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# Cytochemical Demonstration of Estrogen Binding Sites in Breast Cancer by Estradiol Covalently Linked to Horseradish Peroxidase

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**Abstract**—*17β-Estradiol-6-carboxymethyloxime was covalently linked to horseradish peroxidase for the cytochemical demonstration of estrogen binding sites in breast cancer tissue. The affinity of the 17β-estradiol–horseradish peroxidase ($E_2$–HRP) conjugate for the estrogen receptor in a human myometrial cytosol preparation was reduced by a factor of about 16 relative to that of 17β-estradiol. The estradiol concentration of the $E_2$–HRP conjugate used in the incubations was in the range $2 \times 10^{-9}$–$1 \times 10^{-7}$ mol/l which, when the reduced affinity of the conjugate is taken into account, corresponds to $1 \times 10^{-10}$–$7 \times 10^{-9}$ mol/l unbound estradiol. The cytochemical reaction was carried out on cytofuge preparations of cell suspensions of breast cancer tissue. The intensity of the cytochemical reaction was microscopically evaluated by scoring. The results were analyzed in a plot allowing the calculation of an apparent $score_{max}$ and an apparent $K_d$ value. The reaction intensity was reduced to 20–25% of the control level by a 20-fold excess of 17β-estradiol. The cytochemical results correlated positively with the content of estrogen receptors in the cytosol as measured by a validated radioligand method.*

## INTRODUCTION

THE ESTROGEN receptor content in cytosol preparations of human breast cancer tissue as determined by radioligand techniques is of prognostic value [1]. A major drawback of these methods is their inability to detect receptor heterogeneity within the specimen. To solve this problem, histochemical approaches have been attempted. The use of antisera against the receptor appears promising [2–4] while immunohistochemical methods based on the demonstration of receptor-bound estradiol seem to suffer from lack of specificity [5]. Techniques employing tagged ligands are numerous. Many of these probably demonstrate estrogen binding sites but no definite evidence has been produced that these methods demonstrate the estrogen receptor. A main criticism of such methods concerns the high ligand concentration usually employed. This makes histochemical labelling likely to include non-specific binding [6]. Another point of criticism concerns the possibility that during the incubation procedure the soluble cytosol receptor is extracted from tissue sections [7]. With these problems in mind we here report methodological aspects and experiences with a method designed for the cytochemical demonstration of estrogen binding sites in cytofuge preparations of cell suspensions of human breast cancer tissue.

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## MATERIALS AND METHODS

### *Reagents*

Horseradish peroxidase was from Sigma Chemical Company, St Louis, MO, U.S.A. 17β-Estradiol, estradiol and 6-ketoestriol were from Steraloids, Inc., Wilton, U.K. 6-Keto-17β-estradiol was from Makor Chemicals Ltd, Jerusalem,

Israel. The double antibody solid phase (DASP®) was from Organon, Oss, Holland. Other chemicals were from E. Merck, Darmstadt, F.R.G.

*Preparation of the estrogen peroxidase conjugates*

17β-Estradiol- and estriol-6-carboxymethyl-oximes were prepared by a microtechnique as earlier described [8]. The linking of the steroid oxime to horseradish peroxidase (HRP, EC 1.11.1.7) was carried out according to the mixed anhydride method [9, 10]. The estrogen-HRP conjugates were dialyzed against water and stored in 0.05 mol/l phosphate buffer, pH 7.5, at 4°C. The average number of estrogen molecules per HRP molecule was estimated by radioimmunoassay. Anti-estradiol or -estriol antiserum, tritiated estradiol or estriol and dilutions of estradiol- or estriol-HRP ($E_2$- or $E_3$-HRP) conjugates and estradiol or estriol standards were incubated for 18 hr at 4°C in 0.1 mol/l phosphate buffer, pH 7.0, containing 0.12% gelatine. Separation of bound and unbound phases was performed by DASP techniques [11]. After centrifugation the precipitate containing the antiserum-bound steroid was washed twice with 0.9% sodium chloride. Radioactivity was extracted by acidification with 1 mol/l HCl. After centrifugation an aliquot of the supernatant was taken for radioactivity counting. The HRP concentration of the conjugate was measured spectrophotometrically at 412 nm, $E = 11.1 \times 10^4$/mol/cm. Before each experiment an aliquot of the estrogen-HRP conjugate was briefly shaken with dextran-coated charcoal and centrifuged in order to remove free estrogen. The stability of the conjugates was tested by prolonged dialysis against water after which aliquots of the dialyzate were tested for free estrogens by capillary gas chromatography using a 0.2 mm × 25 m vitreous silica well-coated open tubular (WCOT silicone SE-30) column and trimethylsilyl ether derivatives of the estrogens. The conjugate was stable for several months, but if signs of dissociation occurred the batch was discarded.

