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Monoclonal antibodies to rabbit progesterone receptor: Crossreaction with other mammalian progesterone receptors

(hybridoma/uterus/rabbit/human)

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ABSTRACT **A mouse was immunized with purified rabbit uterine cytosolic progesterone receptor (specific activity: 3 nmol of steroid bound per mg of protein). After fusion of its spleen cells with Sp_2-OAg myeloma cells, supernatants of 11 hybrid cultures were found to react in both an immunoenzymatic test and a double-immunoprecipitation test with the progesterone receptor. Clones were obtained from the five hybrid cells that gave the strongest response in both tests. Antibodies from cell culture supernatants and ascitic fluids were characterized. Three are of the IgG1 and two of the IgG2a isotype. Their apparent affinity for the progesterone receptor was measured by immunoprecipitation in physiological salt conditions. The equilibrium dissociation constants were between 0.1 and 4 nM. All five monoclonal antibodies crossreacted with the rabbit nuclear receptor, the human cytosolic receptor, and other mammalian (rat, guinea pig) but not avian (chicken) cytosolic progesterone receptors. There was no interaction with the glucocorticoid receptor and corticosteroid binding globulin.**

The study of steroid hormone receptors has been hampered for many years by difficulties in the purification of these proteins and by the impossibility of using immunological tools for their detection and quantification. Initial progress in this field has been made for the estrogen receptor (1, 2). In the case of the progesterone receptor, preparation of polyclonal antibodies against mammalian receptors (3) and recently against avian receptors (4) has been reported. However for such antigens, which occur in low concentrations and are difficult to purify, the possibility remains of misinterpretations caused by the presence of antibodies directed against proteins contaminating the receptor preparation.

To solve this problem we have undertaken the preparation of monoclonal antibodies against the rabbit progesterone receptor.

MATERIALS AND METHODS

Purification of the Rabbit Uterine Progesterone Receptor. Receptor was purified as described (3). However, to concentrate the receptor and increase the purity a final purification step was added. Receptor eluted from the hydroxyapatite column with 0.2 M sodium phosphate, pH 7.4/30% (vol/vol) glycerol buffer was diluted 1:3.2 in 1 mM sodium phosphate, pH 7.4/30% glycerol buffer. It was applied to a small (0.7-ml) calf thymus DNA-cellulose column. After washing with 10 mM Tris·HCl/1.5 mM EDTA, pH 7.4/30% glycerol buffer (10 ml) and with the same buffer but containing 0.1 M NaCl (10 ml) and finally 5 mM pH 7.4 sodium phosphate buffer (10 ml) the receptor was eluted in 0.8 ml of 5 mM sodium phosphate/0.5 M NaCl buffer, pH 8.3. The specific activity of the receptor preparation that was used for immunization was 3 nmol of steroid bound per mg of protein. Receptor concentration was 750 pmol/0.8 ml.

Immunization. A 3-month-old BALB/c mouse received subcutaneous injections of 375 pmol of receptor. The receptor solution was concentrated 2-fold by lyophilization and emulsified with an equal volume (0.2 ml) of complete Freund's adjuvant. Injections were into the footpads and the back. Two weeks later a second immunization was performed with the same amount of receptor in incomplete Freund's adjuvant. One week later 125 pmol of receptor in 0.2 ml was injected intraperitoneally and the same amount was administered intravenously. Three days later the mouse was sacrificed.

Cell Fusion and Cloning of Hybridomas. Sp_2-OAg myeloma cells (5) and hybridomas were cultured in the ER medium (Eagle's medium reinforced by doubling the amino acids, vitamins and glucose, supplemented with 1 mM pyruvate and 2 mM glutamine) enriched with 10% horse serum (605 Hi; GIBCO). A suspension of spleen cells was prepared in the ER medium. Ten aliquots were prepared and fused separately with $3 \cdot 10^6$ Sp_2-OAg cells on membrane filters in the presence of polyethylene glycol (45%, wt/vol) as described by Buttin *et al.* (6). Twenty hours after fusion the cells were distributed in multiwell dishes. Forty-eight hours after fusion the selective hypoxanthine (50 μM)/azaserine (10 μM) medium (6) was added.

Supernatants of cultures were taken for immunoglobulin assay and detection of anti-receptor antibodies. Cells from the positive wells were cloned by limit dilution (50 cells for 96-well plates). Wells containing single clusters of hybridoma cells were again tested for the presence of anti-receptor antibodies.

