Monoclonal Antibodies against a Component Related to Soluble Estrogen Receptor

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ABSTRACT

Mice were immunized with 1 to 3 µg of cytoplasmic estrogen receptor fragment purified from human myometrium by affinity chromatography. Two RE-antibody-secreting clones were detected from one fusion that were capable of precipitating cytosol RE. Monoclonal antibody D5 (subclass IgG1) reacts with an antigen that is related to RE from immunoprecipitation studies but which can be separated from the hormone binding unit. In the presence of anti-mouse serum, D5 precipitates labeled human cytoplasmic RE complexes from breast tumor, fibroid, myometrial, and endometrial preparations but does not react with nuclear RE from human endometrium or cytoplasmic RE from other species tested. Conversely, antibody C3 (Class IgM) precipitates human cytoplasmic RE and nuclear RE complexes as well as labeled cytoplasmic RE from rat and calf uteri and chick oviduct. Neither antibody reacts with progesterone receptor or androgen receptor from human breast tumor, SHBG from human plasma, or rat α-fetoprotein. With D5, steroid labeling of cytoplasmic RE at 25°C increased the RE immune complex precipitated. D5 precipitates molybdate-stabilized RE from myometrial cytosol when labeled at 25°C but not at 4°C. C5 precipitates molybdate-stabilized RE whether cytosol was steroid-labeled at 4°C or 25°C. For D5, optimal precipitation of RE from human breast tumor was observed when cytosol was steroid-labeled at 25°C in buffers of pH range 5 to 6. Immunochromatographic studies indicate that D5 is associated with a Mr 29,000 component in RE-positive cytosols. Electrofocusing and sucrose density gradient analysis confirmed that D5 antigen is a non-hormone-binding component related to cytosolic RE from breast tumor and myometrium.

INTRODUCTION

The possibility that antibodies directed against receptor proteins might provide an alternative immunocytochemical approach to direct detection of the receptor moiety of steroid-hormone receptor complexes or unliganded receptors prompted several reports which described the production of polyclonal antibodies to receptor proteins (3, 9, 10, 15, 20, 24, 26).

Introduction of a high degree of antigen specificity and antibody homogeneity has been described more recently in publications which present the application of hybridoma technology (18) to the production of monoclonal antibodies raised against RE2 from calf uteri (11) and a human breast cancer cell line (12). Specific monoclonal RE antibodies may provide a basis for direct RE immunocytochemistry in human breast tumors, obviating the necessity of existing steroid ligand-binding procedures while allowing tissue and cellular RE localization to be performed immunocytochemically.

This paper describes the production of monoclonal antibodies to a partially purified RE preparation from myometrium, a normal human tissue which contains cytoplasmic RE. The individual nature of each antibody is reflected by major differences in specificity observed between antibodies produced by hybridomas from the same fusion.

MATERIALS AND METHODS

Chemicals. [2,4,6,7-3H]estradiol-17β (91 Ci/mmol), [16α(125I)]iodoestradiol (1500 Ci/mmol), [1,2,6,7-3H]progesterone (99 Ci/mmol), and 125I-labeled 05 were supplied by Amersham International (U. K.). DEAE Sephaloc and Sepharose-protein A were obtained from Pharmacia Ltd. (Great Britain). A cross-linked immunoadfinity matrix (a gift from Mr. D. R. Sutherland, I. C. R. F.) was prepared as described previously (27) by coupling 10 mg of an affinity purified rabbit anti-mouse Ig to 1 ml of protein A-Sepharose 4B. A control mouse monoclonal antibody, anti-HAL-DR (subclass IgG1), was a gift from Mrs. H. Durbin, I. C. R. F. Ampholines (pH range, 5 to 6) were obtained from KLB Produkter AB, Stockholm, Sweden. Tween 20, bovine serum albumin, ovalbumin, bovine γ-globulin, and human hemoglobin were supplied by the Sigma Chemical Company Limited.

