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DISCREPANCIES BETWEEN ANTIBODY (EIA) AND SATURATION ANALYSIS OF OESTROGEN RECEPTOR CONTENT IN BREAST TUMOUR SAMPLES

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Summary—The use of different techniques for assay of oestrogen receptors (ER) in breast cancer raises the question of their relative effectiveness in measuring concentrations of functional receptors. Data were obtained on soluble receptors from supernatants from 58 primary breast tumour homogenates, using the ligand ([^{3}H]oestradiol) binding assay with dextran-coated charcoal (DCC) separation, either at a single saturating ligand dose, or by Scatchard analysis, and by using the Abbott enzyme immunoassay (EIA) kit. As previous reports have shown, the two methods gave reasonably good correlation ($r = 0.8$), but EIA values were systematically higher than DCC (slope = 3.0). Similar values were obtained when the ER + ve/progesterone receptor (PR) + ve subgroup were examined separately ($n = 34$, $r = 0.86$, slope = 3.0). However the two sets of data were in much better agreement in the ER + ve/PR − ve subgroup ($n = 10$, $r = 0.98$, slope = 1.24). When analysed by isoelectric focusing on polyacrylamide gels (IEF), two major specific binding components were identified, at pI 6.1 and at pI 6.6. Both isoforms were present in 50/66 ER + ve/PR + ve breast tumour samples, but only the pI 6.6 (4S) was present in most ER + ve/PR − ve samples (13/20).

It appears that, compared with DCC, the EIA method gives much higher values for the 8S isoform, whereas the two methods detect the 4S isoform with similar sensitivity.

In assays on the tumour cell lines, T47D and MCF-7, still greater discrepancies, at least 10-fold, were found between EIA and DCC data.

INTRODUCTION

The relationship between tumour hormone receptor content and survival is now well established [1, 2]. In particular, up to 60% of patients whose tumours contain oestrogen receptors (ER + ve) respond well to endocrine therapy [3–5], and this figure can be increased to 75% if progesterone receptors (PR), which are induced by oestrogen action, are also present [6–9]. In ER − ve/PR − ve cases, on the other hand, a positive response is seen in fewer than 10% of cases.

For these reasons, steroid hormone receptors in primary breast tumours are now routinely assayed in many centres. Various techniques are available for this purpose. In general they fall into two categories. Those in the first rely on the specific binding of a radioactively labelled steroid ligand to the receptor using nuclear or "cytosolic" (now taken to reflect receptors only weakly bound to the nucleus) fractions. Free steroid is frequently extracted with dextran-coated charcoal, hence the common designation DCC for these methods. The data presented can consist of the evaluation of receptor concentration alone, using a single saturating dose of ligand, alternatively several concentrations can be used, and analysis by Scatchard plot then provides information on binding affinity as well as receptor concentrations. Variants on this general methodology are also found.

More recently, a new category of receptor assays has been developed which relies on the recognition of specific epitopes on the receptor by monoclonal antibodies. A widely used form of this assay is based on the monoclonal antibody originally developed by Greene *et al.* [10, 11] and commercially available in the form of an enzyme-immunoassay (EIA) kit supplied by Abbott laboratories.

Several studies have validated the newer monoclonal antibody method by comparison of the data obtained with those generated by the DCC assay on the same samples [12–17].

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Some [13, 15], have reported good correlation between the two methods. Other authors have sounded various caveats, and in particular report higher values with the EIA method than with ligand binding assay [16, 17].