Development of Ascites and Preparation of Immunoglobulins. Positive clones were injected intraperitoneally into BALB/c mice previously treated with pristane. Ascitic fluids were harvested and precipitated with ammonium sulfate (50% saturation) and the proteins were dialyzed against phosphate-buffered saline (0.01 M sodium phosphate/0.15 M NaCl, pH 7.4).

Detection of Anti-Receptor Antibodies in Cell Culture Supernatants. Two methods were used to detect the anti-receptor antibodies. In the enzyme-linked immunosorbent assay (ELISA) method the receptor preparation used for immunization was diluted (13.75 pmol/ml of 50 mM potassium phosphate buffer,

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Abbreviations: R5020, 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; ELISA, enzyme-linked immunosorbent assay.

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pH 8). Aliquots (50 μl) were incubated in plastic wells (Nunc immuno 96F). After overnight incubation at 4°C two washes were performed with 100 mM NaCl/2.6 mM KCl/6 mM Na_2HPO_4/1.5 mM KH_2PO_4 buffer, pH 7.4, containing 1‰ Tween-20 and 0.2‰ sodium azide. Bovine serum albumin (1 mg/ml in the wash buffer) was added and incubated for 2 hr at 4°C. Supernatants of cell cultures were diluted 1:3 in the wash buffer and aliquots (70 μl) were incubated for 4 hr at 4°C. After four washes, sheep anti-mouse immunoglobulins conjugated to β-galactosidase (7) were added for 2 hr at 4°C. After five washes the enzymatic activity was measured.

In the double immunoprecipitation method the uterine cytosol (15 mg of protein per ml and 30 pmol of receptor per ml) was prepared from rabbits treated with diethylstilbestrol as described (3). It was incubated for 2 hr at 0°C with 50 nM ^{3}H-labeled 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione (R5020) (specific activity 16 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10} Bq). Aliquots of cytosol (100 μl) were incubated for 18 hr at 0°C with 0.7 ml of cell culture supernatant. Mouse immunoglobulins (10 μg) were added and precipitated by rabbit anti-mouse immunoglobulins. The precipitates were recovered after centrifugation through a cushion of 1 M sucrose and their radioactivities were measured.

Preparation of Steroid–Receptor and Steroid–Corticosteroid Binding Globulin Complexes. Uteri were obtained from rabbits, rats, and guinea pigs. Oviducts were excised from chickens. Liver and plasma were obtained from rabbits. In all cases the animals had been treated with estrogen as previously described (3) to increase progesterone receptor concentration. Human uterine fragments were obtained at hysterectomy, and a preliminary assay of progesterone receptor was performed to select receptor-rich biopsy samples.

Cytosol was prepared (3) in 10 mM Tris·HCl/1.5 mM EDTA/2 mM dithiothreitol buffer, pH 7.4 (3 ml of buffer per g of tissue). The cytosol was incubated with hormones for 2 hr at 0°C. Steroid concentrations were as follows: for rabbit progesterone receptor 50 nM [^{3}H]R5020 (specific activity 87 Ci/mmol) and 1 μM unlabeled cortisol. For rat, guinea-pig, chicken, and human progesterone receptor: 2 nM [^{3}H]R5020 and 1 μM unlabeled cortisol. For rabbit estrogen receptor: 2 nM [^{3}H]estradiol (specific activity 85 Ci/mmol; Amersham). For glucocorticoid receptor: 2 nM [^{3}H]dexamethasone (89 Ci/mmol; Amersham). For the study of corticosteroid binding globulin, plasma diluted 1:12 was incubated with 2 nM [^{3}H]cortisol (specific activity 80 Ci/mmol; Amersham).

Sucrose Gradient Sedimentation. Sucrose gradients (5–20%) were prepared in 10 mM Tris·HCl/1.5 mM EDTA/2 mM dithiothreitol buffer, pH 7.4, containing 10% (vol/vol) glycerol. In some experiments ("high salt") 0.3 M KCl was added. Incubation mixtures (0.135–0.2 ml) were layered on top of 4.9-ml gradients and centrifuged for 14 hr (low salt) or 18 hr (high salt) at 48,000 rpm in a Beckman SW 50.1 rotor.

RESULTS

Detection of Hybridoma Cells Secreting Anti-Receptor Antibodies. The mouse was bled before being killed. Its serum was assayed by the ELISA test against the antigenic preparation. The serum was also incubated with rabbit uterine cytosol containing [^{3}H]R5020–receptor complexes. Sucrose gradient ultracentrifugation and immunoprecipitation analysis showed the presence of anti-receptor antibodies, confirming the results of the ELISA test (data not presented).

