Monoclonal Antibodies against Estrogen Receptor: Interaction with Different Molecular Forms and Functions of the Receptor†

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ABSTRACT: Hybridoma cells have been produced by fusing SP2/O-Ag14 mouse myeloma cells with spleen cells from a mouse immunized with a purified preparation of estrogen receptor from calf uterus. The antibodies, all of the immunoglobulin G (IgG) class, interact with different forms of calf receptor as well as with rat and human receptors. The equilibrium dissociation constant of the antibody–receptor complex was measured in solid phase and in solution. With immobilized antibodies the K_d is 0.06 nM whereas in solution it is 0.5 nM. Only one antigenic determinant is present per molecule of receptor with the antibodies tested. The antibodies JS34/32 are able to form only a 1:1 complex with the 8S form of the receptor, whereas a 2:1 receptor–IgG complex is formed at low antibody concentration with the high-salt or nuclear form of receptor. The antibodies JS34/32 and JS28/32 prevent neither the nuclear uptake of the receptor nor the extraction of the translocated receptor from the nuclei.

Despite great progress in the purification of various forms of estrogen receptor during the past few years, various aspects of the mechanism of action of steroid hormones are still far from being completely elucidated. The use of new techniques in the study of steroid receptors may provide information fundamental for fully understanding their mechanism of action.

Hybridoma technology (Köhler & Milstein, 1975, 1976) allows for the production of large amounts of monospecific immunoglobulins. Because of their specificity and homogeneity, the monoclonal antibodies may be more useful research tools than the antibodies from the serum of immunized animals. Recently, monoclonal antibodies have been widely used in hormone–receptor studies. The molecular structure of the acetylcholine receptor has been extensively studied with monospecific immunoglobulins against determinants of various subunits (Lindstrom et al., 1980; Tzartos & Lindstrom, 1980; Gullick et al., 1981; Conti-Tronconi et al., 1981) or against the cholinergic binding site (Mochly-Rosen & Fuchs, 1981). Monoclonal antibodies have also been used for the purification of nicotinic cholinergic receptor (Lennon et al., 1980). Purification and molecular characterization of β-adrenergic receptor (Frazer & Venter, 1980) as well as informations on structure and function of thyrotropin receptor (Yavin et al., 1981) were obtained with the use of hybridoma technology. Production of monoclonal antibodies against nuclear estrogen receptor from calf uterus (Greene et al., 1980a) and against native human receptor from the MCF-7 cell line has been reported (Greene et al., 1980b). Only some of the latter antibodies, however, were able to recognize determinants present in estrogen receptors from different mammalian species.

We describe here the production of a series of hybridoma cell lines that produce monoclonal antibodies directed against determinants of calf uterus estrogen receptor. The antibodies from all the hybridoma cell lines tested show some interspecies cross-reactivity. These antibodies are a versatile tool for investigating the structure of the receptor and may aid in an understanding of the complex events of activation and translocation of the receptor.

Materials and Methods

17β-[6,7-$^3H_2$]Estradiol (54 Ci/mmol) and 16α-[^{125}I]iodo-17β-estradiol (200 Ci/mmol) were purchased from New England Nuclear Corp. The radioactive estradiol derivatives were routinely checked for purity and contained less than 2% impurities. All other reagents used were of analytical grade and obtained from commercial sources. All incubations were performed in duplicates at 4 °C unless stated otherwise. Protein concentrations were determined by the Coomassie Blue method (Bradford, 1976). TED buffer contained 20 mM Tris-HCl,[1] pH 7.4, 1 mM EDTA, and 1 mM dithiothreitol.

Tissue and Cells. Calf uteri were collected fresh from immature animals, stripped from fat, ligaments, and ovaries and frozen at the slaughterhouse. Parental myeloma cell line (SP2/O-Ag14) and hybridoma cell lines were grown in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS). Preformed tissue culture media and serum were purchased from Gibco.

Receptor Purification. The purification was performed as described in detail elsewhere (Puca et al., 1980). Multiple batches of 250–300 g of calf uteri were processed each time. The cytosol was subjected to an initial heparin–Sepharose purification step. The peak of specific estradiol binding activity eluted with heparin was filtered through an estradiol–Sepharose column, and the receptor was eluted with TED buffer containing 0.5 M NaSCN, 3×10^{-7} M 17β-[6,7-$^3H_2$]estradiol (10.8 Ci/mmol), and 10% dimethylformamide. The eluted receptor was dialyzed and concentrated under vacuum.

