40 to 80 μmol per mole of creatinine, which is 100–200 times greater than the values we observed. Alternatively, if the concentration of chromium in total extracellular water, whose volume is approximately 20 L (23), reflects that in plasma, then we would expect the urinary excretion of chromium to be at least 1000 times greater than the values we observed. We conclude that increased urinary loss is not responsible for the observed decrease in the concentration of plasma chromium.

Further studies will be needed to identify the principal mechanism responsible for the decrease in chromium concentration in plasma and to identify any abnormalities in dynamic responses in diabetic subjects, in whom the basal concentration of chromium in plasma is known to be significantly decreased (15).

References
24. Baron DN (ibid.): 100.

An Enzyme Immunoassay Compared with a Ligand-Binding Assay for Measuring Progesterone Receptors in Cytosols from Breast Cancers

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To assay progesterone receptor (PR), we compared Abbott's enzyme immunoassay (PR-EIA) with a ligand-binding assay involving dextran-coated charcoal (PR-DCC), using cytosols prepared from 109 breast-cancer biopsies. Results by the two PR methods agreed well. Least-squares analysis produced a line of best fit having a slope of 0.88, an intercept on the PR-EIA axis of 16 fmol per milligram of protein, and a correlation coefficient ($r^2$) of 0.87. To evaluate whether accurate PR-EIA measurements could be obtained on stored cytosols, we compared PR-EIA values for fresh cytosols with values for cytosols stored for various lengths of time up to 13 weeks. Agreement was excellent, especially when the samples showing very high binding (>600 fmol per milligram of protein) were excluded. The lines of best fit after least-squares analyses of the remaining values had slopes between 1.0 and 1.1, intercepts <$3$ fmol/mg, and $r^2$ all >$0.91$.

Additional Keyphrases: sample handling monitoring therapy

Steroid receptors have become important as diagnostic indices. Progesterone receptor (PR) status combined with estrogen receptor (ER) status defines at least three biological subgroups (ER+/PR+, ER+/PR−, ER−/PR−), which display different responses to endocrine therapy. In patients with recurrent breast cancer who were treated with endocrine therapy, PR presence improved the predictive accuracy of ER+ by a further 15–20%, to approximately 70% (69). Receptor-negative tumors rarely respond to hormone therapy.

In adjuvant chemotherapy, determination of PR added significantly to the predictive value of ER (2). Others have
found PR to be a more effective guide than ER for prognosis in early breast cancer (stages I and II) (3), and PR is an independent prognostic factor for recurrent disease (4).

The technical problems associated with the traditional assessment of receptor proteins in breast cancer by use of radioactive ligands have been documented in numerous quality-assurance trials throughout the world (5, 6). The development of monoclonal antibodies to estrogen (7) and progesterone (8) receptors and the use of solid-phase enzyme immunoassay (and immunocytochemistry) have opened a new era in receptor analysis and require testing in the field.

Materials and Methods

We assessed 109 specimens from patients with breast cancer for ER and PR by ligand-binding assays, which are described in detail elsewhere (9). In the study, we used specimens received by the laboratory from March to August 1987, excluding only any small biopsies that could not yield sufficient cytosol.

The buffer contained, per liter, 10 mmol of Tris, 1.5 mmol of EDTA, 100 mL of glycerol, 1 mmol of dithioerythritol, and 20 mmol of molybdate. We used a Polytron PT-10 to homogenize the specimens, taking care to keep the temperature of the stainless-steel probe and the specimens as close to 0 °C as practicable. Immediately after centrifuging the homogenates, we stored multiple aliquots of cytosol in liquid nitrogen for up to 13 weeks.

Cytosols (2–4 mg of protein per milliliter) were incubated with six different concentrations of tritiated ligand: 0.1 nmol to 5 nmol of estradiol per liter for ER, and 0.2 nmol to 10 nmol of organon 2058 (Amersham International, Amersham, Bucks, U.K.) per liter for PR. In parallel, we performed incubations that also contained excess nonradioabeled ligand, diethylstilbestrol for ER and cold organon 2058 for PR, to measure the nonspecific, low-affinity binding. Both assays were performed in one working day by incubating ER for 2 h at 14 °C and PR for 4 h at 0 °C. Dextran-coated charcoal (DCC) was used to adsorb the excess ligand.

We used Scatchard plots and least-squares analysis to determine the dissociation constant and the maximum binding for each specimen. To measure protein we used Bradford’s procedure with Coomassie Blue (10), with normal control serum (Gillford, Irvine, CA) as the protein standard.

Final receptor results were expressed as femtomoles per milligram of cytosol protein. A PR result >10 fmol/mg was reported as positive, as was an ER >8 fmol/mg. (This laboratory has been a member of the Australian and New Zealand Quality-assurance program since its inception in 1981.)

To evaluate the PR-EIA kit (Abbott Diagnostics, North Ryde, N.S.W.) we immobilized the PR in the 109 cytosols onto beads coated with an anti-PR monoclonal antibody, exactly as outlined in the instructions accompanying the kit. After 18 h, unbound material was removed by aspiration and washing. A second PR-specific monoclonal antibody, time conjugated to horseradish peroxidase (EC 1.11.17), was added, binding to a different epitope on the PR that had been immobilized on the bead. Addition of enzyme substrate solution (hydrogen peroxide and o-phenylenediamine dihydrochloride) produced a color, the intensity of which was proportional to the receptor in the sample. We added 1 mL of 0.5 mol/L sulfuric acid to the reaction, and measured the absorbance at 492 nm with a spectrophotometer. The standard curve was based on the results for human PR at five different concentrations, assayed concurrently with a control and the specimens. Because the protein concentration of the cytosol for the ligand-binding assay was ideally in the range 2–4 g/L, we diluted each cytosol for the PR-EIA assay with buffer to give a protein concentration near 1–2 g/L, as suggested for the PR-EIA assay.

