PRODUCTION AND DETECTION OF ANTIBODIES AGAINST THE ESTROGEN RECEPTOR FROM CALF UTERINE CYTOSOL

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1. Introduction

With the recent development of biospecific adsorbants, progress has been made towards the purification of steroid receptors [1–5]. Standard biochemical techniques presently used to characterize steroid receptors could be complemented with an immunological approach if antibodies were available. Although the production of antiserum has been attempted [6], unequivocal evidence for antibodies against the receptor has not yet been presented.

The purpose of the present study was to determine if the estrogen receptor from calf uterine cytosol was immunogenic in rabbits, a study which consequently required the development of a method to test antisera for anti-receptor antibodies. The identification of receptor–antibody interactions was complicated by 2 problems: (1) the receptor available for testing antisera was present at concentrations less than 10 nM in partially purified preparations and (2) it was necessary to detect antibodies complexes with an antigen which can only be identified by its saturable, non-covalent and specific binding of a tritiated steroid. The separation of [^{3}H]estradiol receptor from antibody-associated [^{3}H]estradiol receptor and free [^{3}H]estradiol by gel filtration on acrylamide-agarose columns provided a means to solve these problems.

This preliminary report describes the production of antisera using μg quantities of partially purified estrogen receptor and a chromatographic method for detecting small amounts of soluble antibody–receptor complexes.

2. Materials and methods

2.1. *Preparation of aggregated and '4S Trypsin' non-aggregated forms of estrogen receptor (flow sheet in fig. 1).*

Cytosol was prepared from frozen calf uteri as previously described [3,4]. The estrogen receptor was partially purified by precipitation at 4°C with either ammonium sulfate (30% saturation for 1 h) or sodium citrate (0.55 M for 2 h). Following centrifugation at 35 000 *g* for 10 min, the pellets were suspended in TE buffer (50 mM Tris-HCl, 1.5 mM EDTA, pH 7.4) equal to 10% of the initial cytosol volume and treated with trypsin (Calbiochem, 1 X crystallized; 100 μg/ml cytosol for 3–10 h at 4°C). Under these conditions, one can distinguish in these preparations 2 molecular forms of receptor. One is the so-called '4S Trypsin' form of the receptor [7] which has very little tendency to aggregate. The other form sediments with S values greater than 10S and is operationally defined as aggregated receptor. The trypsin-treated solution was then centrifuged (35 000 *g*, 10 min.), and pellets containing the aggregated receptor derived from sodium citrate precipitation (designated AGR-1) were used to immunize rabbits.

Supernatants, containing aggregated and '4S Trypsin' receptor molecules, were chromatographed