Synthesis of 17α-(Carboxymethyl)-17β-estradiol. The ethyl ester of 17α-(carboxymethyl)-17β-estradiol was prepared from 3-*O*-benzylestrone by the published procedures (Mousseron-Canet & Beziat, 1965). The *O*-benzyl protecting group was removed by catalytic hydrogenation over 10% palladium/charcoal and the ester group hydrolyzed in dilute ethanolic NaOH. The 17α-(carboxymethyl)-17β-estradiol was crystallized, after acidification, from an ethanol–water mixture. The structure of 17α-(carboxymethyl)-17β-estradiol was confirmed by mass spectrometry and nuclear magnetic resonance spectroscopy.

Immunization of Mice, Fusion, and Cloning. Three Balb/c mice (3-weeks old) were each injected subcutaneously with

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate-buffered saline; DEAE, diethylaminoethyl; IgG, immunoglobulin G; BSA, bovine serum albumin.

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10 μg of purified receptor in 0.1 mL of PBS emulsified with an equal volume of Freund's complete adjuvant. Subsequently, equal doses of the receptor in incomplete Freund's adjuvant were injected at days 14, 28, and 56. Ten days after the last injection, each mouse received a daily iv injection of the estrogen receptor (10 μg in 0.1 mL of PBS) in the tail for 3 days. The mice were bled and the sera tested for the presence of anti-receptor antibodies. On the day after the last iv injection, one mouse was sacrificed and the spleen removed under sterile conditions. A cell suspension in 5 mL of Hank's balanced salt solution (HBSS) medium was made by repeated passage through a 20-gauge needle. Red blood cells were lysed with 3 volumes of a solution of 0.15 M NH_4Cl and 10 mM $KHCO_3$, pH 7.2 at room temperature for 5 min. Spleen cells (approximately 10^8) were mixed with 5×10^7 myeloma cells (SP2/O-Ag14) and centrifuged at 800*g* for 5 min. The cells were resuspended in 50% poly(ethylene glycol) (PEG-1000) and diluted to a final 5% concentration of PEG over a 8-min period at 37 °C with serum-free DMEM. The cells were recentrifuged and resuspended in DMEM containing 20% FCS at a final concentration of 2.5×10^6 cells/mL. The cells were plated on three 96-well microtiter dishes (Falcon). After incubation for 24 h, the medium was changed to a medium containing hypoxanthine–aminopterin–thymidine (HAT) for hybrid selection. The cells were refed every other day with HAT medium for 2 weeks and with hypoxanthine–thymidine (HT) containing medium for 1 week. The culture medium was then replaced with DMEM containing 20% FCS. Hybrid cells were found in 180 wells. After a further 2 days, the medium from each well was assayed for antibody to estrogen receptor by double antibody immunoprecipitation assay.

In 30 out of the 180 wells the presence of antibody reacting with estrogen receptor was established. This was further confirmed by sucrose gradient density analysis. Cells from the positive wells were cloned in soft agar. Visible clones were collected and transferred to 96-well plates. At confluency, the supernatant of these clones was screened for the specific antibodies with the double antibody immunoprecipitation assay.

Cells from some of the positive clones were injected ip in Balb/c mice (10^7 cells/mouse) primed with 2,6,10,14-tetramethylpentadecane. After 7–10 days, the mice were sacrificed, and the ascites fluid was collected from the peritoneal cavity. Antibodies were purified by ammonium sulfate precipitation (Deutsch, 1967) followed by DEAE-cellulose chromatography (Fahey, 1967).

For characterization of antibody subclass, each vertical row of a 96-well microtiter plate was adsorbed with a different monoclonal antibody. Different subclass-specific rabbit anti-mouse immunoglobulins sera (Zimed Labs) were incubated in each horizontal row. After an appropriate washing, all 96 wells were incubated with peroxidase-labeled affinity purified goat anti-rabbit IgG (Zimed Labs). After an exhaustive washing, the peroxidase substrate, 2,2′-azinobis(3-ethylbenzothiazolinesulfonic acid), was added to determine the specific subclass of immunoglobulins. The incubation and washing procedures were according to the protocol supplied by the manufacturers.

Preparation of Hormone–Receptor Complexes. Cytosol was prepared as described earlier (Puca et al., 1972). Aliquots of cytosol were incubated for 2–4 h with 10 nM 17β-[6,7-$^3H_2$]estradiol. The cytosol was then added to the pellet of an equal volume of a dextran-coated charcoal suspension (1% norite A, 0.1% dextran T-70, and 0.1% gelatin in 10 mM Tris-HCl, pH 7.4), incubated for 5 min at 0 °C and centrifuged at 4000 rpm for 10 min. The same procedure was applied for labeling the cytosol with 16α-[^{125}I]iodo-17β-estradiol. The nondisplaceable binding was less than 5% of the total binding.