Fusion of spleen cells with Sp_2-OAg cells led, after 2 weeks, to the development of hybridoma cells in 130 wells. The 85 wells that contained mouse immunoglobulins were tested for the presence of anti-receptor antibodies. Supernatants from 5 wells were strongly positive by the ELISA test and were able in the double immunoprecipitation assay to precipitate 9- to 17-fold more receptor than supernatants from other wells. The hybrid cells were cloned and injected into mice to produce ascites, and antibodies were further characterized.

Supernatants from six other wells were slightly positive in the ELISA test and in the immunoprecipitation test gave results only about 2-fold above background. These cells were frozen for future characterization.

Immunoglobulins prepared from ascites fluid of the five clones were incubated with [^{3}H]R5020–receptor complexes and centrifuged on sucrose gradients (Fig. 1). Immunoglobulins from two different unrelated hybridomas were used as controls.

All five immunoglobulins displaced the steroid–receptor complexes to higher S values (from 6.5 S to 10 S). Similar results were obtained when steroid–receptor–antibody complexes were analyzed in high-salt gradients: in the presence of antibodies the receptor was shifted from 4 S to 8 S (not shown).

Immunoglobulin Class of Anti-Receptor Antibodies. Immunoglobulin isotypes were characterized with isotype-specific antisera (8) in supernatants of culture media of cloned hybrid cells (Table 1). Three are of the IgG1 κ isotype and two of the IgG2a κ isotype.

Specificity of the Monoclonal Antibodies. Steroid–receptor (or cortisol–corticosteroid binding globulin) complexes were incubated with anti-progesterone receptor immunoglobulins and centrifuged at low ionic strength through density gradients as described for Fig. 1.

The five monoclonal antibodies reacted with cytosolic progesterone receptors from human (Fig. 2), rat, and guinea pig uteri but not with receptors from chicken oviduct (Table 1). However, the stability of the complexes was salt dependent: for instance, the antibody–human receptor interaction was abolished by increasing ionic strength to 0.3 M NaCl. Because a

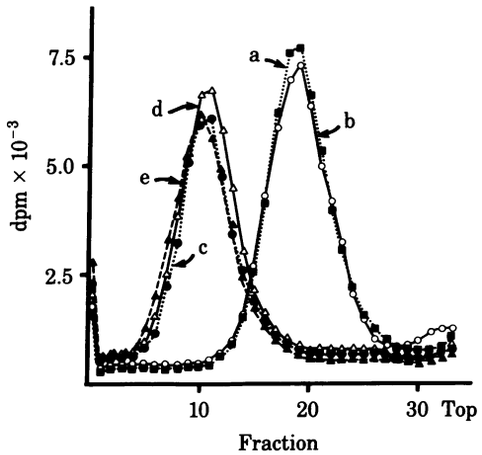

FIG. 1. Study by density gradient ultracentrifugation of the interaction of monoclonal antibodies with the rabbit cytosolic progesterone receptor. Rabbit uterine cytosol was incubated with [^{3}H]R5020. Aliquots (25 μl) were diluted 1:5 with 0.01 M Tris·HCl/1.5 mM EDTA/2 mM dithiothreitol buffer, pH 7.4, and further incubated for 5 hr at 0°C with ammonium sulfate-precipitated immunoglobulins (equivalent to 10 μl of ascites fluid). Subsequently the aliquots (0.135 ml containing 0.2 pmol of steroid–receptor complexes) were centrifuged for 14 hr at 4°C and at 48,000 rpm in a Beckman SW 50.1 rotor. [^{3}H]R5020–receptor complexes were incubated without antibody (curve a), with nonrelated antibody (curve b), with Mi 1-2 (curve c), with Mi 5-31 (curve d), or with Mi 11-5 (curve e). Incubations with Mi 19-5 and Mi 60-10 gave identical patterns and for clarity are not shown.

Table 1. Characteristics and reactivity of the monoclonal antibodies

Monoclonal antibody	Class	K_d,* nM	Reactivity with progesterone receptor			
			Human uterus	Guinea pig uterus	Rat uterus	Chicken oviduct
Mi 1-2	IgG1	1.6	+	+	+	−
Mi 11-5	IgG1	2.6	+	+	+	−
Mi 19-5	IgG1	0.2	+	+	+	−
Mi 60-10	IgG2a	4.0	+	+	+	−
Mi 5-31	IgG2a	0.1	+	+	+	−

*K_d is the apparent equilibrium dissociation constant of antibody–[^{3}H]R5020–rabbit progesterone receptor complexes. Interaction of antibodies with receptors was studied by density gradient ultracentrifugation as described in *Materials and Methods* and Figs. 1 and 2.

high concentration of salt provokes receptor activation the following experiment was performed: [^{3}H]R5020–human receptor complexes were incubated with antibodies of the IgG2a class, precipitated by staphylococcal protein A-Sepharose, and submitted to high salt (0.3 M NaCl). A very rapid solubilization (complete in less than 15 min) of these nonactivated complexes was observed. Moreover, there was no release of hormone from receptor in the presence of the antibodies.