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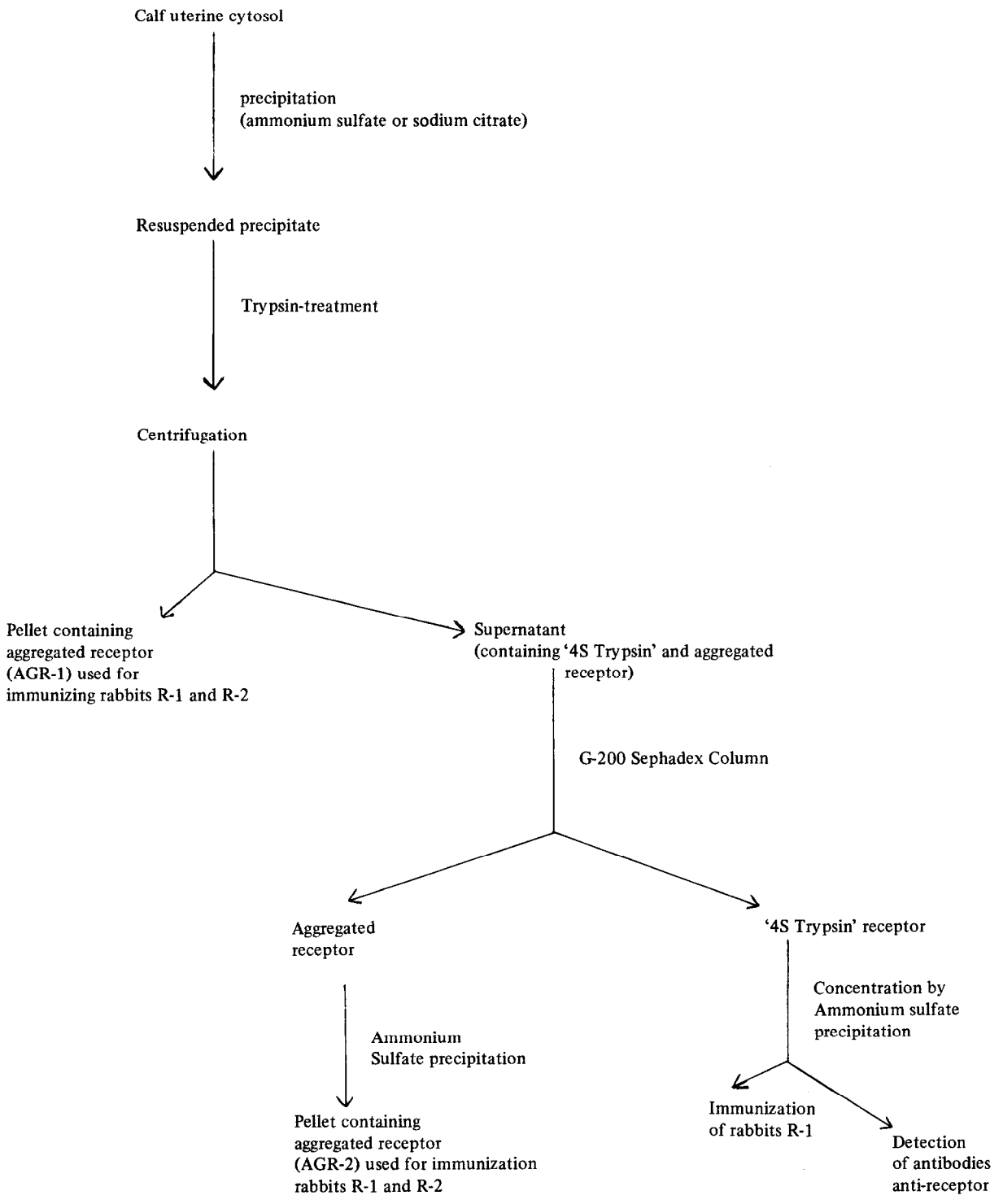

Fig.1. Flowsheet for the preparation of aggregated and '4S Trypsin' forms of the estrogen receptor.

on a Sephadex G-200 column (300–900 ml were filtered on a 16 liter column). Fractions constituting the peak of aggregated receptor activity were pooled and precipitated with ammonium sulfate (45% saturation, 12 h, 4°C). This aggregate-containing precipitate (AGR-2) was also used for immunizations. After pooling the fractions forming the 4S peak activity and precipitating with ammonium sulfate (45% saturation), the pellet containing the '4S Trypsin' receptor was dissolved in TE buffer (final volume was 10% of the pooled fractions volume) and used either for immunization or to test antisera as described below.

Receptor activity was monitored throughout the purification procedure by measuring the specific binding of [^{3}H]estradiol using 2 differential dissociation methods. The techniques involved (1) removal of free and non-specifically bound [^{3}H]estradiol with dextran-coated charcoal [8] and (2) binding of the [^{3}H]estradiol receptor complex on hydroxyapatite columns [9]. Radioactivity ([^{3}H]estradiol 48–55 Ci/mmole, CEA) was counted at 30% efficiency in Bray's scintillation solution.

2.2. *Production of antisera*

Adult male rabbits (new Zealand White and Fauve Bourgogne breeds) were immunized with non-aggregated and/or aggregated receptor preparations. Blood containing pre-immune sera was collected from marginal ear veins; animals were deprived of food for 16–20 h before bleedings were made. Seven rabbits were injected using various immunization schedules. The 2 antisera chosen to run the complete series of controls were produced in rabbits R-1 and R-2; these 2 immunization schedules are described below.