*Estrogen receptor affinity of the estrogen-HRP conjugates*

Myometrial samples derived from menstruating patients undergoing hysterectomy for uterine prolapse were homogenized in 25 mmol/l Tris-HCl buffer, pH 7.5, supplemented with 1.5 mmol/l EDTA, 2 mmol/l dithiothreitol and 10% (v/v) glycerol. The homogenate was centrifuged at 105,000 *g* for 60 min at 4°C. Aliquots of the supernatant cytosol fraction were incubated with [$^3$H]estradiol at $1 \times 10^{-9}$ mol/l along with varying concentrations of 17β-estradiol and estrogen-HRP conjugates for 18 hr at 4°C. Receptor-bound and non-bound estradiol and estrogen-HRP conjugates were separated by the addition of anti-estradiol or -estriol antiserum-DASP complexes. The preparation of liquid scintillation samples was performed as explained above. Displacement curves for each non-labelled ligand were constructed and the relative binding affinities of the compounds were calculated at the 50% competition level.

*Determination of estrogen cytosol receptors*

Determination of estrogen cytosol receptors in specimens from mammary breast cancer tissue was performed by a dextran-coated charcoal method as earlier described [12].

*Tissue specimens*

*MCF-7.* The established estrogen receptor-positive line of human breast cancer cells, MCF-7, was kindly provided by Charles M. McGrath (Michigan Cancer Foundation) and cultivated according to Butler *et al.* [13]. The cells were harvested after 3–6 days and washed by centrifugation in phosphate-buffered saline, pH 7.3, supplemented with 10 mmol/l glucose. Cytofuge preparations were made from the cell suspension. The preparations were dried in air, fixed for 10 min in -20°C acetone and stored at -20°C until used. Fixation with cold acetone reduced estrogen binding by the receptor [7] but enhanced the cytochemical reaction by facilitating permeation of the conjugate through the cell membrane.

*Cell preparations of breast cancer tumors*

Surgical biopsy specimens were teased and the cellular yield was washed by centrifugation in ice-cold phosphate-buffered saline with glucose. Cytofuge preparations were handled as above.

*Incubation technique*

The cytofuge preparations were incubated with different concentrations of the $E_2$-HRP conjugates at 37°C in a total volume of 11 ml in plastic containers. After incubation with the $E_2$-HRP conjugate the cytofuge preparations were washed for 5 min each in three changes of fresh buffer whereafter the cytochemical demonstration of peroxidase activity was carried out for 20 min at 37°C according to Graham and Karnovsky [14]. Triton X-100 was added at 0.025% (v/v) which increased the activity of the peroxidase enzyme [15]. The cytofuge preparation was then washed in fresh buffer and the reaction product was intensified by incubation in 0.5% (w/v) $CuSO_4$ in 0.05 mol/l Tris-HCl buffer, pH 7.6, for 10 min.