Immunoprecipitation Assay. Aliquots of 16α-[^{125}I]iodo-17β-estradiol-labeled receptor (0.15 pmol) were incubated at 0 °C for 4 h with hybridoma cell culture medium or with various dilutions of purified antibodies in final volume of 150 μL in 20 mM phosphate buffer, pH 7.2, containing 0.145 M NaCl (PBS). The immune complex was precipitated by addition of 25 μL of the appropriately diluted goat anti-mouse immunoglobulin (IgG, IgA, IgM) serum and 25 μL of normal mouse serum (1:50 diluted in PBS), followed by an overnight incubation at 4 °C. Dilutions of the goat antisera were optimized for each individual batches. The precipitated immunocomplexes were centrifuged at 2000*g* for 20 min, the pellets were washed twice with PBS containing 1 mg/mL BSA, and the radioactivity in the pellet was counted in a Searle γ counter. The control incubations consisted of a medium or similar concentration of purified immunoglobulins from a control hybridoma derived from the fusion of the same myeloma cell line and spleen cells from a nonimmunized mouse. In routine assays, the immunoprecipitation was performed with 25 μL of a 5% suspension of formaldehyde-inactivated *Staphylococcus aureus* (Pansorbin, Calbiochem) in PBS containing 1 mg/mL BSA.

The antibodies (1 mg) from the clone JS34/32 were coupled to 200 mg of *N*-hydroxysuccinimide ester (Cuatrecasas & Parikh, 1972) substituted Mâtrex-102 beads (Amicon) by incubation in 2.5 mL of 20 mM phosphate buffer, pH 8.0, for 4 h at 24 °C. The unreacted activated groups of the beads were blocked with 10 mM glycine in the same buffer. The immunoadsorbent was repeatedly washed by centrifugation with high ionic strength buffers and resuspended in 2 mL of PBS. The binding assay was performed by addition of 10 μL of the immunoadsorbent suspension to various concentrations of 16α-[^{125}I]iodo-17β-estradiol–receptor complex in a final volume of 0.1 mL. After 16-h incubation at 0 °C, the beads were pelleted and washed twice with PBS containing 1 mg/mL BSA, and the radioactivity in the pellet was determined. Mâtrex beads coupled with nonimmune immunoglobulins were used as control for nonspecific binding.

Sucrose Gradient Sedimentation. Analysis by centrifugation on sucrose gradient was performed by layering 0.2 mL of the sample on 4.3 mL of a continuous 10–30% (w/w) sucrose (Schwarz/Mann, ultrapure) gradient in 20 mM Tris-HCl, pH 7.4, containing 1 mM EDTA and 0.4 M KCl when indicated. After centrifugation with a Beckman SW60Ti rotor in a Sorvall OTD-2 ultracentrifuge (equipped with an ω^2 d*t* integrator), gradients were fractionated by puncturing the bottom of the tube and collecting 10 drops per fraction. The bottom of the tube was cut and also counted. Bovine serum albumin (4.4 S), bovine γ-globulin (7 S), and catalase (11.2 S) were used as standards.

RTF Activation, Nuclear Translocation, and Trypsinization of Receptor. Receptor transforming factor (RTF) was activated by addition of $CaCl_2$ and KCl to the cytosol (Puca et al., 1972) with or without preincubation with 0.1 mg/mL monoclonal antibodies or control antibodies. Analysis of the transformed receptor was performed by DEAE-cellulose chromatography by the published procedures (Puca et al., 1977).

Nuclear translocation was performed according to the method described in the literature (Jensen et al., 1972). In brief, the uterine homogenate in TED buffer was centrifuged at 10000*g* for 20 min. The supernatant was incubated for 4

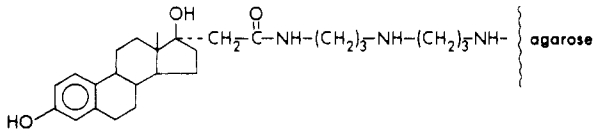

FIGURE 1: Structure of 17β-estradiol affinity matrix.