Buffers. The buffer used in tissue preparation was TED buffer. In the antibody precipitation reactions, the buffer used was PBS/10 mM EDTA adjusted to pH 8.0 with 1 N NaOH (PBS/EDTA).

Culture Conditions. Hybridoma cell culture lines were grown in RPMI (Grand Island Biological Co.), 20% fetal calf serum (Flow Laboratories) containing penicillin (100 units/ml) (Sigma), streptomycin (100 µg/ml) (Sigma); selective growth medium also contained hypoxanthine (13.611 mg/liter), amphotericin (0.176 mg/liter), and thymidine (3.876 mg/liter) (Flow).

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. Samples of breast tumor obtained at surgery were placed immediately into liquid nitrogen and transported to the laboratory where they were stored in a liquid nitrogen refrigerator until the steroid receptor status of the patient was determined. 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.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: RE, estrogen receptor; SHBG, sex steroid binding globulin; DCC, dextran-coated charcoal; PBS, phosphate-buffered saline (153 mM NaCl-3 mM KCl-10 mM NaH2PO4-2 mM KH2PO4, pH 7.4; TED buffer, Tris-EDTA-dithiothreitol buffer (10 mM Tris-HCl-l mM EDTA-1 mM dithiothreitol adjusted to pH 7.4 at 4°C); SDS, sodium dodecyl sulfate.
The MCF-7 and CAMA-1 cells were the stock lines maintained in this institute, and they contained 135 and 58 fmol cytosol RE/mg protein, respectively. 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 tumor, myometrial, and endometrial cytosols and rat uterine and calf uterine cytosols were prepared by tissue homogenization (Polytron, 2 x 20S passes) in 5 volumes of tissue buffer (4°C). Cultured cells were disrupted by hand with a Teflon-glass homogenizer. Rat uterine and human endometrial nuclear RE was prepared as described (6). High-speed supernatant fractions (200,000 × g for 60 min) were labeled with [3H]-steroids over 1.5 h or overnight (4°C) to 5 or 10 nM. Where [3H]-estradiol was used, labeling was performed at 1 nM. In some cases, labeling of RE preparations was performed at 25°C for 1 h, followed by further incubation at 4°C for 1 h.

Cytosols treated with potassium thiocyanate (KCNS) were labeled with [125I]-estradiol for 1 h 4°C prior to addition of KCNS from a 2.5 M stock in TED buffer (adjusted to pH 7.4) and further incubated for 16 h (4°C).

Enriched receptor preparations were obtained after treatment of cytosol with solid ammonium sulfate to 25% saturation. The mixture was centrifuged at 10,000 × g for 20 min and, after reconstitution of pellets in 1/100 volume of PBS/2 mM dithiothreitol (pH 7.4), the solution was centrifuged at 200,000 × g for 30 min. Female human pregnancy plasma containing SHBG was diluted 1/10 with TED buffer prior to labeling overnight with [3H]-estradiol (4°C).

Complexes labeled with radioactive estradiol were treated with a pellet obtained from the same volume of DCC suspension (0.25% w/v) Norit A charcoal in Tris-EDTA buffer containing 0.0025% (w/v) dextran T70 adjusted to pH 7.4, 4°C]. [3H]Progesterone labeled samples were DCC-treated for 5 min (4°C).

Steroid Ligand Binding Assays. Competitive assays for specific [H]- or [3H]-steroid binding were performed on cytosols or enriched fractions obtained after ammonium sulfate fractionation and labeled overnight in the presence or absence of a 500-fold excess of unlabelled competitor, followed by DCC treatment as described above.

**Antigen Isolation.** RE preparations used for immunizations were purified by affinity chromatography from myometrium as described previously (4) using an estradiol hemisuccinate matrix; some preparations were further purified by an electofocusing step.

**Immunization.** Male BALB/c mice (2 months old) were immunized with a total of 5 injections of partially purified estrogen receptor each of between 1 and 3 μg on Day 1, the receptor was suspended in complete Freund’s adjuvant and injected into the xiphistemum. On Days 3 and 7, the antigen was suspended in complete Freund’s adjuvant and injected subcutaneously into the skin of the back. On Day 30, the receptor suspended in Freund’s incomplete adjuvant was injected intraperitoneally. The fusion was performed 4 days after the final immunization.

