Monoclonal antibodies to rabbit progesterone receptor: 
Crossreaction with other mammalian progesterone receptors 
(hybridoma/uterus/rabbit/human)

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Communicated by Jacques Oudin, July 11, 1983

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 Sp2-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 IgGl 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 (0.2 ml) call thymus DNA-cellulose column. After washing with 10 M Tris-HCl/1.5 mM EDTA, pH 7.4/30% glycerol buffer (10 ml)

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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. Sp2-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 H; Gibco). A suspension of spleen cells was prepared in the ER medium. Ten aliquots were prepared and fused separately with 3×10⁸ Sp2-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 limiting 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,

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

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‡‡ Communicated by Jacques Oudin, July 11, 1983
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 [3H]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 SP2-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 [3H]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 antiserum (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-progestosterone 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
Table 1. Characteristics and reactivity of the monoclonal antibodies

<table>
<thead>
<tr>
<th>Monoclonal antibody</th>
<th>Class</th>
<th>$K_d$*</th>
<th>Human uterus</th>
<th>Guinea pig uterus</th>
<th>Rat uterus</th>
<th>Chicken oviduct</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mi 1-2</td>
<td>IgG1</td>
<td>1.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mi 11-5</td>
<td>IgG1</td>
<td>2.6</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mi 19-5</td>
<td>IgG1</td>
<td>0.2</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mi 60-10</td>
<td>IgG2a</td>
<td>4.0</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mi 5-31</td>
<td>IgG2a</td>
<td>0.1</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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

High concentration of salt provokes receptor activation the following experiment was performed:[^3H]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 the 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[^3H]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 density gradients. All five antibodies shifted nuclear[^3H]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[^3H]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-
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 immunoadfinity purification of receptors.

We are grateful to Dr. M. Stanislawski for the determination of the immunoglobulin classes and to Miss E. Barbier for excellent technical help and N. Malpoint for typing the manuscript.