Table I: Purification of Estrogen Receptor

	sp act. (mol/mg of protein)	recovery (%)	purification (x-fold)
cytosol	8.59×10^{-13}	100	0
heparin-Sepharose	1.30×10^{-11}	60	15
estradiol-Sepharose[a]	2.74×10^{-9}	42	3200

[a] Determination of specific activity was performed after dialysis and concentration of eluted fractions.

h at 0 °C with 10 nM 17β-[6,7-$^3H_2$]estradiol. The 10000*g* pellet was resuspended, filtered through 200 μM mesh nylon gauze, centrifuged, and washed once with 10 mM Tris-HCl, pH 8.3, containing 0.4 M KCl and finally with TED buffer. Aliquots of pelleted nuclei corresponding to 1 mL of homogenate were incubated for 1 h at 24 °C with 1 mL of the prelabeled cytosol. The tubes were then centrifuged, and the pellet was washed with TED buffer and resuspended in 10 mM Tris-HCl, pH 8.3, containing 0.4 M KCl. After 1 h at 0 °C, the suspensions were centrifuged, and the supernatant containing estrogen receptor is referred to as nuclear estrogen receptor. The same experiment was also performed after preincubation of the 17β-[6,7-$^3H_2$]estradiol-labeled cytosol with 10 μg/mL monoclonal antibodies.

The trypsinized estrogen receptor was prepared by incubation of the cytosol at 0 °C for 1 h with 10 μg/mL trypsin (Erdos et al., 1971). The reaction was stopped by addition of a 3-fold molar excess of soybean trypsin inhibitor.

Results

Production of Monoclonal Antibodies. The estradiol receptor from calf uteri was purified by a modification of the published procedure (Puca et al., 1980) with two sequential affinity chromatographic steps. The receptor, eluted from a heparin–Sepharose column, was further purified with a novel estradiol–Sepharose derivative (Figure 1). The diaminodipropylamino-Sepharose was coupled to 17α-(carboxymethyl)-17β-estradiol by a water-soluble carbodiimide. The new derivative has a higher affinity for estrogen receptor than the previously described estradiol 17β-hemisuccinate–Sepharose conjugate (Sica et al., 1973), probably because of the intact 17β-hydroxyl group (Korenman, 1969). Data from a typical purification experiment are shown in Table I. If one assumes a molecular weight of 70000 for the purified estrogen receptor (Molinari et al., 1977; Puca et al., 1978) and one estradiol binding site per molecule, the purity of the receptor was a little more than 20%. A full physiochemical characterization of the estrogen receptor purified according to this procedure is described elsewhere (Nola et al., 1979; Sica & Bresciani, 1979). This receptor preparation was used for immunization. After hybridization of the spleen cells of the immunized mouse with a myeloma cell line (SP2/O-Ag14) that does not synthesize immunoglobulins (Shulman et al., 1978), 30 microtiter plate wells out of 180 were detected to contain hybrids producing anti-receptor antibodies. A few of these positive hybrid populations were cloned on soft agar. Five positive clones were injected intraperitonealy into mice and antibodies purified from the ascites fluids. All the antibodies produced were IgG: the clones JS3/35, JS20/27, and JS28/32

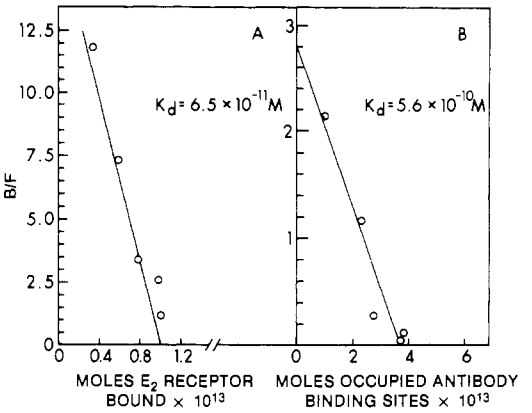

FIGURE 2: Scatchard analysis of receptor–monoclonal antibodies interaction. (A) Various concentrations of 16α-[^{125}I]iodo-17β-estradiol–receptor complex were incubated with a fixed amount of antibodies JS34/32 coupled to Mâtrex-102 (see Materials and Methods) for 16 h at 0 °C. The beads were then pelleted and washed twice, and the radioactivity in the pellet was determined. (B) Aliquots of 17β-[6,7-$^3H_2$]estradiol–receptor complex (0.38 pmol) were incubated for 12 h with five different concentration of purified antibodies JS34/32 in the range 0.5–50 nM at 0 °C in a final volume of 0.25 mL. Aliquots of the reaction mixtures were centrifuged on high-salt sucrose gradient, and the amount of antibodies bound to the receptor was calculated from the area of the peaks of the antibodies–receptor complexes formed.

were producing IgG_1 and the clones JS30/1 and JS34/32 IgG_{2A}. All of them showed a good binding capacity for *S. aureus* protein A. All the clones described here are able to cross-react with estrogen receptor from rat uterus. The clone JS3/35 is directed against an antigenic determinant that is not recognizable in the human receptor, whereas the antibodies from the other clones interact with the receptor from human breast cancer as well as from the MCF-7 cell line. No cross-reactivity is present toward bovine sex steroid binding globulin or bovine α-fetoprotein (data not presented).

