Antibodies to estrogen receptor: Immunochemical similarity of estrophilin from various mammalian species

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ABSTRACT Immunoglobulin obtained from the serum of rabbits immunized with a highly purified preparation of estradiol–receptor complex from calf uterine nuclei has been shown to contain specific antibodies to the receptor protein (estrophilin) by four criteria: (a) precipitation of the radioactive steroid upon addition of goat antibody against rabbit gamma globulin to a mixture of the tritiated estradiol–receptor complex and the immunoglobulin, (b) adsorption of the estradiol–receptor complex by the immunoglobulin linked to Sepharose, (c) adsorption of the estradiol–receptor complex in the presence of the immunoglobulin by Staphylococcus aureus protein A linked to Sepharose, and (d) the ability of the immunoglobulin to increase the sedimentation rate of the estradiol–receptor complex. Antibodies to calf nuclear estrophilin were shown to crossreact with the nuclear receptor of rat uterus, as well as with the extranuclear receptor of calf, rat, mouse, and guinea pig uterus and of human breast cancer. The antibodies do not react with either the nuclear or extranuclear dihydrotestosterone–receptor complexes of rat prostate or with the extranuclear progesterone–receptor complex of chick oviduct. These findings indicate an immunochemical similarity among estrophilins from several mammalian species, as well as between extranuclear and nuclear forms of the receptor, but not among receptor proteins for different steroid hormones.

The action of estrogenic hormones in stimulating growth and function of female reproductive tissues takes place primarily, though perhaps not exclusively, through their interaction with an extranuclear receptor protein (estrophilin) present in characteristic amounts in hormone-dependent or “target” cells (1–3). Association with the steroid hormone induces conversion of the receptor to an active form; the activated hormone–receptor complex is translocated to the nucleus where it binds in the chromatin and in some way enhances the ability of the nucleus to synthesize certain types of RNA.

Despite recent advances in our knowledge about the intracellular interactions of steroid–receptor complexes, detailed understanding of the processes of receptor activation, translocation, and nuclear binding is still far from complete. In the hope that techniques of immunochemistry might provide new approaches to the study of hormone receptors, we have prepared specific antibodies to estrophilin by immunizing rabbits with a highly purified preparation of the estradiol–receptor complex (ER) from calf uterine nuclei. This paper describes evidence for the presence of these antibodies in the serum of the immunized rabbits and for their crossreactivity with hormone–receptor complexes from various sources.

MATERIALS AND METHODS

Reagents. [6,7-3H]Estradiol-17β (57 Ci/mmol) and [2,4,6,7-3H]estradiol-17β (108 Ci/mmol) were obtained from New England Nuclear Co.; unless otherwise noted, E* represents the 6,7-tritiated hormone. Tubercle bacilli and Freund's complete and incomplete adjuvants were purchased from Difco, bordetella pertussis vaccine from Eli Lilly & Co., and Staphylococcus aureus protein A bound to Sepharose CL-4B from Pharmacia. Immunoglobulin from immunized and nonimmunized rabbits was coupled to cyanogen bromide-activated Sepharose 4B by a published procedure (4). Antiserum to rabbit Ig was prepared by immunizing a female goat with purified rabbit immunoglobulin (12.5 mg for primary injection; 5–12 mg for booster injections) in a manner similar to that described for immunization of rabbits with E*R except that the emulsion containing the antigen was injected subcutaneously. Unless otherwise noted, phosphate buffers were prepared from sodium salts, and all buffers contained 0.01% (wt/vol) sodium azide. Phosphate-buffered saline contained 150 mM sodium chloride in 10 mM phosphate, pH 7.8. Buffers using Tris (T), pH 7.4 at 23°, sometimes containing potassium chloride (K) and disodium EDTA (E), are designated according to the millimolarity of their components; e.g., T10K40E1.5 = 10 mM Tris/400 mM KCl/1.5 mM EDTA.

Antigen. The estradiol–receptor complex used for immunization was prepared by incubating crude calf uterine nuclei with 10 nM E* (5.7 Ci/mmol) in calf uterine cytosol at 30° for 60 min. The nuclear complex was extracted in T10K400 and purified as described elsewhere (5–7) by a sequence of salt precipitation, gel filtration through Sephadex G-200, and electrophoresis in polyacrylamide gel. During the gel filtration the receptor loses its tendency to aggregate under low-salt conditions, and its sedimentation rate changes from 5.2 S in sucrose gradients containing 400 mM KCl to 4.8 S in either high- or low-salt gradients. The purified preparation of this “stabilized” E*R retains the ability of the original, aggregating form to stimulate RNA polymerase in isolated uterine nuclei (1, 5). Different preparations were found to contain from 10 to 30% of the radioactivity expected for the pure steroid–receptor complex; this value was used to estimate the amount of estrophilin in the protein used for each immunization, although the actual purity of the receptor preparation may be somewhat higher because some of the receptor probably is bound to nonradioactive, endogenous estrogen from the calf uteri and some may lose its ability to bind hormone by partial denaturation during the course of purification.