One problem may stem from the existence with tumour cytosols of several isoforms of the receptor. In particular, analysis by sucrose density fractionation [6] or by isoelectric focusing [17, 18] shows the presence of several forms. In the nucleus, the activated form of the receptor is thought to be dimeric (5S), while receptor isoforms in the soluble fraction appear as the 8S form on sucrose density gradient (equivalent to the pI 6.1 band in isoelectric focusing studies) and the 4S form (equivalent to the pI 6.5 band) [19]; other more minor forms also exist such as the 50 kDa receptor identified in human and animal uterine tissues, and a 30 kDa form of the receptor (mero-receptor) [20–25]. It is likely that the 8S (pI 6.1) form represents a multimeric assembly of at least two receptor molecules and other proteins, while the 4S form is monomeric [20, 23, 26]. Since the activated nuclear form is dimeric [27], it may be that the capacity to form other multimeric complexes, such as the 8S assembly, also reflects essential properties of a functional oestrogen receptor. In view of discrepancies between DCC and EIA methods the possibility exists that either method under- or overestimates one or more of these isoforms. In view of the different significance which each may hold in the interpretation of ER function, it seemed important to investigate this possibility.

This paper describes studies designed to investigate the ER isoform profile in PR + ve and PR − ve tumour cytosols, and the relationship of the DCC and EIA ER assay data in the same samples.

EXPERIMENTAL

Tissue handling

Human breast tumour tissue was obtained at operation. The tissue was placed on ice for periods of no more than 10–15 min, until tumour tissue could be identified, excised and snap frozen in liquid nitrogen. The sample was stored in liquid nitrogen until processed.

All tissue processing was performed at 4°C. The tissue was homogenized, using a polytron homogenizer, in glycerol phosphate buffer (10% glycerol, 50 mM phosphate, 1.5 mM EDTA, 5 mM monothioglycerol, pH 7.4) 1 : 10 w/v. The homogenate was centrifuged for 60 min at 100,000 *g*, and the supernatant was used for receptor analysis.

Cell culture

The cell lines MCF-7 (obtained from the Michigan Cancer Foundation, U.S.A.) and T-47D (from European Collection of Animal Cell Cultures, Porton Down, England) were grown to confluence in 150 cm^2 flasks in Eagle's (modified) minimum essential medium and Dulbecco's modification of Eagle's medium, respectively, both containing penicillin (100 IU/ml), streptomycin (100 μg/ml), L-glutamine (4 mM), sodium pyruvate (2 mM), 100× non-essential amino acids (10 ml/l) and 5% (v/v) foetal bovine serum. Cells were harvested using trypsin–EDTA solution (0.5% trypsin; 0.02% EDTA) and washed twice in serum-free medium. The cell pellet obtained after centrifugation was frozen under liquid nitrogen. Cytosol for receptor assay was obtained by resuspension of the pellet in glycerol phosphate buffer (1 ml/10^7 cells), sonicated on ice and centrifuged at 100,000 *g* for 1 h at 4°C. All cell culture materials were obtained from Flow Laboratories Ltd, Herts., England.

Scatchard analysis and single saturating dose (SSD) method

Scatchard analysis and SSD method were performed exactly as described previously [28]. For the Scatchard analysis, the data were plotted according to the method of Scatchard [29].

Determination of oestrogen receptor by enzyme immunoassay (EIA)

The EIA was performed according to the instructions provided with the Abbott kit.

Isoelectric focusing

The isoelectric focusing gels were cast in slabs of size 125 × 260 mm and separation was conducted along the short axis. Polyacrylamide gels, contained 12% glycerol, 2 mm thick, with high porosity (T = 5%, C = 3%) were used. A pH 3.5–10 gradient was achieved using 0.7% (w/v) LKB ampholine 3.5–10 and 0.3% (w/v) LKB ampholine 5–8.

Gels were photopolymerized, using riboflavin, at room temperature for at least 8 h.

Isoelectric focusing was performed with a LKB Multiphor II system in a cold room (4–8°C) and the temperature of the cooling

water was kept constant at 4°C. Electrode solutions of 1 M sodium hydroxide (cathode) and 1 M sulphuric acid (anode) were used. Gels were prefocused for 40 min at 20 mA/1200 V/20 W. After DCC extraction, aliquots (270 μl) of the radioactive supernatants (3 mg protein/ml) derived from SSD assays, using the method described above, were loaded in acrylic plastic frames placed on the surface of the gel near the cathode. A mixture of nine natural proteins (Bio-Rad) were used for pH calibration. The runs were carried out for 2 h at 1200 V/20 mA/20 W. After the run, the gels were cut into 2.5 mm slices and each slice was incubated with 5 ml of scintillation cocktail (Packard) for 24 h at room temperature and radioactivity assayed in a Beckman LS7500 counter.

