The Lability of Estrogen Receptor: Correlation of Estrogen Binding and Immunoreactivity

Frederic Clayton and James Wu

We compared the Abbott enzyme immunoassay method for estrogen receptor with a standard dextran-coated charcoal method, and studied the effects of treatments causing denaturation. Estrogen receptor values were slightly higher by the Abbott method, but the methods agreed with regard to receptors being present or absent in 94–98% of 50 cases. Heat lability of immunoreactivity by the Abbott enzyme immunoassay is comparable to the lability of estrogen binding by the estrogen receptor. Estrogen and molybdate substantially stabilize estrogen receptor during the assay, but improper handling of tissue before the assay may cause similar, substantial decreases in estrogen receptor by either method. The Abbott method is easier to use than the dextran-coated charcoal method, requires less tissue, and measures receptor with or without endogenously bound estrogen, but reagent cost is high.

Additional Keyphrases: enzyme immunoassay • dextran-coated charcoal procedures compared • variation, sources of • sample handling • cancer

The marked lability of estrogen receptor is a serious problem, which undoubtedly is responsible for falsely negative results for estrogen receptor. Substantial decreases in estrogen receptor activity have been documented after a delay in cooling the tissue (1), after mastectomy as opposed to biopsy (1, 2), after electrocautery (3), and after prolonged storage except at −70 °C (4). Furthermore, a falsely negative result is clearly the most clinically deleterious error that could occur. One may hope that this problem could be averted by studying immunoreactivity, which in other proteins may be preserved even when biological activity is lost. We have studied the heat inactivation of estrogen receptor by the Abbott enzyme immunoassay (ER-EIA) method in parallel with a conventional dextran-coated charcoal (DCC) (5) technique.

Materials and Methods

Breast carcinoma tissue, promptly frozen and stored at −70 °C until analysis, was homogenized and diluted to approximately 2 mg of protein per milliliter, by use of the method and reagents of the NEN estrogen receptor (125I) RIANEN assay system (New England Nuclear, North Billerica, MA 01821; cat. no. NEA-089). This six-point dextran-coated charcoal procedure measures estrogen receptor binding capacity in tissue cytosol (5). The homogenized and diluted breast tissue extract was then assayed by that method or by the Abbott monoclonal enzyme immunoassay (ER-EIA) for the quantitative measurement of human estrogen receptor in tissue cytosol (Abbott Laboratories, Diagnostics Division, North Chicago, IL 60064) (6) after treatment as described below.

Results

Comparison with the dextran-coated charcoal (DCC) method. Estrogen receptor analysis was performed simulta-
neously for 50 cases by both the DCC and ER-EIA methods, with good correlation (Figure 1). The correlation coefficient was 0.86 and the linear regression by the method of Deming (7) was described by the equation:

\[
estrogen\text{receptor(DCC test)} = 0.86 \pm (0.04) \times estrogen\text{receptor(ER-EIA test)} + 2 (\pm 5)
\]

For cases with estrogen receptor values <70 fmol/mg of protein, the linear regression was described by the equation:

\[
estrogen\text{receptor(DCC test)} = 0.93 \pm (0.06) \times estrogen\text{receptor(ER-EIA test)} - 0.1 (\pm 2)
\]

At two of four concentrations chosen to determine a positive result (5 and 10 fmol/mg of cytosol protein), one case was considered positive by one method and negative by the other. At 3 fmol/mg, three cases were positive by one method and not the other; at 20 fmol/mg, two cases were positive by one method and not the other. In five of seven disagreements, the ER-EIA result was positive while the DCC result was negative. As one may gather from the linear regression analysis, the ER-EIA results were slightly higher than the DCC results, particularly at high receptor concentrations.

**Inactivation in tissue.** To evaluate the heat inactivation of estrogen receptor immunoreactivity and simulate poorly handled tissue, we examined three breast carcinoma cases in the following manner. Tissue for examination was bisected. One portion of the tissue was allowed to thaw to room temperature for 1 h, then refrozen. The other portion was kept frozen. Each was then thawed, homogenized, and assayed by both the ER-EIA and DCC methods in the usual manner. As shown in Table 1, a substantial and similar drop in estrogen receptor values was seen by both methods for the refrozen tissue.

