Failure of Estradiol Immunofluorescence in MCF-7 Breast Cancer Cells to Detect Estrogen Receptors

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ABSTRACT

An indirect immunofluorescence assay was used to detect estradiol in MCF-7 breast cancer cells to determine if the estradiol-specific fluorescence observed represented estrogen receptor-bound estradiol. Appropriate controls were used to demonstrate the immunological specificity of our assay procedures. Initial studies of estradiol binding in MCF-7 cells were performed at 20°C for 1 hr with different concentrations of estradiol. Cytoplasmic and nuclear staining were observed following treatment with 10 nM estradiol, but not with lower concentrations which were nevertheless still sufficient to saturate estrogen receptor. The staining intensity increased with higher estradiol concentration, which is consistent with estradiol binding to lower-affinity binding sites. In order to further determine if estradiol binding by estrogen receptor was being detected, we pretreated MCF-7 cells with 5 nM diethylstilbestrol at 37°C for 1 hr to translocate all estrogen receptor to the nucleus and then administered estradiol at varying concentrations for 4 hr at 4°C. The estradiol was still primarily detected in the cytoplasm, although virtually all of the estrogen receptor was found to be present in the nucleus by standard [3H]estradiol binding assays. Additional immunochemical studies using sucrose gradient analysis to detect antibody-estradiol-receptor complexes clearly established that these complexes could not be detected.

The present results suggest that, although immunochemical assays can specifically detect estradiol in MCF-7 cells, the estradiol is bound to lower-affinity binding sites rather than to estrogen receptor. Saturation analyses of intact viable MCF-7 cells performed at 37°C for 30 min using [3H]estradiol at concentrations ranging from 0.1 to 93 nM revealed an additional lower-affinity estradiol-binding site besides the receptor, perhaps analogous to the Type II sites reported in the rat uterus and human breast cancers.

INTRODUCTION

Only about one-third of patients with metastatic breast cancer will benefit from endocrine treatment or ablative surgery. The original suggestion by Jensen et al. (18) that estrogen receptor assays will benefit from endocrine treatment or ablative surgery.

can be enhanced through the measurement of progesterone receptor, a product of estrogen stimulation (16, 35). But it is clear that the distribution of these receptors in frequently heterogeneous tumor cell populations could seriously affect our conclusions. Furthermore, the ability to detect receptors in a relatively small sample of tumor cells such as a pleural effusion or a needle biopsy would be helpful. A histological method for revealing estrogen and progesterone receptors would therefore be highly desirable.

Such histological assays could be approached through the use of antibodies to the receptors, or indirectly through the use of antibodies to the steroid hormones to detect the receptor-bound steroids, or by using a labeled ligand which will bind to the receptor yet can be detected microscopically. Although an antibody to the estrogen receptor has been prepared (14), its use in immunohistochemical procedures has not been reported. However, several laboratories have reported the use of immunofluorescence (27-34, 37, 39) and immunoperoxidase techniques using antibody to estradiol (13, 20, 21, 28-31), as well as cytochemical techniques using fluorescein-estradiol conjugates (22, 23, 38, 40), to detect the binding of steroid hormones in human or animal estrogen-dependent tissues and tumors. The results of these studies seemed to suggest that immunochemical assays could be used to detect estradiol binding which might correlate with the presence of estrogen receptors, and therefore, be useful clinically.

In preliminary studies, we observed that (a) a correlation existed between the percentage of estradiol-binding cells detected by immunochemical assays and the amount of estrogen receptor present in human breast cancers, (b) estradiol binding was restricted to estrogen target tissues and some tumors derived from target tissues in humans and rats, and (c) the percentage of estradiol-binding cells correlated with the amount of tumor regression in mammary tumor-bearing rats (22-29). However, when we further examined the specificity of the immunochemical assays by using human breast cancer cell lines as model systems, we found that high concentrations of estradiol were required to observe estradiol-specific staining. In addition, when we attempted to use DES as a competitor of estradiol binding, we were not able to detect reproducible inhibition of staining under our routine assay conditions (30).