**Cell Fusion.** Four days after the final immunization, the mouse spleen was removed under sterile conditions. Fusion of the spleen cells with mouse myeloma cells (P3-X63-Ag. 653; Flow Laboratories Ltd.) was achieved using polyethylene glycol 6000 (BDH) according to the procedures of Hogg et al. (13). Fused cells were plated on eight 24-well tissue culture dishes and grown in selective medium containing hypoxanthine-aminopterin-thymidine medium. Wells that contained cell clusters were assayed for RE antibody by the double antibody precipitation technique described below.

**Cloning and Expansion of Hybridomas.** Monoclonal hybridoma cell lines were obtained by limiting dilution and then by plating out at one cell per well density using mouse peritoneal macrophages as feeder cells. Cloned cells were then expanded in suspension culture ultimately without the aid of feeder cells. Cells from positive clones were also injected i.p. into pristane-primed BALB/c mice (10° cells/mouse). After 8 to 12 days, ascites fluid was collected from the peritoneal cavity.

**Detection of RE-Immune Complexes: Double Antibody Precipitation Assay.** Medium from hybridoma culture wells (100 μl) or ascites fluid (25 to 50 μl) was incubated with enriched myometrial RE (20 fmol) labeled with [125I]-estradiol at 4°C for 1.5 h at a final volume of 0.50 ml PBS/EDTA buffer (pH 8.0). After addition of 8 μl normal mouse serum to incubations followed by sufficient sheep anti-mouse serum to precipitate mouse immunoglobulins, the mixture was further incubated for 2 h (4°C) before centrifugation at 5000 × g (15 min, 4°C). Overnight incubation of labeled RE with antibody from culture medium and ascites or overnight incubation with precipitating antisera did not improve the yield of RE immune complex precipitated. [125I]-radioactivity in pellets was counted using a gamma counter. Where samples were labeled with [3H]-steroids, the pellets were dissolved in 0.2 n NaOH (100 μl) and neutralized with 1 n HCl (20 μl) before counting.

**Purification of Monoclonal Antibodies.** Antibody D5 (IgG) from culture medium: IgG was isolated from culture medium initially by a DEAE-Sepharacel ion-exchange chromatography step and then applied to a column of Sepharose CL-4B Protein A. Absorbed IgG was eluted with 0.1 M citrate buffer (pH 6.0) containing 0.02% sodium azide. Fractions containing IgG were adjusted to pH 8.0 with crystals of Tris base.

**Immunofluorescence Assay (IF) for Specificity.** Microscopic examination was performed on 4% paraformaldehyde fixed biopsy fragments or ascites fluid. The antibodies were conjugated with fluorescein isothiocyanate (FITC) and examined using a fluorescence microscope.

**Immunoradiometric Assay.** A solid-phase assay using [125I]-estradiol was performed as described (5) in microtiter wells.

**Immunofluorescence Microscopy.** Immunofluorescence microscopy was performed on formalin-fixed, paraffin-embedded tissue sections using indirect immunofluorescence techniques. Sections were incubated with primary antibody followed by fluorescently conjugated secondary antibody.

**Immunohistochemistry.** Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue sections using a standard avidin-biotin immunoperoxidase technique. Sections were incubated with primary antibody followed by biotinylated secondary antibody followed by avidin-biotin-peroxidase complex and developed with 3,3'-diaminobenzidine as substrate.
Protein Estimations. A colorimetric method was used (21). Ouchterlony Immunodiffusion. The class and subclass of the monoclonal immunoglobulins produced were determined by Ouchterlony analysis (23).

RESULTS
Detection of Antibody-producing Clones
From the successful fusion, 192 culture wells were screened for antibody activity, of which 15 produced an assay result of >30% above background, and these were chosen for subculture. Background levels of precipitation were the same regardless of the type of control medium used. A selection of results as screening proceeded is given in Table 1, which shows improvement in antibody activity produced by lines C3 and D5, while line G2, which initially improved, failed to continue producing antibody activity.