**Inactivation in cytosol extracts.** We also measured heat inactivation in the homogenized tissue extracts by placing them at room temperature or 37°C for various intervals before performing the ER-EIA assay (Figure 2). These results showed a moderate but definite decrease in estrogen receptor immunoreactivity at room temperature and a marked, apparently biphasic loss in immunoreactivity at 37°C. The heat inactivation at 37°C was substantially slowed by the prior addition of 50 nmol of diethylstilbesterol per liter, and paralleled the slow phase of heat inactivation without added diethylstilbesterol, with a half-life of 45 to 51 min. When both 50 nmol of diethylstilbesterol and 10 nmol of sodium molybdate were added per liter, no detectable degradation was seen at 1 h at 37°C.

The amount of estradiol necessary for the estrogen receptor protective effect was also studied (Figure 3). A tumor extract, prepared as described above, was incubated at 37°C for 20 min after the addition of various amounts of betaestradiol. A control contained the same amount of betaestradiol, but was kept at 0–4°C. These samples were then assayed by the ER-EIA method. The results indicated that half-maximal protective effect is achieved at a free estradiol concentration of approximately 120 pmol/L, which suggests a \(K_d\) of 1.2 × 10⁻¹⁰ mol/L.

Table 1. Heat Inactivation of Estrogen Binding and Estrophilln Immunoreactivity in Tissue

<table>
<thead>
<tr>
<th>Case no.</th>
<th>Fresh* fmol/mg</th>
<th>1h refrozen* fmol/mg</th>
<th>% acty retained</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>64/46</td>
<td>20/15</td>
<td>31/33</td>
</tr>
<tr>
<td>2</td>
<td>150/113</td>
<td>21/17</td>
<td>14/15</td>
</tr>
<tr>
<td>3</td>
<td>60/68</td>
<td>24/18</td>
<td>40/26</td>
</tr>
</tbody>
</table>

In each case the numerator is the estrogen receptor result (fmol/mg cytosol protein) by ER-EIA and the denominator that by the DCC method. With both processed immediately upon thawing. Tissue adjacent to the tissue analyzed properly was thawed, left for 1 h at room temperature, and refrozen to simulate poorly handled tissue.

Fig. 1. Estrogen receptor concentrations by ER-EIA and DCC

Comparison of estrogen receptor assays run concurrently by the Abbott ER-EIA method and DCC method. The results correlated well (\(r = 0.95\)). Using criteria of 3, 5, 10, or 20 fmol/mg of protein, only one to three cases in 50 were positive by one method and negative by the other. Five cases with receptor levels greater than the range of the ER-EIA method (250 fmol/L with 2.0 g/L cytosol protein) are not shown.

Fig. 2. Heat inactivation of estrogen receptor immunoreactivity

The tumor cytosol extracts (2.1 mg/mL, 211 fmol/mg protein when run normally) were incubated for various intervals at room temperature (8h), at 37°C (C), at 37°C in the presence of diethylstilbesterol, 50 nmol/L (Δ), or at 37°C in the presence of 10 nmol of sodium molybdate and 50 nmol of diethylstilbesterol per liter (Δ). Each was then assayed by the Abbott ER-EIA method. Heat inactivation at 37°C was biphasic, with substantial inactivation at 5 min, followed by a slow phase (\(h_2\) 45 min) paralleling the slow degradation seen in the presence of diethylstilbesterol (\(h_2\) 51 min). No significant inactivation was seen when both diethylstilbesterol and molybdate were added.

Fig. 3. Half-maximal protective effect

37°C. The heat inactivation at 37°C was substantially slowed by the prior addition of 50 nmol of diethylstilbesterol per liter, and paralleled the slow phase of heat inactivation without added diethylstilbesterol, with a half-life of 45 to 51 min. When both 50 nmol of diethylstilbesterol and 10 nmol of sodium molybdate were added per liter, no detectable degradation was seen at 1 h at 37°C.