Immunization. Six-month-old male New Zealand White rabbits were immunized with the purified nuclear estradiol–receptor complex by the procedure of Vaitukaitis et al. (8). For

Abbreviations: E*, tritiated estradiol; R, estrophilin; E*R, radioactive estradiol–receptor complex; Ig-n, immunoglobulin from serum of rabbit immunized with E*R. Ig-n, immunoglobulin from serum of nonimmunized rabbit. Abbreviations for various buffer solutions are described in Materials and Methods.
the primary immunization, an emulsion, prepared by homogenizing a saline solution containing 20 μg of E*R with an equal volume of Freund's complete adjuvant and an additional 5 mg of dried tubercle bacilli, was injected intradermally at multiple sites on the back, while 0.5 ml of bordetella pertussis vaccine without added antigen was injected intradermally in the thigh. The experiments described in this paper were carried out with immunoglobulin from a rabbit that had received six booster injections over a period of 1 year with an emulsion of the immunogen and Freund's incomplete adjuvant; the last two booster injections, containing 20 and 50 μg of receptor, respectively, were given on December 16, 1976, and January 11, 1977. Blood was collected at 14-day intervals via the marginal ear vein; except for the experiments of Fig. 1, all results were obtained with material from blood drawn on January 31, 1977. A crude immunoglobulin fraction (Ig-i) was prepared from the serum by precipitation with 33% saturated ammonium sulfate in 50 mM phosphate buffer, pH 7.4, according to the procedure of Shiu and Friesen (9). The washed precipitate was redisolved in phosphate-buffered saline (or in some experiments in 20 mM phosphate, pH 7.4) and the dialyzed solution, after clarification by centrifugation, was analyzed for protein by its absorbance at 280 nm and for the absence of other serum proteins by acrylamide gel electrophoresis. Immunoglobulin (Ig-n), prepared similarly from serum of a nonimmunized rabbit (as well as from the immunized rabbit before any antibody titer appeared), was used as a control.

Radioactive Hormone-Receptor Complexes. The estradiol-receptor complexes of uterine cytosol from immature calves, rats, mice, and guinea pigs were prepared by homogenizing the tissues in four volumes of T10 buffer, using a Polytron PT-10 homogenizer with efficient cooling, and making the high-speed supernatant fraction 20 nM in E*; after 60 min at 4° the excess E* was removed with dextran-coated charcoal. The complex of human breast cancer cytosol was prepared similarly except that the tumor specimen, pulverized while frozen in liquid nitrogen, was homogenized in T10 containing 0.5 mM dithiothreitol, and the cytosol fraction was made 0.5 nM in E* (108 Ci/mmole) without the use of charcoal. The crude nuclear complex of calf uteru was prepared by extraction of washed nuclear sediment with T10K400 after its incubation for 60 min at 25° with 20 nM E* in calf uterine cytosol. Rat nuclear complex was obtained by similar extraction of the nuclear sediment from a homogenate in T10 of immature rat uteri excised 4 hr after the subcutaneous injection of 100 ng (20.8 μCi) of 3H in 0.2 ml of saline. The dihydrotestosterone-receptor complexes of rat prostate were kindly supplied by Shutsum Lee. The cytosol complex was prepared by adding 2 nM [3H]dihydrotestosterone to prostatic cytosol, followed by partial purification of the complex by precipitation with ammonium sulfate and filtration through Sephadex G-25, whereas the nuclear complex was obtained by extraction of prostatic nuclei with T10K400 after their incubation for 20 min at 20° with prostatic cytosol containing tritiated dihydrotestosterone. The progesterone-receptor complex of chick oviduct, prepared by adding [3H]progesterone to chick oviduct cytosol and partially purified (as well as activated in respect to nuclear binding) by precipitation with ammonium sulfate, was a gift of Thomas Spelsberg.