Rabbit R-1 (Fauve Bourgogne breed) was injected with 70 mg protein containing 20 μg '4S Trypsin' receptor (in 0.75 ml phosphate buffered saline, pH 7.4) emulsified with 1.5 ml Freund's Complete Adjuvant (DIFCO) at 15 intradermal (back) and 2 subscapular sites. Two booster injections were made at intradermal, intramuscular and subscapular sites using Freund's Incomplete Adjuvant 9 weeks after the initial injection using 3 ml of a '4S Trypsin' receptor preparation containing 60 μg of '4S Trypsin' receptor and a total of 45 mg protein and 7 weeks later using 150 μg receptor in aggregated form (AGR-1). Trial bleedings were made between the 2 boosters and 16 days after the last injection.

Rabbit R-2 received 25 μg aggregated receptor AGR-2 emulsified in Freund's Complete Adjuvant at 8 intradermal, 2 subscapular and 2 intramuscular (thigh) sites. Boosters were given, using Incomplete Adjuvant, after 5 weeks (50 μg receptor as AGR-2), followed 4 days later with an identical injection and 12 weeks later with 150 μg receptor as AGR-1. Trial bleedings were made between the second and third boosters and 17 days after the last injection.

Antisera were also prepared against cytosol preparations which had been exposed to a biospecific adsorbant to remove estrogen receptors, as previously described [4]. Rabbits were injected subcutaneously with cytosol preparations containing less than 5 ng receptor/ml (CSR) emulsified in Freund's Complete Adjuvant for the first injection and in Incomplete Adjuvant for booster injections.

Sheep anti-rabbit globulins were purchased from GIBCO.

All antisera were stored at −20°C without preservatives.

2.3. *Detection of antibodies against the estrogen receptor*

Before rabbit sera were used in these experiments, they were centrifuged at 12 000 *g* for 30 min (4°C)

The following components were incubated in sterile plastic tubes at 4°C with constant agitation for 24 or 48 h: (1) Rabbit antisera or control sera (15–20 μl with a total of 1.1–1.5 mg protein, (2) 100 μl of a preparation containing 7–20 ng of partially purified [^{3}H]estradiol '4S Trypsin: receptor (These amounts were calculated on the basis of a mol. wt. of 60 000 and assuming one steroid binding site per molecule of receptor), (3) sufficient TE buffer to bring the total incubation volume to 120 μl.

Samples (100 μl) of the incubation milieu were filtered on ACA-34 gel (LKB) columns (0.8 × 22 cm) containing a 0.5 cm layer of Sephadex G25 (coarse) at the bottom. TE buffer was used as the equilibrating and working buffer. The flow rate was 2–4.5 ml/h; 0.22 ml fractions were collected. After fractions were transferred into scintillation vials, the tubes were rinsed with 2 aliquots (5 ml each) of scintillation liquid (4 g omnifluor, 1l toluene, 0.3l ethanol) which were added to the vials to insure that total