The amount of estradiol necessary for the estrogen receptor protective effect was also studied (Figure 3). A tumor extract, prepared as described above, was incubated at 37°C for 20 min after the addition of various amounts of betaestradiol. A control contained the same amount of betaestradiol, but was kept at 0–4°C. These samples were then assayed by the ER-EIA method. The results indicated that half-maximal protective effect is achieved at a free estradiol concentration of approximately 120 pmol/L, which suggests a \(K_d\) of 1.2 × 10⁻¹⁰ mol/L.
Inactivation by excessive vortex-mixing was also studied. Aliquots of a tumor cytosol extract were vortex mixed for 10 or 60 s or for 60 s after the addition of 50 nmol of diethylstilbestrol per liter. This resulted in the loss of 13%, 79%, and 56%, respectively, of the estrogen receptor ER-EIA immunoreactivity, relative to a control that was not vortex mixed. Thus, estrogen receptor immunoreactivity was also labile to excessive shearing, although steroid binding again demonstrated some protective effect.

Discussion

These results confirm that estrogen receptor immunoreactivity is labile to inactivation by heat and excessive mixing, and that thermal inactivation of immunoreactivity is comparable to thermal inactivation of estrogen binding. Use of the ER-EIA method therefore has little or no advantage over conventional receptor binding assays for specimens that have been improperly handled. These results are compatible with the known lability of immunohistochemically reactive estrogen receptor when the tissue is not fixed promptly after thawing (8, 9).

Our results also indicate that thermal and shear-induced inactivation of immunoreactivity are significantly decreased by the addition of estrogens, in much the same way that estrogen binding activity is preserved (10). The concentration of estradiol needed for the protective effect is essentially identical to that needed for receptor binding, which suggests that estrogen binding to the receptor site causes the protective effect. This protective effect is enhanced by the presence of molybdate (11), to the extent that receptor degradation is negligible. This observation is of practical value, because estrogen and molybdate are present in the buffers provided by Abbott (Karen Fitzgerald, personal communication). Therefore, during the assay, the ER-EIA method may be less sensitive to inadequate technique than is the DCC method or other receptor binding assays that require the preservation of unbound estrogen receptor for a portion of the procedure.

The lability of estrogen receptor not bound to estrogen suggests that recent results (8, 9, 12, 13) demonstrating the principal or sole location of estrogen receptor to be in the nucleus should be interpreted cautiously. It is plausible that the more-labile estrogen-free receptor, traditionally considered to be present in the cytoplasm, may preferentially lose antigenicity during fixation, processing, and immunohistochemical staining.

Our results indicate that estrogen receptor values by the ER-EIA method correspond to those of the DCC method, in agreement with the literature (14). Depending on whether 3, 5, 10, or 20 fmol/mg of cytosol protein is chosen as the cutoff value for a positive result, only one to three cases in this series of 50 were positive by one technique and not the other. When estrogen receptor values were high, the ER-EIA results were somewhat higher than those obtained by the DCC method. In addition, at high receptor concentrations, the results did not correlate as well as at low. Because both the higher values by ER-EIA and the scatter are least apparent at low estrogen receptor concentrations, the number of cases positive by one method and negative by the other, which is obviously the distinction of clinical importance, should thus be minimized. Although precision was not measured, our results are generally compatible with those claimed by Abbott (6), demonstrating a precision of 2.4 fmol/mg of cytosol protein at low estrogen receptor concentrations.