Immunochemical Interactions. For double antibody precipitation of the crude estradiol-receptor complexes, a solution of E*R (1 pmol), normal rabbit serum (10 μl), and immunized rabbit serum (10 μl) in 1.0 ml of phosphate-buffered saline containing 10 mM EDTA was incubated at 4° for 4 hr, after which sufficient goat antiserum against rabbit Ig was added to precipitate all the rabbit Ig. Controls used 20 μl of normal rabbit serum and no immunized rabbit serum. After 16 hr the mixtures were centrifuged and the pellets were dissolved in 100 μl of 0.1 M NaOH; radioactivity was measured in 10 ml of scintillation mixture containing 10 mM HCl. (Efficiency for 3H was 28%.) For immunoadsorption experiments, a solution of 0.8 pmol of E*R or E* was incubated for 4 hr at 4° with an agitated suspension of either Sepharose-Ig-i or Sepharose-Ig-n (containing 300 μg of protein) or of Sepharose alone in a total volume of 600 μl of T10K400. Similarly, 0.3-pmol aliquots of E*R or E* in T10K400 were incubated at 4° for 90 min with 100 μg of either Ig-i or Ig-n (or with no added immunoglobulin) and then for 4 hr longer with an agitated suspension of 50 μl of Sepharose-protein-A in a final volume of 300 μl of T10K4000. After centrifugation, the beads were washed and the combined supernatant and washings were assayed for radioactivity in Triton X-100 scintillation mixture. (Efficiency for 3H was 90%.)

Results of Studies. Various hormone-receptor complexes [0.05–2 pmol in 150 μl of T10 cytosol or T10K400 nuclear extract] and Ig-i or Ig-n (200 μg except for the experiments of Fig. 1 and Fig. 5, which used 500 μg and 400 μg, respectively) were incubated at 4° for 1–5 hr in a final volume of 220 μl of T10 or T10K400 depending on the gradient to be used. A 200-μl aliquot of each mixture was layered on 3.5 ml of a 10–30% sucrose gradient containing either T10K400E1.5 (low salt) or T10K400E1.5 (high salt) and centrifuged at 2° for 16 hr at 253,000 × g. Successive 100-μl fractions were collected from the bottom and radioactivity was measured in Triton X-100 scintillation mixture. In some experiments bovine plasma albumin (4.6 S), bovine gamma globulin (7.0 S), β-amylase (9.2 S), and catalase (11.3 S) were sedimented in parallel gradients to serve as markers.

Results

Immunoglobulin from the serum of a rabbit immunized with purified estrogen-receptor complex of calf uterine nuclei contains specific antibodies to the receptor protein. This can be demonstrated by comparing this immunoglobulin (Ig-i) with a similar preparation from a nonimmunized rabbit (Ig-n) in the ability to interact with the radioactive estradiol-receptor complex of crude nuclear extract. As shown in Table 1, in the presence of Ig-i, but not of Ig-n, a significant amount of tritiated estradiol in the form of E*R complex is precipitated by antibody to rabbit gamma globulin or bound to protein-A, a substance that reacts specifically with the IgG type of antibody. Similarly, Ig-i linked to Sepharose binds a much greater proportion of E*R complex than does Sepharose-Ig-n or Sepharose alone.

Because the reaction of Ig-i with oestriol under the conditions used appears to form a nonprecipitating product, the
immature rats (0).

(a) 8,0001 tendency reaction of Ig-i In challenge increase in complex dpm of sources reactivity and crossreactivity with receptors from various sources can be studied informatively by determining the effect of the immunoglobulin on the sedimentation of radioactive hormone–receptor complexes in sucrose gradients. As shown in Fig. 1, addition of Ig-i, but not of Ig-n, causes an increase in the sedimentation velocity of the purified 4.8S estrogen–receptor complex used as the antigen in the immunization. The increase in antibody titer with time and with further antigen challenge can be seen by comparing the incomplete effect of Ig-i from serum drawn on December 22, 1976 with the complete reaction of Ig-i from serum drawn on January 17, 1977. In contrast to the E*R complex, free estradiol shows no tendency to bind to the antibody.

The suggestion in Fig. 1 that the reaction of the antibody with the nuclear complex may produce more than one product is confirmed when the interaction of Ig-i with the 5.2S estrogen–receptor complex in crude extract of calf uterine nuclei is examined on a high-salt sucrose gradient (Fig. 2a). Reaction with Ig-1, but not Ig-n, leads to the formation of two new entities, sedimenting at about 8 S and 11.5 S. Two new sedimentation peaks are also produced from the reaction of the antibody with the nuclear estrogen–receptor complex obtained from uteri of immature rats receiving tritiated estradiol in vivo (Fig. 2b). Antibodies to the calf nuclear receptor react with the extranuclear E*R complexes of calf (Fig. 3) and rat (Fig. 4) uteri and of human breast cancer (Fig. 5). In contrast to the nuclear complexes, these extranuclear complexes react with Ig-i to yield a product that sediments as a single entity in either high-salt or low-salt gradients. Similar results were obtained with the extranuclear estrogen–receptor complexes of mouse and guinea pig uterus. However, the nonaggregating 4.5S form of the calf extranuclear complex, which results from the action of the calcium-activated "transforming factor" of uterine cytosol (10), resembles the nuclear complex in reacting with Ig-i to yield two new sedimentation peaks. The same new sedimentation peak is produced when E* is added to a mixture of Ig-i and calf uterine cytosol as when Ig-i is added to the preformed E*R complex. In analogous experiments, with either the nuclear or extranuclear dihydrotestosterone–receptor complexes of rat