We concluded from these studies that immunochemical assays can be used to detect estradiol in tumor cells, but that the observed binding was probably due to multiple classes of steroid-binding sites, not to estrogen receptor. The purpose of the
current study was to further examine the specificity of immuno-
cytochemical assays to determine if the detected estradiol
binding might be due, at least in part, to interaction with the
estrone receptor.

MATERIALS AND METHODS

Antiserum Preparation. Estradiol antiserum was prepared in female,
albino New Zealand rabbits using 17β-estradiol-6-O-carboxymethylox-
ime-BSA (Steraloids, Inc., Wilton, N. H.) as the immunogen (8).

Adsorption of Anti-Estradiol Antiserum. BSA (fraction V; Miles
Laboratories, Elkhart, Ind.) was coupled to glutaraldehyde-activated
polyacrylamide beads (Biogel P-300, ~400 mesh; Biorad Laboratories,
Richardson, Calif.) using the method of Ternynck and Avramescu (43).
The anti-estradiol antiserum was diluted 1:5 with PBS and adsorbed 3
times with an equal volume of the immunosorbent beads. Each ad-
sorption was performed for 1 hr at room temperature.

A portion of the BSA-adsorbed anti-estradiol antiserum was then
adsorbed with crystalline estradiol or 17β-estradiol-6-O-carboxy-
methylxime for 1 hr at room temperature. Excess undissolved steroid
was removed by centrifugation at high speed for 2 min in a Beckman
Microfuge B centrifuge. The adsorbed antisera were tested by radioim-
munoassay to confirm that the adsorptions were complete.

Methods for Evaluation of Antiserum. The anti-estradiol antiserum
was tested for specificity using a radioimmunoassay we developed for
routine use (30). In brief, approximately 10,000 cpm of [2,4,6,7-3H]-
estriadiol (102 Ci/mmol; New England Nuclear, Boston, Mass.) were
incubated with anti-estradiol antiserum diluted 1:6,000 (final dilution)
in radioimmunoassay buffer (0.01 M sodium phosphate:0.15 M NaCl;
0.1% gelatin, pH 8.8) for 18 hr at 4° in the presence of estradiol or
other steroid hormones used at concentrations ranging from 0.1 nm to
1 μM. Following the incubation, excess steroid was removed by ad-
sorption with dextran-coated charcoal [0.25% Norit A (Sigma Chemical
Co., St. Louis, Mo.):0.025% Dextran T-70 (Pharmacia, Uppsala, Swe-
den) in radioimmunoassay buffer] at 4° for 30 min. The charcoal
suspension was separated by centrifugation at 2000 × g for 15 min.
The supernatant was poured into scintillation vials, 8 ml of aqueous
scintillation scintillant (Amersham/Searle Corp., Arlington Heights, Ill.)
were added, and the samples were counted in a scintillation counter.
Results are plotted (1) in Chart 1.

The specificity of the anti-estradiol antiserum was also checked by
incubating the antiserum at 1:8,000 final dilution with 10,000 cpm of
various 3H-steroid hormones to determine the percentage of binding of
those substances (results not shown). The assay incubation conditions
were the same as those described above. The 3H-steroid hormones
used were purchased from New England Nuclear and included
[2,4,6,7-3H]estrone, 85 Ci/mmol; [1,2,6,7-3H]progesterone, 114 Ci/m-
mol; [1,2-3H]dihydrotestosterone, 40 Ci/mmol; and [1,2,6,7-3H]cor-
tisol, 80.6 Ci/mmol.