Affinity of Monoclonal Antibody for Estrogen Receptor. The equilibrium dissociation constant of the estrogen receptor–antibody complex was determined for antibodies from the clone JS34/32 in solid phase as well as in solution. The purified antibodies JS34/32 were coupled to Mâtrex-102 beads as described under Materials and Methods. A fixed amount of beads was incubated with various concentrations of 16α-[^{125}I]iodo-17β-estradiol–receptor complex, and the data were plotted according to Scatchard as shown in Figure 2A. The K_d for this interaction was 6.5×10^{-11} M. The affinity in solution was determined by analyzing the antigen–antibody complex formation by sucrose density gradient sedimentation (Figure 2B). With increasing antibody concentrations, increasing amounts of complex were found. The amount of occupied antibody binding sites was calculated from the moles of labeled receptor bound. The data were plotted according to Scatchard, and the equilibrium dissociation constant was 5.6×10^{-10} M. It was assumed that the antibody preparation was more than 95% pure and that these monospecific antibodies have two binding sites per molecule with the same affinity for the receptor. These antibodies do not show any binding to estradiol but do recognize unoccupied receptor (Table II).

Characterization of Antibody–Receptor Complexes. Different molecular forms of the estrogen receptor showed no significant variation in their affinity for the antibodies from the various clones tested. The analysis by sucrose density gradient centrifugation of the antibody–antigen complexes showed a different sedimentation behavior with different molecular forms. Addition of increasing amounts of antibodies

Table II: Estradiol Binding of Antibodies JS34/32 Coupled to Matrex-102[a]

preincubation with	[^{125}I]iodoestradiol bound (fmol/mg of resin)
cytosol	143
buffer	<0.5

[a] Aliquots (10 μL) of a 10% suspension of Matrex-102 beads coupled with antibodies JS34/32 were incubated with 50 μL of calf uterus cytosol containing 0.145 pmol of receptor or with 50 μL of TED buffer. After 4 h at 0 °C, the samples were diluted with 2 mL of TED buffer and centrifuged at 2000g for 10 min, and the pellets were washed once with the same buffer. The pellets were resuspended in 200 μL of TED buffer in presence of 10 nM 16α-[^{125}I]iodo-17β-estradiol without or with a 500-fold excess of cold hormone. After an overnight incubation, the samples were washed and centrifuged as above and the pellets counted for radioactivity. Nondisplaceable binding was subtracted from the total bound.

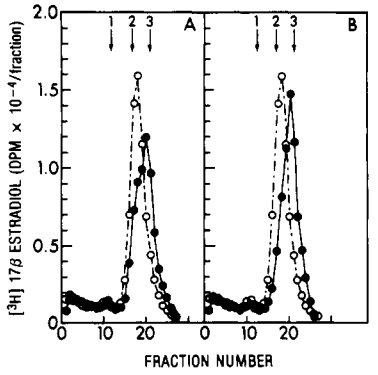

FIGURE 3: Formation of a native 8S receptor–antibody complex. After incubation of the 17β-[6,7-$^3H_2$]estradiol–receptor complex for 12 h at 0 °C with control hybridoma immunoglobin (O) or with antibodies JS34/32 (●), aliquots were centrifuged at 60000 rpm for 13 h in a low-salt gradient. Receptor:antibody molar ratio was 2:1 (A) or 1:2 (B). Migration of various standards, (1) BSA, (2) bovine γ-globulin, and (3) catalase, are marked.