*Evaluation of the reaction product*

Cell nuclei were stained with Mayer's hematoxylin solution. Coded preparations were examined at a magnification of ×1000. The intensity of the cytoplasmic product was scored as follows: 0, cells negative; 0.5, extremely weak; 1, weak; 2, moderate; 3, strong. For each preparation the total score in 100 cells representing 20–30 fields of view was calculated. The total score of a given preparation was corrected for non-specific $E_2$-HRP conjugate binding by subtracting the score value obtained in a preparation similarly handled but incubated in the presence of a 20-fold excess of 17β-estradiol. An apparent $score_{max}$ as well as an apparent $K_d$ value were calculated by plotting the net score values obtained at a given $E_2$-HRP concentration ([$E_2$-HRP]) in a net score/[$E_2$-HRP] vs [$E_2$-HRP] plot.

The precision by which the $score_{max}$ value could be calculated was evaluated using MCF-7 cell preparations. The coefficient of variation from series to series was 11% using a given batch of the $E_2$-HRP conjugate. When the cytochemical results were compared with the estrogen receptor content measured by the radioligand method, the variation between series in which different $E_2$-HRP batches were used was minimized by expressing the $score_{max}$ value as a percentage of that obtained in a MCF-7 cell preparation which was included as a standard in each assay.

## RESULTS

When cytofuge preparations of MCF-7 cells were reacted for the demonstration of estrogen binding sites the majority of the cells contained a brown reaction product in their cytoplasm, the intensity of which was dependent on the concentration of the $E_2$-HRP conjugate. In some cells the reaction product covered the nucleus, but this paper focuses on cytoplasmic labelling only.

Incubation with native HRP (handled as in the conjugation procedure but without added estradiol-oxime) resulted in an extremely weak reaction product in a few scattered cells. The score value of such preparations was less than 10% of that obtained with an equal concentration of the complete $E_2$-HRP conjugate.

*Optimal incubation conditions*

Optimal incubation conditions with the $E_2$-HRP conjugate were evaluated with cytofuge preparations of MCF-7 cells. The effect of the incubation time was studied by incubation in a 0.1-mol/l phosphate buffer, 7.4, at 37°C for 0.5–6 hr. The $E_2$-HRP conjugate was added at $5 \times 10^{-8}$ mol/l. The intensity of the reaction product increased with incubation time until near-maximal levels were achieved at 2 hr.

The intensity of staining was investigated over the pH range 6.4–8.5 in a 0.1-mol/l citrate-phosphate buffer. MCF-7 cell preparations were incubated for 2 hr at 37°C with the $E_2$-HRP conjugate at $5 \times 10^{-8}$ mol/l. At pH values below 7.1 the intensity of the reaction product decreased and the score value obtained at pH 6.4 was 73% of that at pH 7.1. An increase in pH from 7.1 to 8.5 had a smaller effect, increasing the score by 8%. In separate experiments the composition of the buffer was varied. The following buffers were tested at pH 7.4: 0.1 mol/l phosphate buffer, 0.1 mol/l citrate-phosphate buffer, 0.05 mol/l Tris-HCl buffer, 0.14 mol/l barbital-acetate buffer and 0.1 mol/l borate buffer. The phosphate buffer was superior to all the other buffer systems, resulting in the highest score values. Whether the buffer was of the Na or K salt did not influence the results.

*The specificity of the cytochemical reaction*

*Estrogen receptor affinity of the $E_2$-HRP conjugate.* The affinity of the $E_2$-HRP conjugate for the estrogen receptor in a cytosol preparation of human myometrial tissue was evaluated as explained in Materials and Methods. 17β-Estradiol blocked the binding of [$^3$H]estradiol to the cytosol receptor in a concentration-dependent manner (Fig. 1). The curve describing the receptor blocking properties of the $E_2$-HRP conjugate was similar in shape to that of 17β-estradiol, which is taken to indicate that the two agents compete for the same binding sites. As expected, the affinity of the conjugate for the estrogen receptor was lower than that of 17β-estradiol. Assuming that one molecule of estradiol is conjugated to one