Cell Lines and Culture Techniques. The MCF-7 breast cancer cell
line was originally provided by H. D. Soule, Michigan Cancer Founda-
tion, Detroit, Mich. The routine culture conditions were as described
previously (15, 17). For experimental studies, the routine serum sup-
plement was removed and replaced by 5% calf serum stripped of
endoogenous steroids by a 30-min incubation at 4° with a dextran-
coated charcoal pellet (0.25% Norit A and 0.0025% dextran in 0.01
m Tris-HCl, pH 8.0, at 4°, 1 ml/ml serum). Immediately prior to
incubations with hormones, the culture media were removed and re-
placed by KRH glucose solution. The hormones were added at 1000
X concentrated solutions in absolute ethanol.

Cell Harvest. Cells were removed from the surface by a 10-min
incubation at 37° with 1 nm EDTA in Ca++-Mg++-free Hanks’ balanced
salt solution, washed once with Hanks’ balanced salt solution at 4° and
once in PTG or KRH glucose solution. The phosphate buffer was used
if estrogen receptor assays were to be performed while the glucose
solution was used if immunocytochemical assays were to be performed.
at 37°. Duplicate assays were performed using 100-fold excess DES at each concentration of [3H]estradiol to estimate nonspecific binding. Following the incubation, the cells were separated by centrifugation for 5 min at 300 x g and washed 3 times with 2 ml cold KRH glucose. Radioactivity in the cell pellets was extracted with 2 ml ethanol. The ethanol was then mixed with 5 ml tolune-based scintillation fluid (4.0 g PPO, 0.05 g POPOP, and 1 liter toluene) in a Beckman LS 233 liquid scintillation counter with a counting efficiency of 50% for tritium.

Estrogen Receptor Assays. Unfilled cytosol and unfilled nuclear estrogen receptors were measured by a protamine sulfate assay as described previously (45). Briefly, cytosol samples were diluted with PTG to adjust the protein concentration to approximately 1.5 mg/ml; nuclear extracts were diluted 1:8. Aliquots of the cytosol (200 µl) and nuclear extracts (500 µl) were then precipitated with 0.1% protamine sulfate (250 µl). The protamine precipitates were incubated at 4° for 16 hr with 5 nm [3H]estradiol for cytosol and 10 nm [3H]estradiol for nuclear samples. Nonspecific binding was determined by parallel incubations with [3H]estradiol plus a 100-fold excess of unlabeled DES. Following incubation, the free estradiol in solution was aspirated, and the protamine precipitates were washed 3 times with PTG. Radioactivity in the protamine pellets was extracted and counted in 5 ml of scintillation fluid.

Total receptors (unfilled plus estrogen-filled receptor sites) were measured by an estradiol exchange assay (44) in protamine pellets of cytosol and nuclear extracts by incubation with [3H]estradiol or [3H]-estradiol plus DES at 30° for 5 hr. The difference between the values for 30° binding (total receptor) and 4° binding (unfilled receptor) yields the value for filled estrogen receptor sites.

Saturation analyses of cytosol and nuclear estrogen-binding sites were performed by incubating protamine-precipitated cytosol or nuclear extracts with various concentrations up to about 100 nm [3H]-estradiol at 4° for 16 hr. DES at 100-fold excess was used in duplicate assays at each concentration of [3H]estradiol to estimate the amount of nonspecific binding. After incubation, the protamine pellets were washed 3 times with PTG, extracted with 5 ml toluene-based scintillation fluid, and counted. Data were analyzed using the method of Scatchard (41).

Sucrose Density Gradient Centrifugation. MCF-7 cytosol was prepared in PTG containing 0.4 M KCl and adjusted to approximately 5 mg protein per ml solution. The cytosol (200 µl) was incubated with 200 µl [3H]estradiol (1 nm in radioimmunoassay buffer) in the presence or absence of 100-fold excess DES, anti-estradiol antisera (100 µl of a 1:2000 initial dilution prepared in radioimmunoassay buffer), or anti-estradiol antisera which had been preadsorbed with estradiol. The incubation was performed at 4° for 4 hr with continuous low-speed shaking. Two hundred µl of each sample were layered onto 5% to 20% linear sucrose gradients prepared in the PTG-KCl buffer. ['"C]BSA was used as an internal 4.6S marker. The gradients were centrifuged in a Beckman SW 60 rotor at 53,000 rpm (average, 246,000 x g) for 16.3 hr. Fractions (200 µl) were collected and counted in aqueous counting scintillation fluid.

