Immunoradiometric Studies with Monoclonal Antibody against a Component Related to Human Estrogen Receptor

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ABSTRACT

A human specific monoclonal antibody (D5) raised against a M, 36,000 cytosolic estrogen receptor component (RE) partially purified from human myometrium was used to develop a simple, rapid, and sensitive solid-phase immunoradiometric assay (IRMA) for the reactive antigen in tissue cytosols from breast tumors, myometrium, endometrium, and endometrial carcinomas. The IRMA did not detect antigen in RE-negative cytosols from human breast tumors and endometrial carcinomas. RE-positive cytosols from chick oviduct and calf and rat uteri failed to produce an IRMA response.

A pilot study indicated a significant correlation (P < 0.001) between D5 IRMA value and RE sites in breast tumors assayed by [3H]estradiol binding sites. The presence of D5 antigen was dependent on the presence of cytosolic RE but not progesterone receptor. RE-positive patients age 50 years and over demonstrated significantly higher D5 assay values than did patients under 50 years. The data suggest that the D5 antigen is a component of the estrogen receptor or coordinately regulated with the receptor in human cells and that the assay method may have clinical use.

INTRODUCTION

Prior to 1980, detection and quantitation of RE2 in breast tumors and other estrogen target tissues was dependent on indirect measurement of RE through association with bound radioactive estradiol. The development of RE antibodies (3, 8, 12, 18, 19) provided an immunochemical approach for directly detecting the protein moiety of hormone-receptor complexes.

Two monoclonal antibodies (D5 and C3) raised against affinity-purified RE from human myometrium have been partially characterized (5). The human-specific D5 antibody reacts with a M, 29,000 nonglycosylated protein in RE-positive but not RE-negative cytosols and was therefore of potential use in the development of an IRMA. This report describes the development and application of such an assay using 125I-D5 in studies to detect the reactive antigen in human breast tumors and other estrogen target tissues. It further describes a pilot study with human breast tumors comparing the IRMA results with RE measured by the standard, single-concentration, labeled estradiol assay which is used routinely in this laboratory for clinical samples; a highly significant, positive correlation between the two parameters was obtained. Additional data are presented for normal and malignant endometrium, myometrium, and a cultured human endometrial cell line.

MATERIALS AND METHODS

Chemicals. [2,4,6,7-3H]Estradiol-17β (91 Ci/mmol) and [1,2,6,7-3H]progesterone (99 Ci/mmol) were supplied by Amersham International (U. K.). BSA, ovalbumin, γ-globulin, and Tween 20 were obtained from Sigma Chemical Co. (St. Louis, MO).

Buffers. The buffer used in tissue preparation was 10 mM Tris-HCl-1 mM EDTA-2 mM dithiothreitol adjusted to pH 7.4 at 4°C. In some cases, 10 mM NaH2PO4-1.5 mM monothioglycerol containing either 10 or 30% glycerol adjusted to pH 7.4 (4°C) was used. Cytosol or antibody dilutions were made in PBS, pH 7.4.

Antibodies. Monoclonal antibodies D5 (IgG) and C3 (IgM) raised against partially purified (approximately 5% pure) cytosolic human myometrial receptor were prepared as described (5). 125I-Labeled D5 was prepared by the method of Hunter and Greenwood (9). Preparations of 125I-D5 obtained for assay development ranged in specific activity from 4 to 6 mCi/mg protein. Sheep polyclonal antibody G3 raised against myometrial RE was prepared as described (4).

Human Tissue Processing. Uterine tissues obtained at hysterectomy were placed immediately into a Dewar flask containing dry ice for transportation to the laboratory where they were stored at -70°C until required.

Breast tumors obtained at surgery were placed immediately into liquid nitrogen for transportation and storage. Any tissue surplus to receptor assay requirements was subsequently stored at -70°C. At no time prior to homogenization were tissues allowed to thaw. Cell cultures were maintained in Dulbecco’s modified Eagle’s medium containing 10 to 15% fetal calf serum and changed to serum-free medium for 2 days prior to harvesting. The HEC-1A human endometrial cell line was a gift from Dr. J. Fogh (Sloan Kettering Institute for Cancer Research) and contained no detectable RE.