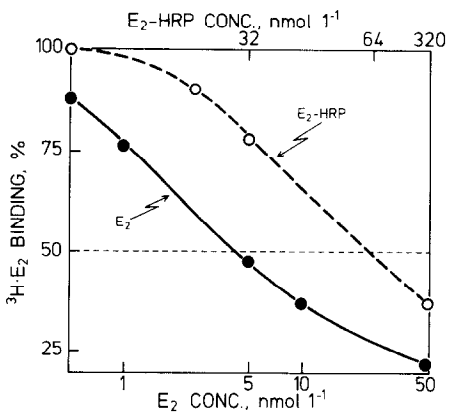


*Fig. 1. Determination of the relative affinity of the estradiol-peroxidase conjugate ($E_2$-HRP) for the cytosol estrogen receptor in a human myometrial preparation. The experiment was carried out as explained in Materials and Methods. A 50% displacement of* [$^3$H]*estradiol was achieved at 60 and 4 nmol/l for $E_2$-HRP and estradiol ($E_2$) respectively. The points represent the mean results of one experiment carried out in duplicate.*

molecule of peroxidase, the affinity of the conjugate was decreased 16 times relative to that of free estradiol.

*$E_3$-HRP conjugate.* At concentrations which gave a strong reaction in MCF-7 cell preparations with the $E_2$-HRP conjugate, only occasional cells contained a weak reaction product after incubation with the $E_3$-HRP conjugate. This is in accordance with the observation that the affinity of the latter conjugate for the estrogen receptor in human myometrial cytosol was 164 times lower than that of free estradiol (not shown).

*Effect of estradiol and norgestrel.* When the reaction was carried out with the $E_2$-HRP conjugate at $5 \times 10^{-8}$ mol/l, a 20-fold excess of 17β-estradiol decreased the intensity of the reaction product to 20–25% of that obtained without added estradiol.

L-Norgestrel (D-3β-ethyl-17α-ethynyl-17β-hydroxy-4-gonen-3-one) has a low affinity for the estrogen receptor but binds with high affinity to sex hormone binding globulin and with less affinity to albumin [16].

In cell preparations from receptor-positive mammary cancer tumors norgestrel at $3 \times 10^{-6}$ mol/l depressed the intensity of the reaction product by 18%.

*Correlation between the cytochemical reaction and the estrogen receptor content*

Incubation of cell preparations from human breast cancer tumors were carried out in 0.1-mol/l phosphate buffer, pH 7.4, for 2 hr at 37°C. At higher concentrations of the $E_2$-HRP conjugate, i.e. $5 \times 10^{-8}$–$1 \times 10^{-7}$ mol/l, some cells were positively labelled whether the specimen turned out to be estrogen receptor-positive or not. In receptor-negative cases, however, the intensity of the reaction rapidly decreased as the concentration of the $E_2$-HRP conjugate was decreased. In contrast, in cell preparations from specimens which turned out to be estrogen receptor-positive the intensity of the reaction was dependent on the $E_2$-HRP conjugate concentration in such a way that a linear net score/[$E_2$-HRP] vs [$E_2$-HRP] plot was obtained and an apparent $score_{max}$ as well as an apparent $K_d$ value could be calculated. Thus the decision whether a specimen was receptor-positive or not was based on an analysis of results obtained at several different concentrations of the $E_2$-HRP conjugate.

Cell preparations of tumor specimens from 22 patients with histologically verified mammary cancer were cytochemically investigated for $E_2$-HRP binding sites. Adjacent specimens were biochemically analyzed for estrogen receptor content. The results are presented in Table 1.