The inability to detect estrogen receptor already bound to endogenous estrogen is a clear disadvantage of conventional estrogen receptor tests. Receptor bound to endogenous estrogen, which often decreases conventional estrogen receptor measurements by 10 to 35% (15, 16), is accurately reflected in the ER-EIA test. We suspect that this may account for the slightly higher values demonstrated by this method. It may also account for the biphasic decay of estrogen receptor immunoreactivity at 37 °C without the addition of estrogen (Figure 2). Unbound estrogen receptor may account for the fast phase of degradation, which was substantially completed in 5 min at 37 °C (Figure 2), and estrogen receptor–endogenous steroid complex may account for the slow phase of receptor degradation, with a decay rate similar to that of estrogen receptor–diethylstilbestrol complex (κt 45 and 51 min, respectively).

Additional advantages of the estrogen receptor ER-EIA method include the fact that less cytosol is required (approximately 0.2 to 0.4 mg of cytosol protein as compared with 3.6 mg by a six-point DCC method) and that substantially less work is involved, an important factor when many samples are run concurrently. Twenty or more ER-EIA tests can easily be done in one run by one technician—a very difficult task with the DCC method. Lower-affinity estrogen binding to other proteins, which may obscure the presence of receptor in the DCC method, is irrelevant to the ER-EIA technique because estrogen binding is not measured.

However, the Abbott ER-EIA test has two important disadvantages. There are, as yet, no commercially available progesterone receptor immunoaasays. Thus, if one does both estrogen and progesterone assays, one may save even more labor and tissue by using a combined iodinated estrogen and tritiated progesterone DCC method. Furthermore, the ER-EIA is very expensive. A 100-bead kit with standards and controls for four runs, sufficient in our laboratory for about 25 assays (in duplicate), costs $700.00. This results in a current reagent cost of $22.00 per case studied, as compared with $10.00 for the DCC method used in our laboratory.

In summary, results obtained with the Abbott assay correlate well with those by conventional DCC assays, and the former has several advantages: ease of use, stability of the receptor during the assay as a result of added estrogen and molybdate, accurate representation of estrogen receptor bound to endogenous steroid, and less tissue needed for an assay. However, the lability of estrogen receptor immunore-
Urine Discoloration after Acetaminophen Overdose

P. M. S. Clark,¹ J. D. A. Clark,² and T. Wheatley²

Three patients with acetaminophen overdose were observed to have dark-brown urine at an early stage of their illness. Subsequently, acute anuric renal failure and hepatic dysfunction developed in all three. p-Aminophenol was identified by chromatographic and colorimetric methods in the urine of each case and is thought to be responsible for the discoloration.

Additional Keyphrases: p-aminophenol • enzymatic analysis • chromatography

Case Histories

Case 1: A 51-year-old woman was admitted 20 h after she had ingested 75 g of acetaminophen, 320 mg of codeine phosphate, and an unknown quantity of nitrazepam. Dark-brown urine was observed 16 h later, when she was already oliguric. She rapidly developed acute anuric renal failure, (plasma creatinine 337 µmol/L) and a moderately increased (776 U/L) serum alanine aminotransferase (EC 2.6.1.2, ALT, normal range <40 U/L) measured at 37°C. Despite supportive measures she died the following day.

Case 2: A 54-year-old man, admitted 15 h after ingesting 50 g of acetaminophen and 100 mg of diazepam, developed urinary discoloration 48 h after the overdose. At this stage he had both renal and hepatic dysfunction, which continued to deteriorate (plasma creatinine 466 µmol/L, normal range 35–125 µmol/L; serum ALT 7570 U/L), and he died four days after the overdose.

Case 3: A 26-year-old woman presented 36 h after ingesting 40 g of acetaminophen. Initial investigations confirmed hepatic damage and impaired renal function (plasma creatinine 224 µmol/L, serum ALT >6000 U/L) and she was observed to have brown urine. Despite full supportive management she developed anuric renal failure (plasma creatinine 908 µmol/L) and hepatic encephalopathy, but after ventilation and hemodialysis she recovered and was discharged three weeks later. Subsequent follow-up revealed normal renal and hepatic function.

Owing to the delay in presentation, none of these patients was treated with N-acetylcysteine. Their serum acetaminophen concentrations on admission were 4.8, 2.0, and 0.3