RE and Other Steroid-labeled Complexes. Human breast tumors and myometrial cytosols were prepared as described (13). Complexes labeled with radioactive steroid binding were performed on cytosols labeled overnight in the presence or absence of diethylstilbestrol. Progesterone receptor assays were performed as described (13). Complexes labeled with radioactive estradiol were treated with an equal volume of DCC suspension in 10 mM Tris-1 mM EDTA buffer (pH 7.4 at 4°C) buffer, breast tumors in phosphate buffer (4°C). Some frozen human breast tumors were pulv erized prior to buffer extraction of the tissue powder. Human endometrial nuclear receptor was prepared as described (6). High-speed supernatant fractions were obtained after centrifugation at 200,000 x g for 60 min. In some cases, 5,000 x g supernatant fractions were used. Human endometrium and cultured cells were disrupted by hand with a Teflon-glass homogenizer, and a 200,000 x g supernatant fraction was obtained.

Steroid Ligand Binding Assays. Competitive assays for specific 3H-steroid binding were performed on cytosols labeled overnight in the presence or absence of a 500-fold excess of unlabeled competitor. For estrogen receptor, cytosols were labeled to 4 nm [3H]estradiol in the presence or absence of diethylstilbestrol. Progesterone receptor assays were performed as described (13). Complexes labeled with radioactive estradiol were treated with an equal volume of DCC suspension in 10 mM Tris-1 mM EDTA buffer (pH 7.4 at 4°C), [3H]estradiol-labeled samples were DCC-treated for 10 min (4°C), [3H]progesterone-labeled samples...
were DCC-treated for 5 min (4°). DCC mixtures were centrifuged at 1,000 x g for 5 min (4°).

**Protein Estimations.** A colorimetric method was used (17).

**IRMA for D5 Antigen.** Consequent to the experiments described in "Results," the following final method was adopted. Individual microtiter wells of 0.40 ml total volume (Dynatech Laboratories Ltd., Billingshurst, West Sussex) were coated with 200 μl of BSA in PBS (100 μg/ml) over 18 h at 4°. After removal of the contents by aspiration, wells in their holder were washed by immersion three times in PBS/0.05% Tween 20 (500 ml) and, after inversion, were tapped firmly onto a tissue pad to remove residual washing buffer. A maximum 25 μl of test samples, diluted to 200 μl in PBS, was normally applied to the coated microtiter wells, usually in triplicate, and incubation was continued for 1 h at 22°. Wells were aspirated and washed as described above before dispensing 125I-D5 (approximately 0.05 μg protein) in 200 μl PBS into each well and allowing incubation to proceed for 1 h at 22°. After washing as described previously, bound 125I-D5 in separated wells was quantitated by γ-counting.

**Histology.** Histological grade of ductal tumors was assessed according to the method of Bloom and Richardson (1).

**RESULTS**

Development of IRMA: Detection of D5-specific Antigen

The final protocol is detailed in "Materials and Methods." The following describes experiments which resulted in that method.

Treatment of Solid Phase

High specificity in IRMA can be achieved by coating the microtiter wells with one monoclonal antibody and then using a labeled second monoclonal antibody which recognizes a different epitope on the antigen (20). This approach worked, in that C3 (IgM) coating of wells enhanced antigen uptake from breast tumor cytosols as compared with uncoupled wells (Chart 1A); polyclonal RE antibody GI/3 gave a similar result to C3 alone, whereas D5 coating inhibited the subsequent binding of 125I-D5 as expected (Chart 1A). In the course of control experiments to establish the best conditions for antigen interaction with plastic wells, the surprising result was obtained that BSA coating of the plate also enhanced antigen recognition by 125I-D5 (Chart 1A). This effect was specific in that bovine γ-globulin, ovalbumin, or rabbit serum were ineffective, and it was only seen with RE-positive cytosols (Chart 1B). The addition of BSA to RE-positive cytosol prior to the incubation of cytosol with BSA-coated microtiter wells had no effect on subsequent addition of 125I-D5, which suggests that direct interaction between BSA and D5 antigen does not occur. Experiments with 125I-labeled myometrial cytosol proteins indicated that BSA was not enhancing general protein uptake to the plastic surface (data not shown). As a result of these experiments, all subsequent experiments used microtiter wells that had been coated with PBS (100 μg BSA/ml).

**Cytosol Incubation Step**

RE-positive and RE-negative breast tumor cytosols were incubated in BSA-coated wells either at 4° or 22° for different times before the 125I-D5 step. Responses observed after 18 h at 4° were very similar to those obtained over 1 h or 18 h at 22°; the shorter incubation at 22° was adopted routinely.