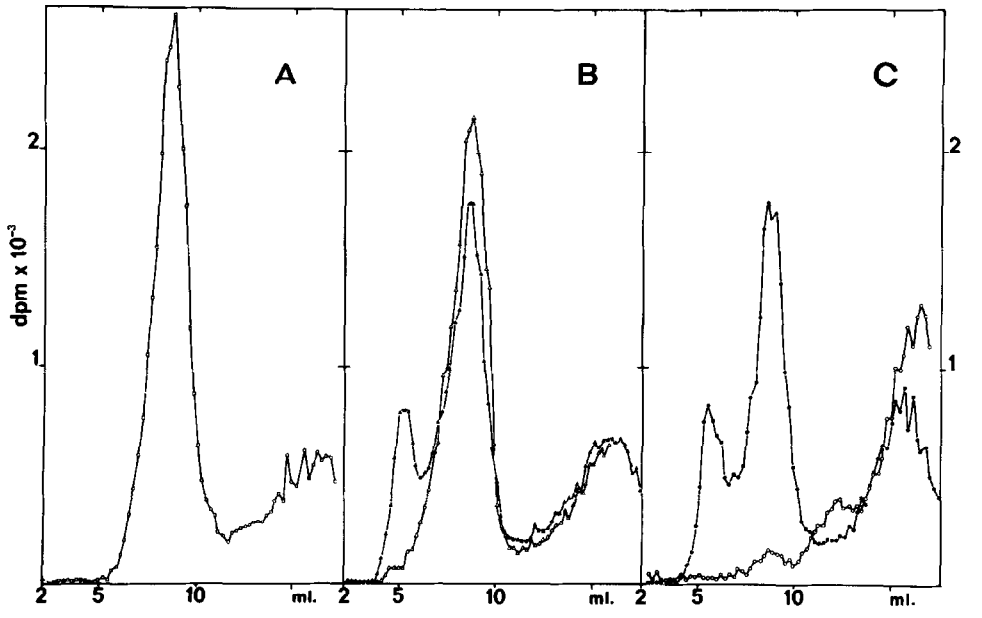

Fig.2. Gel filtration of [^{3}H]estradiol receptor complexes on ultrogel ACA-34. Procedural details are described in Materials and methods; each point on the graph represents the total radioactivity in one fraction (200 μl). (A) chromatography of 100 μl (7 ng) of a preparation of partially purified, '4S Trypsin' receptor incubated with [^{3}H]estradiol. (B) chromatography of [^{3}H]estradiol receptor complex incubated for 48 h at 4°C with anti-receptor antiserum R-1 (▲–▲–▲) or pre-immune R-1 serum (△–△–△). (C) Chromatography of hormone receptor complexes (using [^{3}H]estradiol diluted with non-radioactive hormones) incubated for 24 h at 4°C with anti-receptor antiserum R-2. Prior to exposure to antiserum, the receptor preparation was incubated with [^{3}H]estradiol isotopically diluted with non-radioactive estradiol (○–○–○) or non-radioactive androstanolone (●–●–●).

fraction radioactivity was counted (no counts were observed in a third rinse volume of scintillation liquid). Counting efficiency was 28–30%; results were expressed as dpm which were calculated from the cpm using an external standard.

2.4. *Protein measurements*

The proteins were measured according to the technique of Lowry et al. [10].

3. Results

Fig.2A shows the effluent profile of the '4S Trypsin' estrogen receptor bound to [^{3}H]estradiol. The three different '4S Trypsin' receptor preparations used to test antisera gave similar results.

Incubation of the [^{3}H]estradiol receptor complex with antiserum R1 effected the displacement of 21% of the macromolecular bound dpm from the 4S peak into the void volume (fig.2B). Similar results were obtained with 5 other antisera. After incubation of the [^{3}H]estradiol receptor complex with pre-immune serum, 5% of the bound radioactivity was observed in the void volume.

There was little or no bound radioactivity in the void volume or '4S' peak when the receptor was incubated with [^{3}H]estradiol plus a 10^3-fold excess of non-radioactive estradiol before the steroid receptor complex was exposed to antisera. A similar isotopic dilution with non radioactive androstanolone (5α-androstane-17β-ol-3-one) did not interfere with the shift in dpm (fig.2C).

Little void volume radioactivity (3% of the macromolecular-bound dpm) was detected when [^{3}H] estradiol receptor complex was treated with antisera against non-receptor cytosol proteins (fig.3). A similarly small percentage of bound dpm was displaced when the [^{3}H]estradiol complex was incubated with sheep anti-rabbit globulins and preimmune rabbit

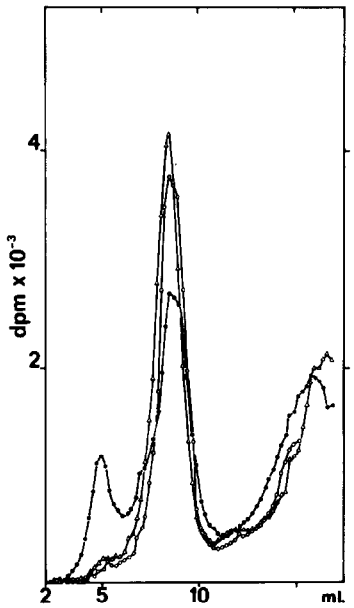

Fig.3. Chromatography of [^{3}H]estradiol '4S Trypsin' receptor complex incubated for 24 h at 4°C with anti-receptor antiserum (•—•—•), with anti-serum against non-receptor cytosol antigens (△—△—△) or with pre-immune serum R-2 plus sheep anti-rabbit globulins (○—○—○).

serum (fig.3). Precipitin reactions were observed in agarose double diffusion plaques [11] between anti-receptor antisera and antigens in the preparations containing '4S Trypsin' receptor and between sheep anti-rabbit globulins and pre-immune rabbit serum.