Protein and DNA Assays. Protein concentrations were measured using the method of Lowry et al. (25) with BSA standards. The amount of DNA was determined using the method of Burton (2).

RESULTS

All of the immunocytochemical studies presented here used rabbit anti-estradiol antisera which had been adsorbed with BSA, since the use of unadsorbed antisera may result in a positive staining pattern due to the presence of cross-reacting cellular antigens and/or BSA from the tissue culture medium (Table 1). In order to prove immunocytochemical specificity for estradiol, a portion of the primary antisera was further adsorbed with either estradiol or estradiol-6-O-carboxymethylxime. Staining was greatly reduced or abolished (Table 1). As a negative control, PBS was used in place of the primary antisera to detect any nonspecific binding of the secondary antisera and/or autofluorescence.

For most routine studies, MCF-7 cells were exposed to estradiol at room temperature for 1 hr. Under these conditions, estrogen-specific immunofluorescent staining was observed in both cytoplasm and nuclei of cells treated with 10 nm estradiol or higher doses (Table 2). In general, the staining intensity increased with higher estradiol concentration in both the cytoplasm and nucleus.

When MCF-7 cells were treated with estradiol at 4°, cytoplasmic (or plasma membrane) staining was predominant. Nuclear staining was also observed at 4° but this was associated with cytoplasmic staining (Fig. 1; Table 3). Warming to 37° after an initial incubation with estradiol at 4° caused a relative increase in the nuclear fluorescence (Fig. 2; Table 3).

These results suggested temperature-dependent translocation of estradiol to nuclei, as expected for receptor. Because of the extremely high concentration of estradiol required, however, we examined estradiol immunofluorescence in MCF-7 cells pretreated with 5 nm DES at 37° to induce complete nuclear translocation of the estrogen receptor. DES was chosen as the ligand because it binds to the estrogen receptor but is not detected by the anti-estradiol antisemur at this concentra
tion. Table 4 presents the receptor distribution measured in cytoplasm and crude nuclei before and after DES treatment. We have shown elsewhere that the receptor associated with crude nuclei before estrogen treatment is removed to the soluble fraction by purification of the nuclei, while after treatment, all receptor is completely localized in purified as well as

### Table 1

<table>
<thead>
<tr>
<th>Adsortion</th>
<th>% of original antisemur titer after adsorption</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>100</td>
<td>3+</td>
<td>3+</td>
</tr>
<tr>
<td>BSA</td>
<td>86.7</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>Estradiol</td>
<td>2.8</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>BSA and estradiol</td>
<td>2.2</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>BSA and estradiol 6-O-carboxymethylxime</td>
<td>&lt;0.1</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

### Table 2

<table>
<thead>
<tr>
<th>Concentration of estradiol treatment</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1 nm estradiol</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10 nm estradiol</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>100 nm estradiol</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>1 µE estradiol</td>
<td>3+</td>
<td>2+</td>
</tr>
</tbody>
</table>

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crude nuclei (5). The results in Table 4 confirm that virtually all cytoplasmic receptor was translocated to nuclei by the DES treatment. Nevertheless, after we then exposed the DES-treated cells to estradiol, the most intense fluorescent staining was still located in the cytoplasm (Figs. 3 and 4; summarized in Table 5). The results of this experiment demonstrate clearly that the estradiol-specific fluorescent staining observed was not due to receptor-bound estradiol.