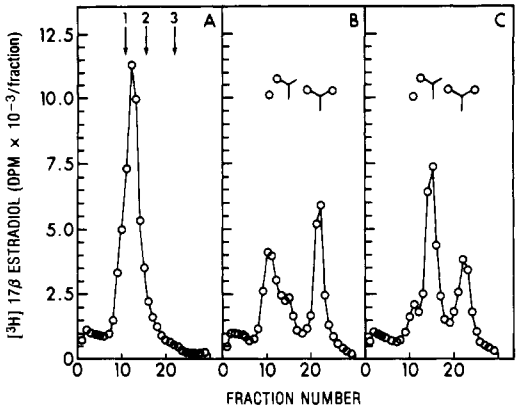

FIGURE 4: Formation of receptor–antibody complexes in the presence of 0.4 M KCl. Aliquots of 17β-[6,7-$^3H_2$]estradiol–receptor complex (0.75 pmol) were incubated with control hybridoma immunoglobulins (A) or with antibody JS34/32 at a receptor:antibodies molar ratio of 1:1 (B) or 1:7 (C) in the presence of 0.4 M KCl. After 10-h incubation at 0 °C, the samples (0.2 mL) were centrifuged on a sucrose gradient containing 0.4 M KCl for 12 h at 50000 rpm. Arrows in (A) show migration of standards: (1) BSA; (2) bovine γ-globulin; (3) catalase. Symbols on top of panels B and C show the type of complex in the corresponding peak: (O) estrogen receptor; (Y) monoclonal antibody.

(clone JS34/32) to the "native" receptor produced a progressive shift of the receptor peak in a low-salt gradient from the 8S to the 11S region. This suggests the formation of a 1:1 molar complex between the receptor and antibody (Figure

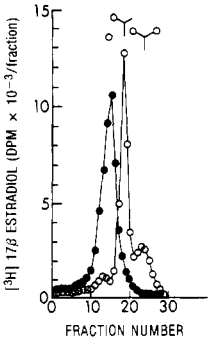

FIGURE 5: Formation of receptor–antibody complexes in the presence of a large excess of antibodies. Aliquots of 17β-[6,7-$^3H_2$]estradiol–receptor complex (0.85 pmol) were incubated in presence of 0.4 M KCl with control hybridoma immunoglobulins (●) or with antibodies JS34/32 (O) at a molar receptor:antibodies molar ratio of 1:50. Samples (0.2 mL) were centrifuged at 60000 rpm for 13 h on a sucrose gradient containing 0.4 M KCl. Symbols on top show the type of complex in the corresponding peak: (O) estrogen receptor; (Y) monoclonal antibody.

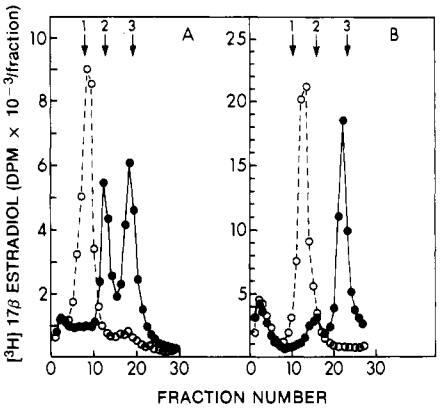

FIGURE 6: Interaction of trypsinized and nuclear receptor with antibodies JS34/32. Experimental details of preparation of trypsinized or nuclear receptor are described under Materials and Methods. (A) Aliquots of the trypsinized receptor–17β-[6,7-$^3H_2$]estradiol complex (0.4 pmol) were incubated with 0.5 μg of control hybridoma immunoglobulins (O) or with 0.5 μg of antibodies JS34/32 (●) in a final volume of 250 μL. After 4 h at 0 °C, the samples (0.2 mL) were centrifuged for 14 h at 48000 rpm on a sucrose gradient in TED buffer. (B) Aliquots of the nuclear receptor–17β-[6,7-$^3H_2$]estradiol complex (3.7 pmol) were incubated with 2.5 μg of control hybridoma immunoglobulins (O) or antibodies JS34/32 (●) in a final volume of 250 μL. After 4-h incubation at 0 °C, the samples (0.2 mL) were centrifuged on a sucrose gradient containing 0.4 M KCl for 12 h at 60000 rpm. Arrows show migration of standards: (1) BSA; (2) bovine γ-globulin; (3) catalase.

3). When the incubation and the gradient separation is performed in high ionic strength (0.4 M KCl), where the receptor dissociates reversibly to a 5S form (Puca et al., 1972), the shift is from the 5S to the 10–11S region, suggesting the formation of a 2:1 complex of receptor to antibody (Figure 4). Upon increasing the concentration of antibodies, the complexed receptor migrates as a symmetric and sharp 7.5S peak, which we assume to be composed of antibody–receptor complex in 1:1 molar ratio (Figures 4 and 5). The sedimentation values of the antibody–receptor complexes are consistent with the theoretical values calculated according to the published procedure (Martin & Ames, 1961), assuming a M_r for the native receptor of 240000 for the low-salt form (8S) and of 118000 for the high-salt form (5S) (Puca et al., 1972). The s value in the same condition for the antibody JS34/32 labeled with [^{35}S]methionine, in absence of the receptor, was 6.8 S. A low-salt stable 4S form derived from a mild tryptic treatment of the cytosol (Figure 6A) and the 5S