*Table 1. Correlation between the intensity of the cytochemical reaction for estrogen binding sites in cytofuge preparations of human breast cancer tissue and the estrogen receptor content measured by a radioligand method*

| Estrogen receptor content, fmol/mg protein | $Score_{max}$, of that in MCF-7 cells | | |
|---|---|---|---|
| | Negative <10 | Low 10–70 | High >70 |
| <3 | 3 | 1 | |
| 3–50 | 1 | 5 | 3 |
| >50 | 1 | 8 | |

There were four patients with a receptor content below 3 fmol/mg protein. Three of these patients were cytochemically classified as negative for estrogen binding sites (apparent $score_{max}$ below 10% of that in MCF-7 cells) while the specimen from the fourth patient was strongly positive (apparent $score_{max}$ over 70%). This biopsy specimen was exceedingly fibrotic, with few cancer cells which might have distorted the biochemical analysis. There were nine patients with intermediate levels of estrogen receptor (3–50 fmol/mg protein). Five of these were correctly reported on cytochemically while in three patients the number of estrogen binding sites was overestimated, and in one underestimated. Finally, tumor material from altogether nine patients contained high levels of estrogen receptor (over 50 fmol/mg protein). The cytochemical assay correctly detected eight of these patients and underestimated one.

In the total of 18 estrogen receptor-positive cases the apparent $K_d$ value obtained with the $E_2$-HRP conjugate for cellular binding sites was $5 \pm 2 \times 10^{-9}$ mol/l (mean ± S.D.). Taking into account the reduced receptor affinity of the conjugate, this corresponds to a value of $3 \pm 1 \times 10^{-10}$ mol/l. This is in close agreement with the $K_d$ value obtained for estradiol for the cytosol estrogen receptor determined by radioligand methods.

## DISCUSSION

On the following grounds we consider the cytochemical reaction obtained with the $E_2$-HRP conjugate in estrogen receptor-positive cell preparations specific for estrogen binding sites: (1) the conjugate was not non-specifically absorbed to cellular components; (2) 17β-estradiol competed effectively in the reaction but L-norgestrel did not.

The question arises as to whether the cellular estrogen binding sites visualized by the present cytochemical reaction represent the estrogen receptor. Arguments in favor of this are: (1) although the affinity of the $E_2$-HRP conjugate was reduced, it competed with 17$\beta$-estradiol for the same binding sites in a cytosol preparation of myometrial tissue; (2) the reaction intensity obtained with the $E_2$-HRP conjugate greatly exceeded that obtained with an $E_3$-HRP conjugate which had a low affinity for the cytosol estrogen receptor; (3) the highest $E_2$-HRP concentrations used in the assay, $1 \times 10^{-7}$ mol/l, corresponding to $7 \times 10^{-9}$ mol/l free estradiol, would saturate the estrogen receptor but only to a limited extent react with non-receptor estrogen binding proteins [6]. At low $E_2$-HRP concentrations, corresponding to $1 \times 10^{-10}$ mol/l free estradiol, non-specific binding was involved apparently only to a very minor extent; (4) the apparent dissociation constant of the $E_2$-HRP conjugate for binding sites in receptor-positive cell preparations ($5 \times 10^{-9}$ mol/l, corresponding to $3 \times 10^{-10}$ mol/l free estradiol) was of the same order of magnitude as that obtained for 17$\beta$-estradiol for the estrogen receptor but clearly lower than the $K_d$ value obtained for type II estrogen binding sites ($8 \times 10^{-8}$ mol/l [6]); (5) in patients with breast cancer, a positive correlation was obtained between the intensity of the reaction product and the estrogen receptor content determined by a validated biochemical cytosol assay.

Thus in cytofuge preparations of mammary cancer cells the present cytochemical reaction appears to demonstrate the estrogen receptor. However, because of the blocking effect of L-norgestrel (about 20%), because 17$\beta$-estradiol left some of the reaction unblocked (about 25%) and because we know that the affinity of the conjugate for the estrogen receptor is decreased (about 16 times) but not if the affinity for other estrogen binding proteins is decreased to the same extent, the reaction may also demonstrate non-receptor binding sites. Final usefulness will depend on whether the reaction is able to predict clinical response to therapy.

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