No interaction was observed with glucocorticoid receptor from rabbit liver, corticosteroid binding globulin from rabbit plasma, or free progesterone. At low ionic strength a very slight increase in sedimentation (a reproducible increase of 1–2 gradient fractions) was observed when the Mi 60-10 and Mi 1-2 antibodies were incubated with [^{3}H]estradiol–estrogen receptor complexes. This slight change in sedimentation properties was abolished by increased ionic strength.

Nuclear progesterone receptor was prepared from rabbit uteri (3), incubated with the antibodies, and centrifuged through

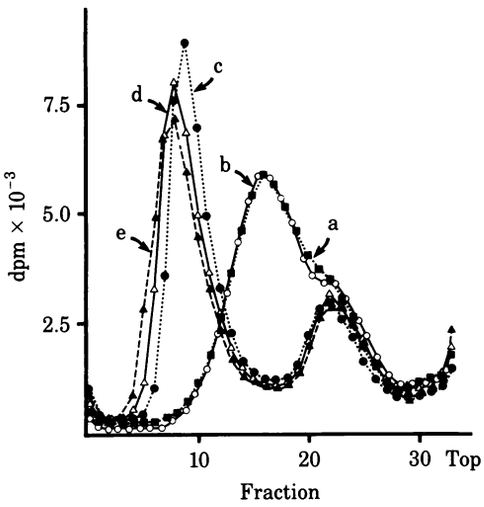

FIG. 2. Study by density gradient ultracentrifugation of the interaction of monoclonal antibodies with the human cytosolic progesterone receptor. Human uterine cytosol was incubated with [^{3}H]R5020. Incubation with monoclonal antibodies and density gradient analysis were performed as in Fig. 1 except that the cytosol was not diluted and the volume was 0.2 ml containing 0.2 pmol of steroid–receptor complexes. [^{3}H]R5020–receptor complexes were incubated without antibody (curve a), with nonrelated antibody (curve b), with Mi 19-5 (curve c), with Mi 5-31 (curve d), or with Mi 60-10 (curve e). Incubations with Mi 1-2 and Mi 11-5 gave similar patterns and for clarity are not shown.

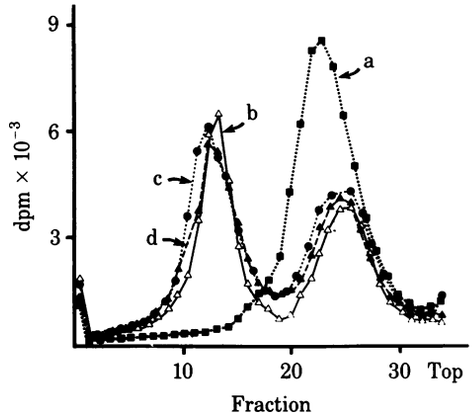

FIG. 3. Study by density gradient ultracentrifugation of the interaction of monoclonal antibodies with the rabbit nuclear progesterone receptor. Nuclear receptor was prepared as described (3) after incubation of uteri with [^{3}H]R5020. Nuclear extract (0.6 pmol of steroid–receptor complexes in 0.194 ml) was incubated for 4 hr at 0°C with ammonium sulfate-precipitated immunoglobulins (equivalent to 6 μl of ascites fluid). Aliquots of the incubation mixtures were centrifuged for 18 hr at 4°C and at 48,000 rpm in a Beckman SW 50.1 rotor. Specific activity of [^{3}H]R5020 in this experiment was 77 Ci/mmol; high-salt (0.3 M KCl) buffer was used. [^{3}H]R5020–receptor complexes were incubated with Mi 19-5 (curve a), Mi 1-2 (curve b), Mi 11-5 (curve c), or with nonrelated antibody (curve d). Incubations with Mi 5-31 and Mi 60-10 gave identical patterns and for clarity are not shown.

density gradients. All five antibodies shifted nuclear [^{3}H]R5020–receptor complexes from 4 S to 8 S (Fig. 3). Some complexes, however, remained in the 4S region even in presence of antibodies.