We also wished to know whether estradiol attached to receptor could be detected by antibody at all. Using high-salt sucrose gradient sedimentation to detect the formation of complexes, we found that, while there was competition between the 4S estrogen receptor and 7S anti-estradiol antibody for the limited amount of [3H]estradiol, there was no evidence of a double complex consisting of antibody-estradiol-estrogen receptor (Chart 2).

In order to determine if the fluorescent staining observed might represent a class(es) of estradiol-binding sites distinct from the estrogen receptor, we performed a whole-cell binding assay using a wide range of [3H]estradiol concentrations (Chart 3A). The Scatchard analysis (Chart 3B) reveals the presence of at least 2 classes of binding sites, with one class exhibiting the high-affinity properties of the estrogen receptor and the other a much lower affinity. The lower-affinity sites are detected mainly at 10 nM or higher concentrations of [3H]estradiol. It should be noted that this is the lower limit for immunofluorescence detection of estradiol in MCF-7 cells. In addition, the saturation analysis (Chart 3A) reveals that the nonspecific binding at the estradiol concentrations at which maximum fluorescence staining was observed represents nearly one-half of the total [3H]estradiol binding by the cell. These lower-affinity sites in MCF-7 cells in some ways resemble the type II binding sites described in rat uterine cells (4, 7), which are discussed further below.

DISCUSSION

Because of the great usefulness of estrogen receptor determinations in selecting therapy for breast cancer patients, there has been considerable interest in the immunocytochemical localization of estradiol, assuming that the estradiol detected is bound to the estrogen receptor. However, rigorous criteria must be applied in control experiments to demonstrate both that the immune reaction is specific for estradiol and that the estradiol detected is indeed receptor bound.

As we reported previously (30), it is absolutely essential to perform the immunocytochemical assays using anti-estradiol antiserum which has been adsorbed with BSA and tested for completeness of adsorption. The use of unadsorbed antiserum can result in false-positive results due to the presence of BSA (in tissue culture cells) or human serum albumin which is present in tissue sections in interstitial spaces, on cell membranes, and/or intracellularly, and which contributes to the

Table 3
Effects of temperature on immunofluorescence staining for estradiol in MCF-7 cells

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>Temperature</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>4°</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1 nM estradiol</td>
<td>4</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>10 nM estradiol</td>
<td>4</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>100 nM estradiol</td>
<td>4</td>
<td>2+</td>
<td>1+</td>
</tr>
<tr>
<td>None</td>
<td>37°</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>1 nM estradiol</td>
<td>37°</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>10 nM estradiol</td>
<td>37°</td>
<td>1+</td>
<td>2+</td>
</tr>
<tr>
<td>100 nM estradiol</td>
<td>37°</td>
<td>1+</td>
<td>2+</td>
</tr>
</tbody>
</table>

Table 4
Effect of DES on intracellular distribution of estrogen receptors in MCF-7 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cytoplasmic ER&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Nuclear ER</th>
</tr>
</thead>
<tbody>
<tr>
<td>None/none</td>
<td>0.364</td>
<td>0.177</td>
</tr>
<tr>
<td>DES/none</td>
<td>0.020</td>
<td>0.027</td>
</tr>
<tr>
<td>DES/1 nM E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.019</td>
<td>0.019</td>
</tr>
<tr>
<td>DES/10 nM E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.002</td>
<td>0.007</td>
</tr>
<tr>
<td>DES/100 nM E&lt;sub&gt;2&lt;/sub&gt;</td>
<td>0.016</td>
<td>0.013</td>
</tr>
</tbody>
</table>

<sup>a</sup> ER, estrogen receptors; E<sub>2</sub>, estradiol.