Protein estimation

These were carried out using the method of Lowry *et al.* [30] using bovine serum albumin as the standard. Receptor concentrations calculated as femtomole ligand bound per milligram of protein.

RESULTS

Comparison of receptor concentration values obtained with the DCC assay (SSD and Scatchard assays were used interchangeably) and with the EIA assay on cytosols from 58 primary tumours gave an overall correlation coefficient $r = 0.8$ but with a slope of 3.0 (Fig. 1). Seven of these tumour cytosols were

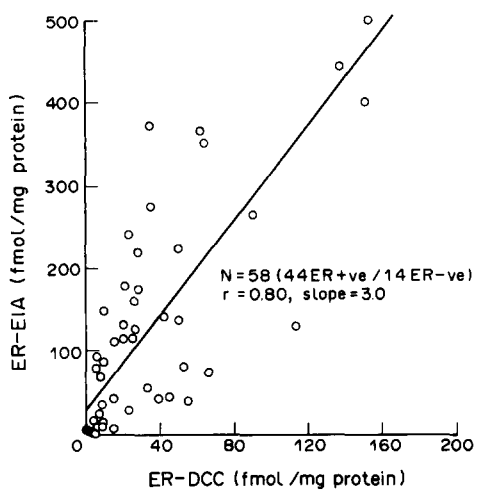

Fig. 1. Correlation between ER concentration data obtained in supernatants from 58 primary breast tumour homogenates by the EIA and DCC methods. Although the correlation is reasonably good ($r = 0.8$), EIA values were in general higher, and the slope of the curve is 3.0.

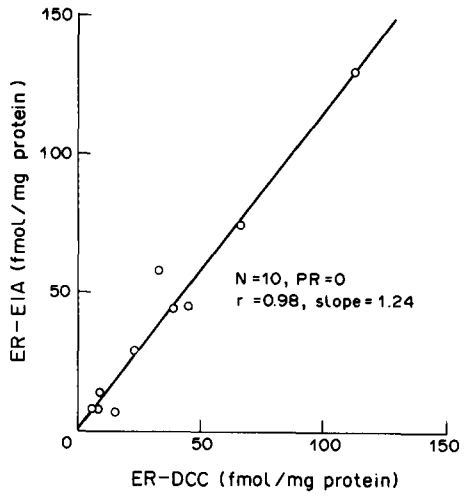

Fig. 2. Correlation between ER concentration data from the ER + ve/PR − ve subgroup included in Fig. 1. Here the values obtained by the two methods are in good agreement, $r = 0.98$, slope = 1.25.

negative by both DCC and EIA, 4 were negative by DCC but positive by EIA and 3 were negative by EIA but positive by DCC. The possibility that the differences observed were attributable to the different buffers being used in each assay was investigated. However, performing EIA with DCC buffer in place of the supplied diluent did not significantly alter the concentration of receptor measured by EIA ($r = 0.96$, slope = 1.22, $N = 14$).

Samples which were ER positive by DCC were divided into PR + ve and PR − ve subgroups. For the PR − ve group, correlation between DCC and EIA values was much clearer; with $r = 0.98$, and the slope was 1.24, ($n = 10$; Fig. 2), whereas the data for the PR + ve group showed good correlation, $r = 0.86$ ($n = 34$) but, as for Fig. 1, gave considerably higher values by EIA than by DCC, and the slope was 3.0. (Fig. 3).