Purification of Monoclonal Antibodies
D5 (IgG1 subclass) was purified from hybridoma culture medium using DEAE-ion exchange chromatography followed by a Sepharose-protein A chromatography step ("Materials and Methods"). However, with C3 (class IgM), activity was lost when culture medium or ascites fluid was subjected to ion-exchange chromatography. This problem was overcome by direct immunoaffinity chromatography of ascites fluid on a Sepharose-protein A coupled rabbit anti-mouse matrix (27), which gave high purification of C3 (data not shown).

Properties of Monoclonal Antibodies: Immunoprecipitation of Steroid-labeled RE Complexes
Reactivity of Human Cytosolic RE from Myometrium
Preliminary tests were performed on an ammonium sulfate fraction of cytosol RE. The following conditions affected immunoprecipitation:

Temperature. RE was more reactive with D5 when labeled at 25° than 4° (Chart 1), whereas reactivity with C3 was only slightly enhanced at the higher receptor labeling temperature (data not shown). RE labeling temperature effected little change in background levels precipitated with anti-HLA-DR control antibody (Chart 1).

Cytosol Steroid Labeling Conditions. While D5 did not react significantly with cytosolic RE labeled at 4° in low ionic strength buffer, addition of 0.4 M KCl or 0.5 M KClCN during labeling ("Materials and Methods") and prior to incubation with antibody potentiated the reactions (Table 2). In the absence of salt, steroid labeling of cytosol at 25° in low ionic strength buffer was effective in producing precipitable immune-complexed RE (Table 2). Cytosol containing molybdate-stabilized RE reacted with C3 but not with D5. An ammonium sulfate cytosol fraction containing molybdate-stabilized RE reacted with D5 at 25° but not at 4°. Overnight dialysis of the molybdate preparation failed to induce immune-complex formation with D5 but, after steroid labeling of the molybdate-stabilized RE fraction at 25°, precipitation of RE-D5 immune-complex was obtained (Table 3).

Antibody Dissociation Constant (Kd). Scatchard plots for both antibodies were obtained using variable amounts of an

| Table 1
<table>
<thead>
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<th>Antibody screening data</th>
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<td>Immunoprecipitation assays on culture medium were performed as described in &quot;Materials and Methods.&quot; The assay input of 3H-estradiol-labeled RE was 60,000 cpm.</td>
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</table>

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Temperature</th>
<th>RE-antibody complex formed*</th>
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<tr>
<td>Cytosol</td>
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<td>-</td>
</tr>
<tr>
<td>TED</td>
<td>4°</td>
<td>+</td>
</tr>
<tr>
<td>TED + 25 mM Na3MoO4</td>
<td>4°</td>
<td>+</td>
</tr>
<tr>
<td>TED + 400 mM KCl</td>
<td>4°</td>
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<td>25°</td>
<td>+</td>
</tr>
<tr>
<td>TED</td>
<td>4°</td>
<td>-</td>
</tr>
<tr>
<td>TED + 400 mM KCl</td>
<td>25°</td>
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* Data obtained from immunoprecipitation assays.

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ammonium sulfate fraction of labeled cytoplasmic myometrial RE as ligand at constant antibody concentration. D5 and C3 bound labeled RE with a \( K_d \) of \( 1 \times 10^{-9} \text{ M} \) and \( 2 \times 10^{-11} \text{ M} \), respectively (Chart 2). Similar results were obtained when RE steroid labeling was completed in the presence or absence of the antibodies.