Table 5
Effect of DES pretreatment on the immunofluorescence staining by antiestradiol serum in MCF-7 cells

<table>
<thead>
<tr>
<th>Hormone treatment</th>
<th>DES</th>
<th>Estradiol</th>
<th>Cytoplasm</th>
<th>Nucleus</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5 nM</td>
<td>None</td>
<td>None</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>5 nM</td>
<td>1 nM</td>
<td>1</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>5 nM</td>
<td>10 nM</td>
<td>2</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>None</td>
<td>1 nM</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<td>None</td>
<td>10 nM</td>
<td>1</td>
<td>1+</td>
<td>1+</td>
</tr>
<tr>
<td>None</td>
<td>100 nM</td>
<td>2</td>
<td>1+</td>
<td>1+</td>
</tr>
</tbody>
</table>
immunocytochemical staining observed. Most reports from other laboratories with one possible exception (21) neglect to mention whether or not the anti-estradiol antibody was adsorbed with BSA. This adsorption is especially important in the light of our unpublished observations that direct staining of human breast cancers with fluorescein-labeled antiserum to human serum albumin appears to reveal cellular heterogeneity similar to that reported with existing immunocytochemical assays used to detect estradiol.

To prove specificity of the antiserum, normal serum of the same species is sometimes substituted to detect any nonspecific binding of immunoglobulins in the primary or secondary antisera. This does not fully demonstrate immune specificity, however, since the normal serum source animal was not exposed to potential antigens such as BSA or impurities in the immunogen which might give rise to antibodies cross-reacting with cellular or plasma antigens. The only acceptable control, therefore, is to adsorb the test antiserum itself with the antigen being tested for, in this case, estradiol (or estradiol-6-carboxymethyloxime). A marked reduction or total elimination of fluorescence staining in specimens as we find in this study indicates specificity for estradiol.

Even though some of the immunocytochemical studies reported previously included controls which did demonstrate immune specificity, there were still a number of points of uncertainty concerning the validity of these methods for detecting estrogen receptor. These included the availability of receptor-bound estradiol to antibody, the concentration of estradiol necessary to visualize the response, and the ability of other known receptor ligands to compete for the visualized estradiol binding. We therefore initiated the present investigation in order to (a) seek evidence of antibody-estradiol-receptor immune complexes, if any, and (b) determine if the intracellular localization of visualized estradiol following experimental treatment was consistent with the detection of estrogen receptor. These experiments were performed using the MCF-7 breast cancer cell line, a well-described model system for in vitro studies of steroid hormone action (6, 15, 17, 24), and included the use of appropriate assay controls to demonstrate immune specificity. The results confirm that estradiol-specific fluorescence staining can be observed in breast cancer cells, but that the immunocytochemical assays are not detecting estradiol bound to the estrogen receptor.

First, it is clear that, if antibodies are to recognize receptor-associated estradiol, either the antibodies must be able to bind estradiol even in the receptor binding site, or else the receptor must first release the bound hormone. Despite several attempts using various incubation times and temperatures, different buffers, and high- and low-salt-containing gradients, we consistently failed to detect any evidence of antibody-estradiol-receptor immune complexes in sucrose gradients. This is essentially the same observation reported previously by Castaneda and Liao (3), who also did not find any evidence of these immune complexes using sucrose gradient analysis. These experiments were performed on cytoplasmic receptors, leaving open a possibility that nuclear receptors would behave differently; this seems unlikely, however, considering their immunological (14) and kinetic similarity.
In addition, the results of several studies which were based on radioimmunoassay methods to detect competition between cytosol estrogen receptor and antibody for binding of estradiol also suggest that a complex of antibody-estradiol-receptor was not formed (3, 10-12). These experiments involved the use of insolubilized antibodies to estradiol, which were incubated with a mixture of [3H]estradiol and cytosol from breast tumors or other estrogen target tissues. Under these conditions, although the antibody would bind free [3H]estradiol and [3H]estradiol bound to nonspecific sites, it could not successfully compete for [3H]estradiol bound to the estrogen receptor, which presumably reflects a lack of immune complex formation. Similar competition experiments have been performed in our laboratory, and we also failed to observe any evidence of reproducible competition suggestive of immune complex formation (data not shown).