![Chart 1. Effect of different coating treatments prior to cytosol incubation on assay response with 125I-D5. In A, aliquots (25 μl) of RE-positive breast tumor cytosol in 200 μl of PBS were incubated (1 h at 22°) in microtiter wells previously coated with the reagent indicated over 18 h at 4° ("Materials and Methods"). Data indicated 125I-D5 bound to wells after addition of 0.04 μg 125I-D5 for 1 h at 22°. In B, aliquots of RE-positive or RE-negative breast tumor cytosol were incubated for 1 h at 22° in microtiter wells pretreated as follows: Lane a, uncoated; coated with BSA at (Lane b) 5000 μg/ml, (Lane c) 500 μg/ml, (Lane d) 250 μg/ml, or (Lane e) 100 μg/ml; or coated with (Lane f) ovalbumin (5000 μg/ml), (Lane g) bovine γ-globulin (5000 μg/ml PBS), or (Lane h) non-immune rabbit serum (1:10 dilution in PBS). After cytosol incubations, antigen bound to wells was detected using 0.05 μg 125I-D5 as well as 125I-D5 in separated wells was quantitated by γ-counting.](chart1)

**Effect of Cytosol Protein Concentration**

Cytosol containing a constant amount of RE but different protein content was incubated in BSA-coated wells for 1 h at 22°. After PBS/Tween washing, microtiter wells were incubated with 125I-D5 for a further 1 h at 22° and bound 125I-D5 was counted.

**Antigen Detection with 125I-D5**

D5 Concentration. Aliquots from RE-positive breast tumor cytosol (77 fmol/ml) mixed with increasing amounts of protein from an RE-negative tumor cytosol which had previously demonstrated negative IRMA response. Constant results were obtained up to an input of 175 μg protein/well, but additional protein reduced the assay response (Table 1). These results are repeatable.

**Effect of Cytosol Protein Concentration**

Assay response was studied using a constant amount of RE-positive breast tumor cytosol (77 fmol/ml) mixed with increasing amounts of protein from an RE-negative tumor cytosol which had previously demonstrated negative IRMA response. Constant results were obtained up to an input of 175 μg protein/well, but additional protein reduced the assay response (Table 1). These results are repeatable.

**Antigen Detection with 125I-D5**

D5 Concentration. Aliquots from RE-positive breast tumor cytosol (698 fmol/ml) and dilutions from it were assayed with different amounts of antibody. A concentration of 0.05 μg 125I-D5/well combined optimal assay response with economic use of antibody (Chart 2) although, in later work, this figure varied slightly depending on the specific activity of the antibody.

**D5 Incubation Step.** The incubation times and temperatures...
IRMA DETECTION OF Estradiol Receptor Component

Optimization of $^{125}$I-D5 concentration. One, 5, or 25 μl of breast tumor cytosol (898 fmol/ml) diluted to 200 μl in PBS were applied to BSA-coated (100 μg/ml) microtiter wells and incubated 1 h at 22°C. After washing, wells were further incubated with (A) 0.01 μg, (C) 0.05 μg, or (B) 0.10 μg $^{125}$I-D5 for 1 h at 22°C, washed, and counted.

Chart 2. Optimization of $^{125}$I-D5 concentration. One, 5, or 25 μl of breast tumor cytosol (898 fmol/ml) diluted to 200 μl in PBS were applied to BSA-coated (100 μg/ml) microtiter wells and incubated 1 h at 22°C. After washing, wells were further incubated with (A) 0.01 μg, (C) 0.05 μg, or (B) 0.10 μg $^{125}$I-D5 for 1 h at 22°C, washed, and counted.

Table 2

<table>
<thead>
<tr>
<th>Incubation</th>
<th>Assay response $^{125}$I-D5, bound cpm</th>
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<tbody>
<tr>
<td>Time (h)</td>
<td>Temperature</td>
</tr>
<tr>
<td>1</td>
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</tr>
<tr>
<td>4</td>
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</tr>
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<td>4</td>
<td>22°C</td>
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<tr>
<td>4</td>
<td>37°C</td>
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a Cytosol (25 μl + 175 μl PBS) was applied to microtiter wells (triplicates) and incubated at 22°C before the D5 incubation step.

were studied using breast tumor cytosol (Table 2). These results were repeatable. For convenience and binding characteristics, 1 h at 22°C was chosen for routine purposes.

Specificity of IRMA Response

RE-rich samples (>20 fmol/mg protein) always gave a positive assay response (Chart 3), with the exception of nuclear RE from human endometrium, which was negative. While control samples (PBS, serum, pleural effusion fluid) gave background counts, low IRMA values were obtained with all RE-poor (<5 fmol/mg) cytosols, including those from breast tumors and endometrial carcinomas, where the IRMA values obtained probably reflected the limitations on sensitivity of the $[^3H]$estradiol ligand binding assays. These data are in contrast to RE-positive cytosols from breast tumors, myometria, and endometria, where high IRMA responses were observed (Chart 3). Only background IRMA counts were obtained when samples of RE-rich cytosols from chick oviduct, cat uteri, or immature and mature rat uteri were analyzed.