Apparent Affinity of Anti-Progesterone Receptor Antibodies to Hormone–Receptor Complexes. A constant amount of immunoglobulins was incubated with various concentrations of [^{3}H]R5020–receptor complexes. Antibody-bound complexes were measured by immunoprecipitation with a second antibody, and free complexes were calculated as the difference between total and antibody-bound complexes. Scatchard plot analysis (9) allowed us to determine equilibrium dissociation constants. As shown in Fig. 4, linear relationships were observed; the equilibrium dissociation constants ranged between 0.1 and 4 nM (Table 1).

DISCUSSION

Generation of monoclonal antibodies is an important step towards the experimental approach to many unsolved problems regarding steroid hormone receptors: cellular and subcellular localization, detection and quantification of receptor protein even in possible non-hormone-binding forms, characterization of messenger RNA for receptor, improvements of purification by immunoabsorption, and definition of the functional domains of the receptor molecule. The preparation of several different antibodies should also facilitate these various approaches of receptor structure and function.

The apparent affinity of the monoclonal antibodies for the progesterone receptor was relatively high (K_d = 0.1–4 nM). It was tested under physiological salt conditions because the binding to antigen of monoclonal antibodies is sometimes highly sensitive to ionic strength conditions (11). The preparation of high-affinity antibodies may be due to the characteristics of the detection test used. Immunoprecipitation of nonpurified receptor involving low concentrations of both antigen and anti-

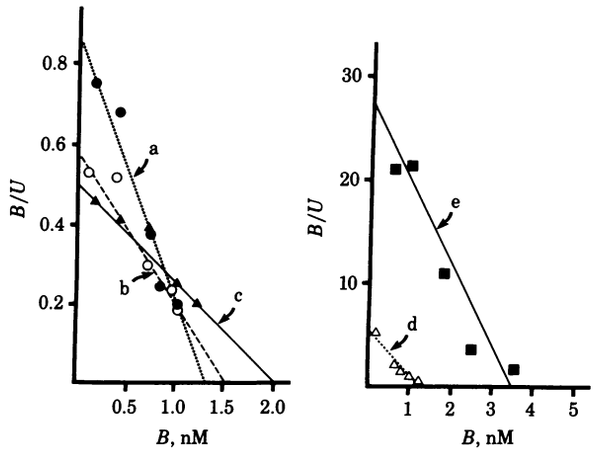

FIG. 4. Affinity of the monoclonal antibodies to the rabbit progesterone receptor. Rabbit uterine cytosol was prepared in 0.01 M sodium phosphate/0.15 M NaCl buffer, pH 7.4, and incubated with 50 nM [^{3}H]R5020 and 1 μM unlabeled cortisol. A parallel incubation was performed in the presence of 1 μM unlabeled R5020 to measure nonspecific binding. Aliquots of the incubated cytosol were then diluted (1:1 to 1:20) by the same buffer containing ovalbumin (2 mg/ml). Ammonium sulfate-precipitated immunoglobulins (100 μl, corresponding to 0.15 μl of ascites fluid) were added to 200-μl aliquots of diluted cytosol. After 18 hr at 0°C, carrier mouse immunoglobulins (10 μg) were added, followed by anti-mouse immunoglobulin serum. The incubation was for 5 hr at 0°C and the precipitates were recovered and their radioactivities were measured. Nonspecific immunoprecipitation was measured by performing parallel incubations but with an unrelated monoclonal antibody. The concentration of steroid–receptor was measured by the dextran/charcoal adsorption method (10). Nonspecific binding was corrected for by measuring radioactive complexes in the incubated cytosol containing excess unlabeled R5020. *B*, steroid–receptor complexes bound by immunoglobulins; *U*, unbound complexes (total concentration of steroid–receptor complexes − *B*). [^{3}H]R5020–receptor complexes were incubated with the following antibodies: curve a, Mi 1-2; curve b, Mi 11-5; curve c, Mi 60-10; curve d, Mi 19-5; or curve e, Mi 5-31.

bodies may select for high-affinity antibodies.

Recently, preliminary communications have been presented by two laboratories (12, §) describing the preparation of a monoclonal antibody against the avian progesterone receptor.

None of the five monoclonal antibodies that have been studied in the present work interacts with the avian receptor, but all crossreact with various mammalian receptors. Their species specificity is thus very similar to that already described for goat polyclonal antibodies (3). The affinity of these antibodies towards the different mammalian receptors in various ionic strength conditions has not yet been studied in detail. This sensitivity to ionic strength might be of interest for the immunoaffinity purification of receptors.

§ Radanyi, C., Renoir, M., Yang, C. R. & Baulieu, E. E. (1982) in *The Endocrine Society*, 64th Annual Meeting, June 16–18, San Francisco, p. 92 (abstr.).

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