An alternative explanation for antibody recognition of receptor-associated estradiol is that the receptor may release the estradiol or at least loosen its hold during fixation (19). If this were true, however, it seems unlikely that the freed hormone would remain precisely at the original site or even in the original cell to await the antibody. Freed hormone would presumably wash away during washing steps if not fixed to cellular structures. It therefore seems most likely that the intracellular estradiol detected by these methods is in fact bound to structures other than the estrogen receptor; we will discuss this possibility further below.

At least 3 types of estrogen-binding components in cells can be distinguished. The estrogen receptor itself, or type I binder, has the highest affinity, with a dissociation constant (Kd) for estradiol on the order of 10^{-10} M. This means that half of the receptors carry hormone at that estradiol concentration, and that nearly all are filled by the time the estradiol concentration reaches 1 nM. Above 1 nM, the receptors are saturated, so that no more estradiol can bind to them no matter how much is added. Other types of sites exist, however, which have lower affinity but higher capacity for estradiol binding. Such sites have been described in the rat uterus by Clark et al. (4, 7), who termed them “type II” estrogen-binding sites. Panko et al. (36) have found large amounts of type II binding in many human breast cancers. Below 1 nM estradiol, few of these sites are occupied, but at higher concentrations, type II binding becomes substantial with most binding sites occupied following exposure to 100 nM estradiol.

There is also another order of estrogen binding within cells, comprised of soluble molecules like albumin and probably also certain molecules associated with cell membranes and other cellular structures. Although no one of these has a high affinity for estradiol, their total binding capacity is very great, and binding to these sites at very high estradiol concentrations may become substantial. These sites we refer to as type III (or nonspecific) estrogen-binding sites.

Finally, it is conceivable that essentially free estradiol could be detected within cells. Whether the observed intracellular fluorescence includes free estradiol was not directly established in our experiments, but we have observed previously that MDA-231 breast cancer cells and many tumor cells obtained from patients with breast cancer failed to exhibit detectable estradiol-specific fluorescence following treatment of the cells with up to 1 μM estradiol (30). Therefore, the free estradiol which would be expected to have diffused into these cells was not detected, presumably having been removed from the frozen sections or fixed cells during washing procedures. When fluorescent staining is present, as with MCF-7, it therefore probably represents estradiol which is bound nonspecifically to cellular structures or specifically to classes of steroid-binding sites.

The binding of estradiol to estrogen receptor, and subsequently to type II and type III sites as the concentration increases, is diagrammed in Chart 4. It is obvious that, if too high a concentration of estradiol is used to incubate specimens for immunocytochemical assays, the probability increases that those assays will detect estradiol-binding sites other than the estrogen receptor. In fact, we have found that, with 1 nM estradiol, which should be sufficient to nearly saturate the estrogen receptors, we cannot detect estradiol-specific immunofluorescence at all. It seems likely, therefore, that most immunocytochemical methods, some of which use as high as 1 nM estradiol, are detecting primarily type II or even type III nonspecific binding rather than the receptor itself.

In the rat uterus, the only tissue in which type II binding has been well studied, type II is present in cell nuclei and increases greatly after estrogen treatment (8, 15). Unlike the estrogen receptor, however, the type II sites present in the cytoplasm (which may be totally distinct from those in the nuclei) are not depleted by estrogen treatment. Nor would the location of type III sites be expected to be affected by estrogens. In this report, we attempted to make use of this distinction to discover which binding was detected by the immunofluorescence procedure. We treated MCF-7 human breast cancer cells with 5 nM DES and proved by radioligand binding assay that all of the true estrogen receptor had in fact been translocated to the nuclei. Nevertheless, the cytoplasmic immunofluorescence was not reduced, which is consistent with the detection of estrogen bound to some sort of type II or even type III component rather than to estrogen receptor. Kurzon and Sternberger (21) recently reported similar results with their immunocytochemical assay in rat uterus.