Table III: Formation of RTF-Transformed Estrogen Receptor[a]

	fmol of transformed receptor/pmol of total receptor
cytosol alone	93.5
cytosol preincubated with control antibodies	87.5
cytosol preincubated with antibodies JS34/32	88.3

[a] Aliquots of labeled cytosol were incubated for 4 h with 0.1 mg/mL antibodies JS34/32, control antibodies, or buffer alone. The samples were then incubated for 45 min at 0 °C in presence of 0.4 M KCl and 4 mM $CaCl_2$. Control incubations were performed in absence of $CaCl_2$. The samples were then filtered through a Sephadex G-25 column equilibrated in TED buffer with 10 mM KCl (pH 8.3). An aliquot of the void volume peak containing approximately 0.6 pmol of receptor was applied to a 3-mL DEAE-cellulose (DE-52) column equilibrated with the same buffer. The column was washed with 2 mL of buffer and the transformed receptor eluted with 7 mL of 0.12 M KCl in TED (pH 8.3). The amount of receptor transformed was calculated as the difference between the radioactivity eluted from the test column and the radioactivity of the control column, normalized to picomoles of total receptor in the cytosol.

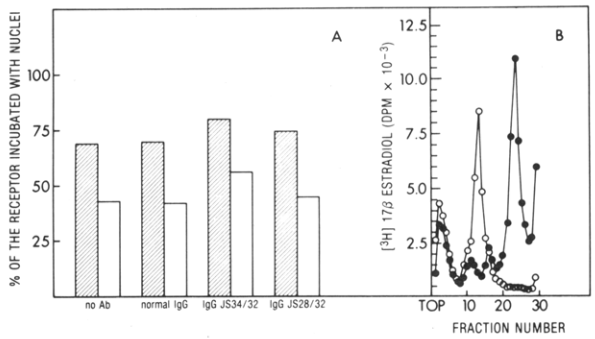

FIGURE 7: Nuclear translocation in the presence of monoclonal antibodies. Experimental details are described under Materials and Methods. (A) Comparison of the nuclear uptake (hatched bars) and extraction of the translocated receptor (open bars) from the nuclei after preincubation of the cytosol with different antibodies as specified. (B) Low-salt sucrose gradient analysis of the translocated receptor extracted from the nuclei after preincubation with control hybridoma immunoglobulins (○) or with antibodies JS34/32 (●). Centrifugation was at 60 000 rpm for 13 h.

form extracted from nuclei after in vitro nuclear translocation (Figure 6B) are both recognized by the antibody JS34/32. These modified forms of the receptor also showed the formation of an antibody–receptor complex of 1:2 or 1:1 molar ratio upon increasing the antibody concentration.

Properties of Monoclonal Antibodies. While the antibodies do recognize unoccupied receptor, certain other functions of the receptor are not affected when it is complexed with the specific monoclonal immunoglobulin. The proteolytic transformation of the native receptor by a specific, Ca^{2+}-activated protease, receptor transforming factor (Puca et al., 1972), is not affected by the presence of the antibodies (Table III). The in vitro translocation of the receptor to the nuclei is not modified by the presence of antibodies JS34/32 or JS28/32 (Figure 7A). After such nuclear uptake of the receptor antibody complex, it is possible to extract the complex with 0.4 M KCl as determined by sucrose gradient analysis (Figure 7B). Furthermore, the nuclear translocation–extraction process is not affected even when two receptor molecules are complexed with one antibody molecule.

Discussion

Since the first successful purification of native estrogen receptor from calf uteri, several attempts have been made to produce antibodies in rabbit or guinea pig. The immune serum from these animals contained a low titer of antibodies that were also able to displace the estradiol molecule from the receptor binding site (unpublished observations). Therefore, any biochemical characterization of these antibodies was not possible in absence of specific labeling of the receptor. Iodination of the purified receptor (by chloramine T or the Bolton–Hunter method) prevents the receptor from being recognized by polyclonal or monoclonal antibodies. Although the method of purification of estrogen receptor from calf uteri was already efficient, a minor modification in the steroid molecule for affinity chromatography made it possible to obtain a receptor preparation with very good antigenic properties.

