EIA determines ER with both filled and unfilled sites. The latter assumption is supported by Thorsen, who found a significant a number of filled ER in cytosols [24].

Comparison of the two techniques in nuclear extracts showed the same relationship as in cytosols with a slope of the regression line of 1.1 ± 0.1. Two other studies comparing nuclear ER determined by EIA and hydroxyl apatite assays (HAP) showed a considerably steeper regression line with a slope of 1.7 [25] and close to 3 [26], respectively. Thus a lower exchange is obtained with HAP or a degradation of ER has taken place during the exchange due to the high incubation temperature (30°C), compared with the Bresciani method used in this study.

A good correlation between ER(C) and ER(N) by both methods was found, but the number (9/56) of ER(C) positive, ER(N) negative tumors was significant. The linear correlation suggests a state of equilibrium between ER(C) and ER(N), but the high frequency of ER(C) positive, ER(N) negative tumors suggests that ER(C) might not be the optimal indicator of hormonal sensitivity, because the discrepancies could reflect tumors containing only unfilled ER or tumors with a defect in the binding of filled ER to chromatin. A comparison of EIA and DCC in the above mentioned nine tumors gave a slope of 2.0 of the regression line compared with 1.26 for the whole material. As EIA measures both filled and unfilled ER and DCC only unfilled ER, the difference in slopes implies that these nine tumors contain filled but low salt extractable (unbound) receptors. This points to tumors with a defect translocation/nuclear acceptor mechanism, which are assumed not to be hormone responsive. This is further supported by the finding of a PgR negative status in all three of these tumors where PgR was measured. Low salt extractable, filled ER has been demonstrated by Thorsen performing an exchange assay on ER negative tumors [24] and by Nicholson *et al.* [27], who compared EIA and DCC in cytosols from 70 breast tumors finding nine DCC negative but EIA positive tumors. Seven of the nine cytosols turned out positive on application of an exchange assay.

In the present study 16% of the tumors were ER(C) positive, E(N) negative (95% confidence limits 7–27%), which is broadly in agreement with the values (2–38%) reported by others [3, 28–30]. The suggested clinical implication is that patients with ER in both fractions respond better to hormonal therapy than those with ER in only one fraction [3, 5].

PgR is assumed to be a protein synthesized due to estrogenic stimulation of the target tissue [31, 32], the presence of PgR thus being an indicator of an intact estrogen response pathway. The presence of ER(C) positive, PgR negative tumors has been assumed to be due either to a defect nuclear ER binding site or to the absence of endogenous estrogen [4]. The qualitative association between cytosolic ER and PgR in this study with 15% ER(C) positive but PgR negative tumors is in accordance with the findings reported in the literature [5, 33, 34]. There was no difference in this respect between DCC and EIA assays. The quantitative association between cytosolic ER and PgR was not outstanding with rather low correlation coefficients.

From a theoretical point of view, a closer relationship was to be expected between ER(N) and PgR as also confirmed in the present study.

The association between immunohistochemical ER staining *in situ* and the biochemical assays was of particular interest as we found the staining to be exclusively localized in the nuclei. Our results show the histochemical score to be equally well correlated

with cytosolic and nuclear ER, supporting the present picture of both low- and a high-salt extractable ER residing in the nucleus, and that cytosolic ER is a purely operational entity. A similar close relationship between immunohistochemical and binding assays has been found in all studies applying monoclonal antibodies to ER [35–37]. Surprisingly, immunohistochemical results were closer related to DCC than EIA results even when the latter were corrected for the upper limit of EIA of 250 fmol. To demonstrate ER, binding of the same monoclonal antibody (H222) is necessary in both assays. The most likely explanation is that tissue processing (fixation, embedding etc.) destroys antigenic sites and precludes immunohistochemical detection. This is supported by the rather high number of EIA positive, histochemically negative tumors (Table 3). Furthermore we have optimized the score system to ensure correlation with DCC results.

In this study we found all assays excluding ER(N(EIA)) to be significantly correlated with tumor differentiation. ER positive tumors are therefore more likely to express histological evidence of tumor differentiation than ER negative tumors. The prognostic value of ER might be related to this association. In the present study no clinical data were available to elucidate this point, and it was not possible to determine if a combination of both markers was better than each marker alone. A similar association between tumor differentiation and receptor status has been found by several investigators [38–40], and a single study suggests an independent prognostic value of ER, PgR and differentiation parameters [41].

The EIA assay and DCC assays apparently produce broadly comparable ER results in high- and low-salt tissue extracts, although the former assay tends to slightly overestimate the actual ER content. This may be explained by the EIA assay measuring ER that has preserved its antigenic site but lost the ability to bind hormone and thus has lost its physiological properties. Until sufficient clinical data has been procured on the prognostic and predictive value of EIA, we recommend ER analysis using the DCC assay.

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