Chart 2. Scatchard analysis of RE monoclonal antibody interaction. In A, aliquots of ammonium sulfate-fractionated myometrial RE labeled at 25° with 2 nM \(^{125}\text{I}-\text{estradiol and treated with DCC were incubated with antibody D5 (1.3 \mu g) for 1.5 h (4°). RE-antibody complexes were precipitated after addition of non-immune carrier serum followed by sheep anti-mouse serum (2 h, 4°). In B, aliquots of DCC-treated RE labeled at 4° were incubated with C3 (2.0 \mu g) and treated as described for A.}

Chart 3. Immunoprecipitation of cytoplasmic RE from human breast tumor. Data shows immunocomplexed RE precipitated when increasing amounts of an ammonium sulfate fraction from human breast tumor cytosol labeled with \(^{125}\text{I}-\text{estradiol at 25° and treated with DCC were incubated with D5 (\bigcirc) or a monoclonal antibody against HLA-DR (\bigcirc) at 4° for 1.5 h prior to addition of precipitating antiserum.}

Chart 4. Interaction of monoclonal antibody with RE in breast tumor cytosol. A, cytosol was steroid-labeled to 1 \mu g (4°) in TED buffer; B, labeling was performed at 4° in TED buffer containing 0.4 M KCl (pH 7.4). C, cytosol was steroid-labeled at 25° in TED buffer while, in D, labeling was performed in TED buffer containing 0.4 M KCl. Aliquots of labeled DCC-treated cytosol were incubated with D5 (13 \mu g protein) (4°) or the same amount of protein from a control anti-HLA-DR antibody (C) for 1.5 h prior to precipitation of RE immune complex as described.
(data not shown). Also, incubation of myometrial RE with a mixture of both antibodies produced an additive effect on precipitation of RE-immune complex in excess of levels observed when only one antibody was used.

**Reaction with Cytoplasmic Breast Tumor RE**

Under conditions of antibody excess, D5 formed immune complexes with cytoplasmic RE from human breast tumors prepared after ammonium sulfate fractionation (Chart 3). C3 exhibited similar properties (data not shown). However, with cytosol RE prepared in high-salt (0.4 M KCl) buffer or TED buffer alone, antibody D5 only complexed low amounts of RE steroid-labeled at 4°C (pH 7.4) (Chart 4, A and B). Further investigations of RE steroid-labeling environment indicated that optimal reaction with D5 occurred when the receptor was steroid-labeled at pH 5 to 6 for 1 h at 25°C in high- or low-salt buffer (Chart 4, C and D) prior to antibody incubation. C3 reacted with breast tumor cytosol RE whether labeled at 25°C or 4°C (data not shown). Additional evidence on formation of soluble RE-immune complexes was obtained after ['H]estradiol-labeled breast tumor RE was incubated with either D5 or control antibody prior to batch incubation with protein A-Sepharose. Only steroid-labeled RE which had been preincubated with D5 was bound to the protein A-Sepharose to a significant extent (Chart 5).

**Specificity**

Enriched cytoplasmic RE preparations were obtained after ammonium sulfate fractionation of cytosols obtained from different species and tissues ("Materials and Methods"). D5 precipitated human cytoplasmic RE but failed to react with other species tested (Table 3). Human endometrial nuclear RE was not precipitated by D5 whether steroid-labeled at 25°C or 4°C. C3 interacts across species with all forms of labeled RE tested (Table 3). Neither antibody formed complexes with SHBG, human breast tumor progesterone receptor or androgen receptor, rat α-fetoprotein, ['H]estradiol alone, or a human breast tumor preparation which was negative for RE by the ['H]estradiol binding assay.

**Characterization of D5 Antigen: Relationship to RE Hormone-binding Unit**

**Identification of Immunoreactive Antigens by Immunoblotting**

Proteins from three breast tumor cytosol preparations were separated by SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose paper ("Materials and Methods"). After incubation with ['H]estradiol-labeled-D5, the blot demonstrated a single strongly reacting ([M, 29,000 ± 1500 (SE)] component in the two RE-positive samples (Fig. 1a, Tracks B, and C) that was barely detectable in an RE-negative cytosol (Fig. 1a, Track A). This M, 29,000 component was also detected in cytosols from two RE-positive mammary tumor cell lines (Fig. 1b, Tracks A and B) and RE-positive endometrium (Track D) but not in RE-positive nuclear extract from human endometrium (Tracks E and F) or cytosol from RE-negative human endometrial carcinoma cell line HEC-1A (Track C). The MCF-7 cytosol also contained a lower-molecular weight component (Track A) which has occasionally been detected in myometrium but not breast tumor.