When ER data obtained by the two methods from cell lines was compared, even greater discrepancies were observed. In MCF-7 cells, typical values for ER concentrations were 22 ± 6 fmols/mg protein ($n = 6$) by DCC assay and 192 ± 40 fmols/mg protein by EIA ($n = 4$). For T47D cells typical values were 11 ± 5 fmols/mg protein ($n = 5$) and 139 ± 10 fmols mg/protein ($n = 3$) for DCC and EIA, respectively.

Isoelectric focusing

Analysis of 86 ER positive primary tumour cytosols was carried out using isoelectric focusing electrophoresis in polyacrylamide gel. The specific binding components were present as

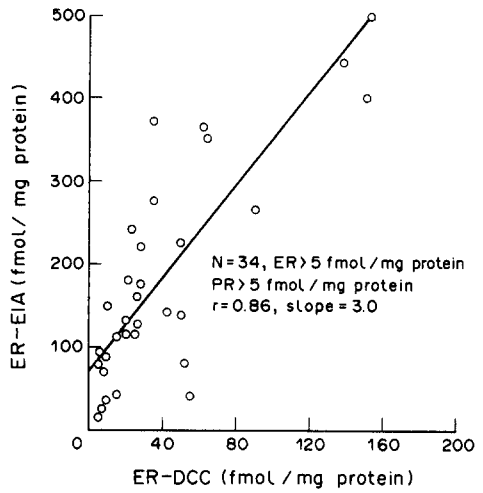

Fig. 3. Correlation between ER concentration data from the ER + ve/PR + ve subgroup included in Fig. 1. The values for correlation between the two sets of data are similar to those for the whole groups (cf. Fig. 1), $r = 0.86$, slope = 3.0.

two isoforms with pI 6.1 and 6.6, respectively (Fig. 4). The pI 6.1 isoform was present in PR + ve tumours but more rarely found in PR − ve tumours (Table 1) thus the presence of PR was significantly correlated with the presence of both isoforms together ($P < 0.001$).

DISCUSSION

In the management of breast cancer, the prognostic outcome of the disease and its likely response to endocrine therapy are taken to be

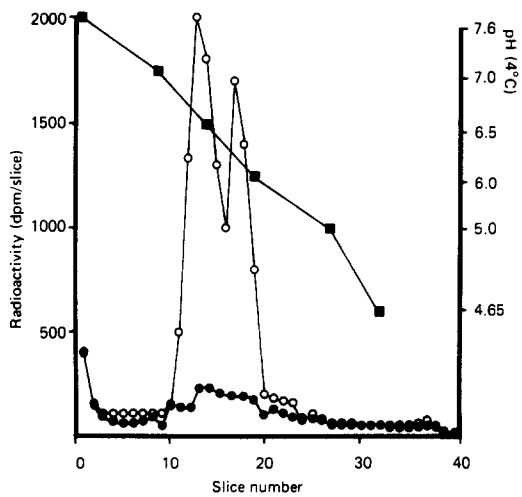

Fig. 4. Isoelectric focusing analysis of ER in a supernatant from a primary breast tumour [open circles represent the radioactive profile in the absence of the specific competitor diethylstilboestrol(DES), and solid circles in the presence of DES]. The ER present as two isoforms, with pI of 6.1 and 6.6. The pI 6.6 isoform is almost invariably the greater in abundance.

Table 1. Distribution of the two major isoforms in 86 ER + ve human breast cancer

	pI = 6.6 + pI = 6.1	pI = 6.6 only
PR + ve tumours	50/66 (75.7%)	16/66
PR − ve tumours	7/20 (35%)	13/20

reflected, at least partly, in steroid hormone receptor status [31]. It is important to be assured that the chosen method for steroid receptor assay produces reliable data which can be interpreted in functional terms. A functional receptor is one which binds steroid, then becomes tightly bound in the nucleus, and elicits activation of specific genes. In the past, the importance of these triple events has been acknowledged by the adoption of methods which seek, with greater or lesser success, to examine them precisely. Thus labelled steroid hormone has routinely been used to assess specific, high affinity, steroid binding components, using either SSD or Scatchard methods. In some laboratories, free soluble ("cytosolic") receptors have been assayed separately from receptors bound to the nucleus [32, 33]. As an index of functional ER, the presence of PR has been taken to reflect the activation of the PR gene by oestrogen bound to its receptor [34].