Preparation of Breast Tumor Cytosol

Studies reported above were performed with cytosols obtained by ultracentrifugation (200,000 × g) of Polytron-prepared tissue homogenates. Breast tumor cytosols prepared from pulverized frozen tissues (“Materials and Methods”) and centrifuged for 15 min either at 5,000 × g or 200,000 × g for 1 h gave identical IRMA responses. Inclusion of molybdate plus leupeptin in the buffers did not affect the IRMA results.
5). There was a highly significant positive correlation between IRMA response and unoccupied RE sites ($r = 0.758$, $P < 0.001$, $n = 41$). Tumors from both young (<50 years) and old (>50 years) fitted onto this regression line.

**Correlation with Progesterone Receptor.** No correlation was observed between assay response and cytosolic progesterone receptor content ($r = 0.009$, $n = 40$; Chart 6). Comparison of receptor phenotype with IRMA value confirmed that assay activity was related to RE but not to progesterone receptor values (Chart 7).

**RE Status, Patient Age, and Histology.** Data summarized in Table 3 indicate that breast tumor cytosols containing RE levels $>$20 fmol/mg protein registered a significantly higher IRMA value than those cytosols with $<$10 fmol RE/mg protein ($x^2$ test, $P < 0.001$). Patients age 50 years and over demonstrated a significantly higher IRMA value than patients under 50 years ($x^2$ test, $P < 0.001$), although insufficient data was available to confirm whether this observation was related to age or menopausal status. Although there was a tendency for the better-differentiated grade 2 to have a higher proportion of IRMA positive samples than the grade 3 tumors, this difference did not reach statistical significance. Too few lobular tumors have been analyzed for comment as to potential differences with the ductal tumors.

**DISCUSSION**

D5 was raised against a proteolytically degraded, estradiol receptor protein from human myometrium that had been partially purified by affinity chromatography. The antibody was detected by its ability to precipitate $^{125}$I-estradiol bound to receptor in a double antibody precipitation assay. These and other data indicate that D5 can, under certain conditions, react with the estradiol binding subunit of the soluble but not nuclear receptor (5). Paradoxically, D5 will not react with RE on sucrose gradients, and the D5 antigen can be separated from RE by isoelectric focusing and SDS-acrylamide gel electrophoresis (5). The latter technique indicated the absence of the antigen in RE-negative cells which complemented the $^{125}$I-estradiol immunoprecipitation data (5). The exact nature of the D5 antigen is the subject of further investigation but, at present, the data are best explained by a model in which the D5 antigen is a non-hormone binding component of the estradiol- but not other steroid-binding recep-
IRMA DETECTION OF ESTRADIOL RECEPTOR COMPONENT

Our histochemical data would support that conclusion, as do the results presented in this paper. A positive assay (˃3000 cpm 125I-D5 bound) has never been obtained with RE-poor cytosols (<30 fmol/ml; <15 fmol/mg protein) from either breast or endometrial tumors; serum and pleural effusion liquid were also negative. Furthermore, a highly significant quantitative relationship exists between the IRMA result and the conventional estradiol-binding assay used for routine clinical purposes in this laboratory. This relationship is also reflected in the data on patient age and possibly tumor grade, both parameters that are known to influence cytosolic RE levels (2, 13). Interestingly, the quantity of D5 antigen is not related to progesterone receptor, which would suggest that the antigen is not simply a product of estrogenic stimulation. At present, we have no data on the hormonal regulation of the D5 antigen.

As we have not fully defined our antigen, its relationship to those recognized by the other monoclonal antibodies raised against steroid-receptor components remains in doubt. It is clearly different from the estradiol-binding unit recognized by the antibodies of the Chicago group (11, 14), and both the size and receptor and tissue specificity of our antigen would seem to distinguish it from the units which do not bind hormone reported for chick oviduct progesterone receptor (7, 10).

The solid-phase IRMA described here is both convenient and quick; it can also easily be adapted to an enzyme-linked assay (data not presented). The quantitative relationship with breast tumor RE indicates that the assay may have clinical use, although this application will require more thorough testing than has been described here; such studies are in progress in collaboration with Amersham International.

REFERENCES


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