**Table 3**

*Interaction of monoclonal antibodies with RE preparations*

Prepared after ammonium sulfate fractionation.

<table>
<thead>
<tr>
<th>Antibody preparation</th>
<th>Breast tumor (cytoplasmic)</th>
<th>Cytoplasmic</th>
<th>Molybdate-stabilized</th>
<th>Endometrium (nuclear)</th>
<th>Fibroid (cytoplasmic)</th>
<th>Rat (Uterus; cytoplasmic)</th>
<th>Cell (Uterus; cytoplasmic)</th>
<th>Chick (Oviduct; cytoplasmic: 4°)</th>
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<tbody>
<tr>
<td>D5</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>C3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>ND</td>
<td>–</td>
</tr>
</tbody>
</table>

* RE species.

* Steroid labeling temperature.

* ND, not done.

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ANALYSIS OF ESTROGEN RECEPTOR-RELATED PROTEIN

Sucrose Gradient and Electrofocusing Studies

We have been unable to demonstrate immune complex formation by shifts on sucrose gradients or on Ultrogel AcA 22 (data not shown) using either antibody with a range of cytoplasmic RE preparation and under a variety of experimental conditions, including those described above, which produce optimal precipitation of RE antibody complexes. However, analysis of steroid-labeled myometrial cytosol fractions obtained after ultracentrifugation, by immunoradiometric assay, indicated the presence of two broad peaks of D5-reactive material sedimenting at approximately 3.3S and 11S (Chart 6, A to C). The sedimentation characteristics of these components were not affected by either salt or molybdate. Neither component coincided with specifically bound 125I-estradiol, although some overlap was observed. In a separate experiment, reaction of cytosol incubation with 125I-D5 prior to sucrose gradient analysis indicated a quantitative shift of the immunoglobulin from 7S to 9S (Chart 6D). The asymmetry of the 9S peak is compatible with the view that 125I-D5 has reacted with both the 3.3S and 11S forms of the antigen.

Much clearer separation of estradiol binding and D5-reactive material was obtained by isoelectric focusing (Chart 7). The pi of the D5-reactive component was 6.60 as compared to 6.40 for the labeled RE complex from breast tumor cytosol. Separation of the two components was also obtained by high-pressure liquid chromatography (data not shown).

DISCUSSION

In this study, monoclonal antibodies against an RE-related component were derived from a mouse immunized with small amounts (1 to 3μg) of an RE preparation of low purity (5% pure) from a normal human tissue. Previously reported quantities of purified RE used for immunization were, for polyclonal antibodies, 0.04 μg (24), 20 μg (3, 10), or 60 to 150 μg (8) and, for monoclonal RE antibodies, 36 to 65 μg (11), 150 μg (12), and 10 μg per injection (22). Hybridomas obtained by fusion of splenic lymphocytes from a second mouse or from two other mice immunized with RE of higher purity (40%) obtained an additional preparative electrofocusing step, failed to produce detectable levels of antibody after primary screening of clones. Throughout the early stages of this work, the antibodies were detected by their apparent ability to bind the radiolabeled steroid receptor complex.

Our studies with RE from human myometrium emphasized that preparation of the RE sample and the conditions of RE steroid labeling used before antibody incubation dictated the degree of RE-antibody interaction obtained with one of the antibodies (D5). The yield of RE-D5 complex precipitated by antiserum was increased if RE components were either fractionated by ammonium sulfate, labeled at 25° rather than at 4°, or labeled in the presence of 0.4 M KCl or 0.5 M KCNS. Cytoplasmic RE from human breast tumors reacted with D5 in a similar fashion to myometrial RE, except that optimal precipitation occurred after 125I-estradiol labeling at lower pHs. Molybdate inhibited RE precipitation by D5 but not C3; C3 also differed from D5 in that variation in steroid-labeling conditions had little effect on the yield of RE-C3 complexes precipitated.