The use of monoclonal antibodies to ER therefore poses special problems. There is no *a priori* reason to suppose that all, or any, of the criteria for the identification of *functional* ER are necessarily met in a monoclonal antibody assay—it may bind to ER fragments or to otherwise inactivated receptor species. Accordingly, the validation of antibody methods, such as the EIA used here, by comparing its data with that obtained by proven methodology, is essential, not only at its introduction into a laboratory, but continuously throughout the period of its use.

Since these antibodies to ER have been available, there have been differing reports on this relationship [12–17]. Certainly all authors are agreed that the *correlation* between DCC and EIA is reasonably good, and this conclusion is born out by the present data. It is clearly arguable that this is the only critical point of validation, since it is the *presence* of ER which has diagnostic relevance, irrespective of concentration [1, 2]. However, the possibility that non-steroid binding ER fragments can be detected indiscriminately by EIA cannot be overlooked, and a number of authors have reported higher values with EIA than with DCC. Our data

support the view that this does occur, and it is conceivable that under some conditions these may exceed the concentrations of functional, steroid binding, ER.

It is also inescapable that the two methods may detect the various isoforms with different sensitivity. Overall, a much higher value is obtained with EIA than with DCC (Fig. 1), but critically, this does not apply when PR are absent (Fig. 2). In other words, the data in Fig. 2 suggest that when a *non-functional* ER, which cannot induce PR, is present, there is a 1:1 stoichiometry between steroid and Ab binding. Conversely, when a functional ER is present, which can induce PR, the stoichiometry suggests 1:3 steroid:Ab binding.

The activation of ER is thought to involve the formation of the nuclear dimeric form [35–37], and the property to do this may be reflected in the oligomeric forms, such as the 8S component, found in supernatant fractions. This is borne out by the present studies, which, following earlier work [38], strongly suggest that the presence of PR is correlated with the presence in the soluble supernatant fraction of both the 4S (monomeric) and 8S (oligomeric) isoforms while the 8S form is absent in PR − ve tumours. Accordingly it is tempting to deduce that steroid and Ab bind with 1:1 stoichiometry to the monomeric form, but with 1:3 stoichiometry to the oligomeric form.

This finding has profound importance for our interpretation of steroid receptor assay data. It has always been an essential assumption in steroid binding assays that the specific binding sites are freely available to added labelled steroid, given appropriate conditions for establishing equilibrium. It now appears that this may not be the case. If the oligomeric 8S form is an assembly containing two or more complete ER molecules (with other components), the best explanation of the present data is that while all of the appropriate binding sites may be available to the antibody, fewer are available to the steroid. This is an unexpected finding since the antibody is supposed to recognize the steroid binding domain. Indeed the stoichiometry of the relationship suggests one steroid binding site per oligomer to 3 or more antibody binding sites: suggesting in turn more than the usually accepted ER dimer (plus other protein) assembly.

There are several possible explanations for this finding. One is that the 8S oligomeric structure sterically hinders access of free steroid to the binding sites. A variant on this possibility would be that, in contrast to the monomeric form, steroid saturating the binding sites is contained within the 8S structure with sufficiently high affinity to preclude exchange with free steroid. It should be noted that, while the correct explanation is not readily apparent, these results are consistent with the observations of others, who have previously shown that an assay based on antibodies to bound steroid on the receptor fails to react with the 8S form as readily as the 4S [39].

These phenomena underlying these observations require further elucidation. Meanwhile it is not presently clear which of the two methodological procedures, EIA or DCC gives values which most accurately reflect functional receptor content of tissues.

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