The screening for anti-receptor antibodies producing clones was performed with a crude receptor preparation in light of the high specificity of the radiolabel for the receptor. Although the selection of clones that were able to interact with the receptor was performed with crude material, the possibility that the antibodies were also able to recognize determinant present in other cytosolic components was excluded. This was evident from binding assays performed with varing receptor concentration; the total binding at saturation was always in good correlation with the number of antibody binding sites used in the assay, assuming 1 mol of estradiol bound per mol of receptor. Furthermore, the fact that the antibodies were able to react with the receptor form produced by mild tryptic proteolysis was a convincing indication that the antibodies were directed toward the steroid binding subunit rather than to another component of the large native receptor present in the crude cytosol. As described in the literature (Erdos et al., 1971), this modified receptor is neither able to aggregate nor able to interact with other cytosolic components.

The monoclonal antibodies obtained exhibit a very high affinity for the estrogen receptor. There is, however, a consistent and appreciable difference in the equilibrium dissociation constant for the receptor–antibody complex as determined in solid phase and in solution. The measured K_d of 0.5 nM in solution is about 1 order of magnitude higher than that obtained in solid phase. The lower affinity in solution cannot be attributed to a dissociation of the complex during the sucrose gradient separation. Other experiments showed high stability of these complexes, and furthermore, no dissociation is evident from the shape of the peaks in the gradient profile. This difference in affinity could be explained by the different local concentration of the reagents when one of the two is immobilized on solid phase. Similar variations have been observed by other authors for the interaction of monoclonal antibodies with β nerve growth factor (Zimmermann et al., 1981) and somatotropic–lactogenic hormones (Ivanyi & Dunbar, 1981).

The sucrose gradient centrifugation is a reliable method to characterize the type of complexes formed. Most of the experiments described above have been performed with the antibodies from the clone JS34/32. These antibodies interact with all the different molecular forms of the estrogen receptor from calf uterus with comparable affinity. However, certain differences in the type of complexes formed with different molecular forms of the receptor confirm some of the hypotheses about the receptor conformation. The so-called native 8.6S form of the receptor always forms a 1:1 complex with the monoclonal antibodies irrespective of whether antibodies or the receptor is in excess. This may suggest either that the native receptor has two determinants per molecule but the antibody is able to internally cross-link them or that the large

size of the 8S form is able to sterically hinder the accessibility of the second binding site on the antibody. If the former would be true, the internal cross-linkage by the bivalent immunoglobulin would be expected to prevent the dissociation of the native receptor (from 8S to 5S form) in 0.4 M KCl. In fact, the antibody-complexed native receptor does dissociate in high-salt conditions (data not presented). The basic assumption in both of the above hypotheses that the high-salt 5S form of the receptor has a single determinant per molecule is supported by the titration experiments performed in high salt or with "low salt stable" forms of the receptor. The high-salt as well as the low-salt forms of the receptor always form a 2:1 complex with antibody when the concentration of the latter is limiting or a 1:1 complex in presence of large excess of antibody. This result confirms not only that these are true monoclonal antibodies but also that all the 4S or 5S forms of the receptor have only one antigenic determinant per molecule for the antibody tested. As the formation of complexes greater than 11–12S is not observed in sucrose gradient experiments, the presence of multiple antigenic sites capable of intermolecular bridging is excluded in all forms of the receptor. These data strongly suggest that the native, large form of the receptor is not a dimer, tetramer, or octamer of the same subunit (Puca et al., 1977) but rather an heteroassociation of proteins. Further investigation with different clones may provide more insight.

The interaction of the monoclonal antibody (clones JS34/32 and JS28/32) does not affect most of the physiological interactions of the estrogen receptor in vitro. The proteolytic activation by RTF is also effective when the receptor is complexed to antibodies. In addition, the nuclear translocation in vitro is unaffected even when the receptor is complexed with the antibodies. It is interesting to note that the translocated antibody–receptor complex can be extracted from the nuclei under the same conditions as can be used for the receptor alone. The analysis of the extracted complex shows a receptor:antibody ratio of 2:1 as expected from the conditions of preincubation. The observation that such a large complex (estimated M_r >270 000) is able to translocate to the nucleus (at least in the in vitro conditions) suggests that the penetration of the nucleus by the transformed receptor does not happen by virtue of its size being smaller than that of the native form, as previously suggested (Puca et al., 1977; Gorsky & Gannon, 1976). Furthermore, the process of activation or transformation induces a specific modification of the receptor molecule in a domain that is itself not modified by the binding of the monoclonal antibodies tested. These antibodies are therefore a potential valuable tool for the comprehension of the molecular events that follow the binding of the hormone to the receptor in the cytoplasm of target cell.

Acknowledgments

We thank Ann P. Leighton for excellent technical assistance and Dr. Pedro Cuatrecasas for helpful discussions and encouragements.

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