We also assessed PR-EIA results for cytosols stored for three weeks, five to seven weeks, and eight to 13 weeks, and compared these with results obtained for the fresh cytosols. The comparisons were analyzed by least-squares analyses.

Results

The age of the patients at the time of biopsy ranged from 31 to 86 y, with 30% of them under 50 y. There were 56 ER+PR+, 14 ER+PR−, 11 ER−PR+, 28 ER−PR−. ER+ accounted for 64% and PR+ for 61%, which is indicative of a population with such a high proportion of younger women.

PR-EIA compared with the ligand PR-DCC method. We compared the standard PR-DCC assay with the PR-EIA assay, using 109 freshly prepared cytosols (Figure 1). Least-squares analysis produced a line of best fit for which the equation was PR-EIA = 0.88 PR-DCC + 16; r² = 0.87. At high receptor concentrations the agreement between the assays was not as accurate as at lower concentrations. The standard PR-EIA curve measured up to 500 fmol per milligram of protein. Higher values could only be estimated by using diluted standards after completion of the assay. Such estimates were “generally within 20% of experimental values obtained by inclusion of a 500 fmol/ml standard” (PR-EIA instruction booklet, page 13). A PR concentration of 400 fmol/mg is regarded as “very high” and, clinically, it probably is academic whether the value is 400 or 900 fmol/mg.

We used a decision matrix (JJ) to relate the results of the new test (PR-EIA) with the standard assay, which has well-proven validity. The matrix (Table 1) expresses the binary
outcome (positive or negative) as a ratio. The PR-positive cutoff value for both tests was defined as greater than 10 fmol per milligram of protein. Sensitivity was defined as the true-positive (TP) ratio, and specificity as the true-negative (TN) ratio. From Table 1, the TP ratio or sensitivity TP/(TP + FN) was 0.99, and the TN ratio or specificity TN/(TN + FP) was 0.81. The accuracy of the new test was the ratio of the correct outcomes to all outcomes, 0.92. Ideally all the ratios should approach unity. It should be noted that six of the eight false-positive PR-EIA results were in the range 11 to 18 fmol/mg.

**Evaluation of PR-EIA measurements on stored cytosols.**  
We compared the PR-EIA values for 60 cytosols stored for three weeks in liquid nitrogen with those for fresh cytosolic PR-EIA. After excluding the four cytosols whose PR-EIAs exceeded 600 fmol/mg, the line of best fit had a slope of 1.11, an intercept of 0.9, and an $\rho^2$ of 0.96. PR-EIA values for fresh cytosols were also compared with those for 42 cytosols stored from five to seven weeks (slope 1.0, intercept 1.7, and $\rho^2$ 0.96) and for 29 cytosols stored from eight to 13 weeks (slope 1.1, intercept $-3.3$, and $\rho^2$ 0.91). Cytosols were only measured once during each of the time intervals.

The slope of each line approached unity and the intercepts were extremely small, indicating good agreement between PR-EIA as measured in fresh and stored cytosols.

Table 2 compares the PR-EIA in stored cytosols with different quantitative levels of PR-EIA in fresh cytosols. There was concurrence in 98% cases. The six asynchronous results all became "positive" during the storage periods, changing from 8 to 12, 10 to 11, 10 to 12, 4 to 19, 10 to 12, and 6 to 12 fmol/mg. No PR-EIA positive cytosol became "negative" during storage.

**Discussion**

The Abbott PR-EIA kit was easy to use, with clear instructions and a consistent positive human control PR. The PR-EIA relied on one (duplicate) measurement of the cytosol. The standard ligand-binding PR comprised a six-point assay with, as well, a measure of the dissociation constant ($K_d$).

In our experience the two methods were similar with regard to "hands-on" time. Both methods rely on an optimal homogenization technique, cytosol preparation has been shown to be the most critical step in valid ER and PR assays (12). Both methods also rely on adequate measurements, preferably involving a standardized protein method together with a standard protein source.

The conventional ligand assay required more sophisticated mathematical manipulation to generate the Scatchard plots, and may entail more subjective decisions than does the single-point PR-EIA assay read from a standard curve. However, that very complexity may provide a greater scope in decision-making.

Storage of cytosol for the PR-EIA assay enabled a larger number of specimens to be assayed in a single run than would be possible if fresh cytosols were required. We have shown the possibility of nearly halving the labor time (i.e., the cost) as well as reducing the cost per assay.

Use of monoclonal antibodies allows direct antigenic recognition of receptor molecules. It is important to be aware of the possibility of detecting nonfunctional, denatured, or nonligand binding in specimens, all of which are clinically irrelevant for determining hormone dependence of a tumor.

Whether the PR-EIA performs as accurately in tissue other than breast cancer remains to be proved, and until information is accumulated, research laboratories must be wary in using this method to assess tissue other than breast cancers.

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**References**