![Fig. 1](image1.png)

**Fig. 1.** Sedimentation pattern of highly purified estradiol–receptor complex of calf uterine nuclei in low-salt sucrose gradients in the presence of Ig-n (O) or Ig-i (O, December 22, 1976 bleed; •, January 17, 1977 bleed). Dashed curve (▲ - ▲) indicates sedimentation of free estradiol in the presence of Ig-i. The E*R complex sediments at the same position in the absence of immunoglobulin as it does in the presence of Ig-n or of Ig from earlier bleeds of the immunized rabbit.

![Fig. 2](image2.png)

**Fig. 2.** Sedimentation pattern in high-salt sucrose gradients of: (a) E*R in extract of calf uterine nuclei after incubation with E* in calf uterine cytosol, and (b) E*R in extract of uterine nuclei from immature rats receiving E* in vivo, in the presence of Ig-n (O) or Ig-i (●).

![Fig. 3](image3.png)

**Fig. 3.** Sedimentation patterns of E*R in calf uterine cytosol in sucrose gradients containing (a) high salt and (b) low salt in the presence of Ig-n (O) or Ig-i (●).
prostate or the extranuclear progesterone–receptor complex of chick oviduct, no displacement of the respective sedimentation peaks was observed in the presence of Ig-i.

DISCUSSION

Although suggestive evidence has been reported for the presence of antibodies to estrophilin in the serum of rabbits immunized with partially purified estradiol–receptor complex from rat (11) or calf (12) uterine cytosol, to our knowledge the present findings represent the first clear-cut demonstration of antibodies to a receptor protein for a steroid hormone. The crossreactivity of antibodies to the purified estrophilin from calf nuclei with extranuclear as well as nuclear estradiol–receptor complexes from calf and rat uterus indicates an immunochimical similarity between the two forms of estrophilin and provides supporting evidence for the concept of a two-step interaction mechanism in which the nuclear receptor is derived from the hormone-induced translocation of the extranuclear receptor (13, 14). The apparent formation of two different products from the reaction of Ig-i with nuclear complexes but only one with cytosol complexes suggests that the nuclear form of the receptor may contain more immunochemical determinants per molecule than are present or accessible in the extranuclear form. The significance of this observation in relation to the phenomenon of receptor activation is not yet clear, but it is noteworthy that the “calcium-stabilized” 4.5S form of the extranuclear receptor (10), which shares with the nuclear form the ability to bind to chromatin and to enhance RNA synthesis in isolated uterine nuclei,1 likewise forms two new sedimentation peaks when treated with Ig-i.

The fact that the radioactivity of estradiol can be used as a marker for the receptor protein implies that the interaction of the antibody with the receptor does not distort the structure of the latter to such an extent that it loses its ability to bind hormone. It also appears that the interaction with antibody does not block the hormone-binding site, inasmuch as addition of radioactive estradiol to a mixture of Ig-i and the uncomplexed receptor in calf uterine cytosol gives rise to the same shifted sedimentation peak as that produced by adding Ig-i to the preformed E*R complex.

The ability of the antibodies to estrophilin from calf uterus to crossreact with estrogen–receptor complexes from rat, mouse, and guinea pig uteri and from human breast cancer indicates an immunochimical similarity among the estrophilin from all these mammalian species. It remains to be seen whether estrogen receptor proteins from other organs, such as anterior pituitary, or from nonmammalian tissues, such as chick oviduct and frog liver, show the same reactivity with the antibodies. Although more examples need to be investigated, the lack of reactivity of the Ig-i with the dihydrotestosterone receptor of rat prostate or the progesterone receptor of chick oviduct would suggest a lack of immunochemical similarity, at least in the determinants involved with our antibody, among receptor proteins for different steroid hormones.

Antibody to receptor protein provides a reagent that should prove useful in many aspects of receptor research. The crossreactivity of Ig-i with estrophilin from human breast cancer opens the possibility of a simple radioimmunoassay for the receptor content of breast cancers as a guide to therapy (15–17). Ig-i linked to Sepharose or other support may prove to be an efficient tool for the purification of estrophilin, which offers insight into the biologic action of the hormone may come from the application of immunochimical techniques for precise intracellular localization of nuclear and extranuclear receptor through electron microscopy.

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1 S. Mohla, E. R. DeSombre, and E. V. Jensen, unpublished data.