Table 1 summarizes results showing the distribution of radioactivity after gel filtration of [^{3}H] estradiol labeled '4S Trypsin' receptor preparations incubated with various sera.

Table 1
Distribution of bound radioactivity after gel filtration of [^{3}H]estradiol receptor complexes incubated with various sera

Reagents incubated with [^{3}H]estradiol receptor	mg serum protein	Incubation time (h)	Macromolecular bound d.p.m.	% bound d.p.m. in void volume
Tris-HCl EDTA buffer	–	48	15,172[d]	6%
Anti R-1[a]	1.4	48	26,025[e]	21%
Pre-immune R-1[a]	1.5	48	24,912[e]	5%
Anti R-1 ([^{3}H]estradiol diluted with non-radioactive estradiol)[b]	1.4	48	3,431[e]	
Anti R-1 ([^{3}H]estradiol diluted with non-radioactive androstanolone[b]	1.4	48	23.318[e]	20%
Anti R-2[a]	1.1	24	39,448[f]	22%
Anti CSR[c]	1.1	24	35,020[f]	4%
Pre-immune R-2[a]+ sheep anti-rabbit globulin	1.5	24	32,158[f]	3%

Procedural details for receptor-sera incubation and gel filtration of complexes were described in Materials and methods. Bound radioactivity was calculated by summing the dpm present in the first 11 ml of effluent.

[a] Sera obtained from rabbits R-1 (bled 16 days after the second booster) and R-2 (bled 17 days after third booster) immunized with receptor preparations.

[b] To detect saturable binding of estradiol, the receptor was incubated, prior to exposure to antiserum, with [^{3}H]estradiol isotopically diluted with non-radioactive estradiol (E_2) or non-radioactive androstanolone.

[c] Antiserum produced in rabbit CSR against cytosol preparations treated with the biospecific adsorbant to remove receptor.

[d,e,f] Three different partially purified preparations of '4S Trypsin' receptor containing 7,14 and 20 ng receptor/100 μl, respectively.

4. Discussion

The displacement of 20–22% of the bound radioactivity from the '4S' peak into the void volume after incubation of [^{3}H]estradiol receptor with antiserum was interpreted as representing the formation of antibody–receptor complexes. The presence of dpm in the void volume was due to the displacement of a macromolecule which is probably the '4S Trypsin' receptor as judged by its elution volume on an Ultrogel column, and its high affinity, specific and saturable binding of estradiol. In addition, the manner in which the antigens were prepared, and the inability of androstanolone to compete for the estradiol binding site make it highly unlikely that antibodies have been raised against a contaminant such as an oestradiol binding plasma protein. Results obtained after incubation of the receptor with pre-immune sera, antiserum against non-receptor cytosol antigens (CSR) or a combination of pre-immune serum and sheep anti-rabbit globulins indicated that most of the displaced radioactivity effected by anti-receptor sera was due to antibody–receptor interaction.

The total amount of bound dpm (void volume plus '4S' peak radioactivity) recovered after exposure of the [^{3}H]estradiol receptor to antisera was equal to or greater than the total bound dpm recovered when the [^{3}H]estradiol receptor was exposed to pre-immune serum or other antisera (not produced against receptor preparations). These results suggested that, under the conditions used in this study, the antibody–receptor complex did not precipitate, but remained soluble. Precipitation of the complex would require a second step to separate antibody-bound receptor from free receptor. Methods such as the double antibody technique were considered to risk perturbing the interaction between receptor and its steroid and to increase the probability of non-specific aggregation or trapping of the [^{3}H]estradiol receptor. Filtration on Ultrogel columns avoided the potential problems of classical precipitation methods and permitted the detection of small amounts of soluble antibody–receptor complexes.

Thus, these results show that multispecific antisera (forming precipitin bands with cytosol antigens in double diffusion in gel tests) which were produced in rabbits immunized with partially purified '4S Trypsin' receptor plus aggreated receptor (rabbit R-1) or with aggregated receptor only (rabbit R-2) reacted with the '4S Trypsin' receptor. Work is in progress to render the antisera specific for the estrogen receptor, which should provide an important tool for characterizing these proteins.

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