The immunoprecipitation studies indicated that D5 would only react with cytosol RE from human tissues and that nuclear RE was not recognized; C3 recognized all types of RE, regardless of species. Neither antibody would precipitate other steroid binding proteins (androgen receptor, progesterone receptor, SHBG, and α-fetoprotein).

All of these data indicate that D5 recognizes an epitope either on “activated” cytosol but not nuclear RE or on an antigen that, under activating conditions, itself complexes with cytosol RE. These alternatives also apply to the protein A results. At present, our results are more compatible with the second explanation. This conclusion is based on the experiments in which the antigen was detected with 125I-D5. A separation of D5 antigen and 125I-estradiol...
Chart 6. Detection of D5-reactive components in cytosols from human myometrium. In A and C, cytosol was prepared in Tris-EDTA-dithiothreitol buffer containing 25 mmo sodium molybdate. In B, cytosol was prepared in Tris-EDTA-dithiothreitol buffer only. Aliquots (200 µl) from [3H]-estradiol-labeled cytosols (A to C only: diethylstilbestrol absent, •; diethylstilbestrol present, O) were DCC-treated and applied to: A, a low-salt sucrose gradient containing 25 mmo sodium molybdate; B, a low-salt gradient (molybdate-free) and C, a high-salt gradient containing 25 mmo sodium molybdate. Unlabeled molybdate cytosol (200 µl) was incubated with 0.5 µg [3H]-D5 (•) for 5 h (4°C) and layered onto a high-salt sucrose gradient (D) containing 25 mmo molybdate. [3H]-D5 alone (0.5 µg + 1 mg bovine serum albumin in 200 µl of molybdate Tris-EDTA-dithiothreitol buffer) was added to a second gradient (C). Ovalbumin (3.7S) and γ-globulin (7.0S) sedimentation markers were applied to a separate gradient. Samples were centrifuged for 17 h (4°C) as described ("Materials and Methods"). After fractionation of gradients γ-counting, 250 µl of PBS were mixed with each fraction of the non-diethylstilbestrol competed gradients A to C, and 200-µl aliquots were applied to microtiter wells for immunoradiometric assay (A).

estradiol binding was obtained by sucrose gradient analysis, isoelectric focusing, or high-pressure liquid chromatography. Furthermore, the molecular weight in SDS gels is 29,000, as compared to the 65,000 to 70,000 usually reported for the estradiol binding subunit (16, 25). The M, 29,000 antigen does not appear to contain carbohydrate residues.

Our results indicate that the M, 29,000 antigen is human-specific antigen and both qualitatively and quantitatively closely related to RE content (5). Thus far, it has only been detected in RE-positive tissues or cells, although it has a cytoplasmic location as compared to the nuclear locus of the estradiol binding unit (17).

The physiological relevance of the M, 29,000 protein remains to be elucidated, but it is clearly related to the estradiol receptor in a specific manner. Two other examples have been described of monoclonal antibodies raised against steroid receptor-containing preparations which react with proteins that do not bind steroid. Our antibody differs from these in some important respects. The antibody raised against chick oviduct progesterone receptor recognizes a M, 90,000 protein that is common to

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androgen, estrogen, glucocorticoid, and progesterone receptor (14), whereas our M, 29,000 antigen is only related to estrogen receptor. Furthermore, it has been suggested that the M, 90,000 protein has no connection with the progesterone receptor (1). The monoclonal antibody against highly purified B subunit of the chick oviduct progesterone receptor recognizes a M, 108,000 protein that has many similarities to the immunogen, except that it does not bind hormone (7). This antibody will, however, react with progesterone receptor on sucrose gradients, which again distinguishes it from our own antibody. Elucidation of the function of the M, 29,000 protein must await its further characterization; these studies are in progress.

REFERENCES


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