The results of our saturation analyses of MCF-7 cells with [3H]estradiol, which were performed to detect other classes of binding sites, show that type II sites may be present in MCF-7 cells. Whether the type II sites that we detected represent basal levels of this class of binding sites or include specific induction in nuclei following estradiol treatment was not established in this study. Because saturating conditions were not demon-
strated for this class of binding site, we were not able to estimate the actual number of binding sites or the dissociation constant. However, from the amount of total binding observed following incubation with 10 nM \(^{3}H\)estradiol, and by subtracting the amount of type I binding (estrogen receptor), we estimated that nearly half of the total estradiol binding was to lower-affinity but competitive estradiol-binding sites. Whether this level of binding can explain the immunofluorescence results for MCF-7 is not yet known.

One necessary criterion for immunofluorescent detection of receptor-associated estradiol is elimination of staining with an excess of DES or antiestrogens, which would fill the receptor sites but cannot be detected by the antibody. Other investigators have reported such apparent competition, and this has been considered evidence for the receptor specificity of their procedures. However, the concentration of DES reportedly used has varied from 1 \(\mu M\) to 1 mm, which is in vast excess of the concentration necessary to saturate estrogen receptors. Since type II estradiol binding is also inhibited by DES (4), and sufficiently high concentrations of DES might even reduce some estradiol binding to type III sites, this “competition” does not invalidate our conclusion that immunocytochemical assays are probably detecting types II or III sites rather than the estrogen receptor. In addition, we have observed that, at DES concentrations as high as 1 \(\mu M\), there is a marked decrease in the binding of antibody to estradiol (Chart 1). If high tissue concentrations of DES were present, the appearance of competitive inhibition of estradiol binding to cellular macromolecules might therefore represent, at least in part, inhibition of antibody activity. In addition, such high concentrations of DES and other steroid hormones approach the limit of solubility in aqueous solutions, and one cannot rule out a decrease in estradiol solubility in the presence of high DES concentrations. Inhibition by appropriate competitors therefore argues for estrogen receptor binding only if the estradiol concentration itself is appropriate (around 1 nM) and the competition excess is not too great (preferably 10 nM but no more than 100 nM for DES).

We have concluded from our study that, although immunocytochemical assays can be used to detect estradiol in breast cancer cells, the hormone specific fluorescence staining observed is not due to direct binding of antibody-estradiol-receptor, but rather must be due to the detection of estradiol bound to lower-affinity binding sites.

REFERENCES


Fig. 1. Immunofluorescence photomicrograph of a frozen section of MCF-7 cells. The cells were incubated at 4° for 4 hr in the presence of 10 nM estradiol as described in Table 3. The antiserum for this experiment and all of the following photomicrographs was antiestradiol antiserum which had been preadsorbed with BSA. x 325.

Fig. 2. Immunofluorescence photomicrograph of a frozen section of MCF-7 cells incubated with estradiol at 37°. The cells were first incubated with 10 nM estradiol at 4° for 4 hr followed by warming the cells in the presence of hormone to 37° for 30 min as described in Table 3. x 325.

Fig. 3. Immunofluorescence photomicrograph of a frozen section of MCF-7 cells incubated with DES and estradiol. The cells were first incubated with 5 nM DES at 37° for 1 hr, washed and cooled to 4°, and then incubated with 10 nM estradiol at 4° for 4 hr as described in Table 4. x 325.

Fig. 4. Immunofluorescence photomicrograph of a frozen section of MCF-7 cells incubated only with DES. The cells were incubated with 5 nM DES at 37° for 1 hr, washed and cooled to 4°, and then incubated with KRH glucose buffer at 4° for 4 hr as described in Table 4. x 325.
Failure of Estradiol Immunofluorescence in MCF-7 Breast Cancer Cells to Detect Estrogen Receptors


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