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Antibodies to rabbit progesterone receptor: Crossreaction with human receptor

(breast cancer/pituitary/vagina/nuclear receptor)

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ABSTRACT **Progesterone receptor from rabbit uterine cytosol was purified to a specific activity of ≈2 nmol of bound hormone per mg of protein. A goat was immunized with this preparation and, after two injections of 0.7–0.8 nmol, yielded antireceptor antibodies. The antiserum reacted with both cytosolic and nuclear rabbit progesterone receptor and also with progesterone receptor from other rabbit tissues (vagina and pituitary). A crossreaction was observed with progesterone receptors from other mammalian, especially human, tissues (cytosolic receptor from rat and guinea pig uterus, cytosolic receptor from human breast cancer, and nuclear receptor from human endometrium). On the contrary, there was no interaction with a nonmammalian receptor (chicken oviduct progesterone receptor). The antibodies did not crossreact with other rabbit steroid receptors (uterine estradiol receptor and liver glucocorticoid receptor) or with nonreceptor progesterone-binding proteins (transcortin from plasma and uteroglobin from uterine fluid).**

The functional properties and physiological or pathological variations of steroid receptors have been extensively studied (for review, see refs. 1 and 2). However, little is known about their structure and biosynthesis. This is mainly due to difficulties in purification of the receptors and in obtaining antibodies to them. Some preliminary results have been described on the production of antisera against estrogen (3) and glucocorticoid (4) receptors. However, more detailed characterization of antibodies (5, 6) and even production of monoclonal immunoglobulins (7) have been described only for the estrogen receptor. We report here the preparation and some of the properties of antibodies against the progesterone receptor.

MATERIALS AND METHODS

Buffer. Tris/EDTA buffer (0.01 M Tris·HCl/1.5 mM EDTA/2 mM dithiothreitol, pH 7.4) was used except when stated. Immunoglobulins were kept in 0.01 M sodium phosphate/0.15 M NaCl, pH 7.4.

Steroid. ^{3}H-Labeled R5020 (specific activity, 85 Ci/mmol; 1 Ci=3.7 × 10^{10} becquerels) was obtained from New England Nuclear.

Purification of the Receptor. The method of purification will be reported in detail elsewhere; it will only be summarized here. New Zealand rabbits weighing 1 kg were treated during 15 days with daily subcutaneous injections of 100 μg of diethylstilbestrol in 0.5 ml of sesame oil. Uterine cytosol was prepared and incubated with 0.1 μM ^{3}H-labeled R5020 (specific activity, 2 Ci/mmol). The hormone–receptor complex was precipitated by 10% (wt/vol) Polymin P (Badische Anilin and Soda Fabrik, Ludwigshafen, Federal Republic of Germany), extracted with 0.2 M KCl, and reprecipitated by 33% saturated ammonium sulfate. The pellet was dissolved and chromatographed on DNA-cellulose. After elution with buffer containing 1 M NaCl, the receptor was adsorbed on a column of phenyl-Sepharose (Pharmacia) and eluted with buffer containing 30% glycerol and 40% ethylene glycol. The eluate was applied to a column of hydroxylapatite and finally eluted with 0.2 M sodium phosphate at pH 7.4. The total recovery of receptor was 5–6%; the specific activity was 1900–2100 pmol of bound R5020 per mg of protein.

Immunization of the Goat and Preparation of Immunoglobulins. A female dwarf goat (25 kg) was immunized with an emulsion consisting of 10 ml of 0.2 M sodium phosphate buffer at pH 7.4 containing 700–800 pmol of freshly purified receptor and 10 ml of complete Freund's adjuvant (Calbiochem) containing 37.5 mg of bacillus Calmette–Guérin (BCG; Pasteur Institute, Paris). About 100 intradermal injections were performed on the back of the animal. Simultaneously, perthydral (1 ml, Pasteur Institute) was injected intramuscularly. The second immunization was performed 10 weeks later, the only difference being the use of incomplete Freund's adjuvant. Blood was obtained 6 weeks prior to the first immunization (preimmune serum) and 14 days after each immunization. Serum was precipitated twice with ammonium sulfate (40% of saturation). It was then dialyzed against phosphate-buffered saline and kept frozen at −80°C.

Sucrose Gradient Sedimentation. Sucrose gradients (5–20%) were prepared in Tris/EDTA buffer containing 10% glycerol and 0.3 M KCl. Incubation mixtures (0.15 or 0.2 ml) were layered on top of 4.9-ml gradients and centrifuged 20 hr at 48,000 rpm (except when stated) in a SW 50.1 rotor. Bovine serum albumin (10 mg/ml) was run as a standard protein in a parallel tube.

RESULTS

Detection of Anti-Progesterone-Receptor Antibodies. To detect the anti-progesterone-receptor antibodies we studied the migration of this protein bound to ^{3}H-labeled synthetic progestin R5020 during density gradient ultracentrifugation. However, at low ionic strength there was a nonspecific interaction between the receptor and immunoglobulins from nonimmunized animals. The sedimentation coefficient was shifted from 7.5 S to 9.6 S (not shown). At 0.3 M KCl this interaction was abolished and the receptor migrated at 4.0 S in presence

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Abbreviations: R5020, 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione; iIg, immunoglobulins obtained after the second immunization of the goat (containing antireceptor antibodies); nIg, immunoglobulins obtained from the goat prior to immunization.

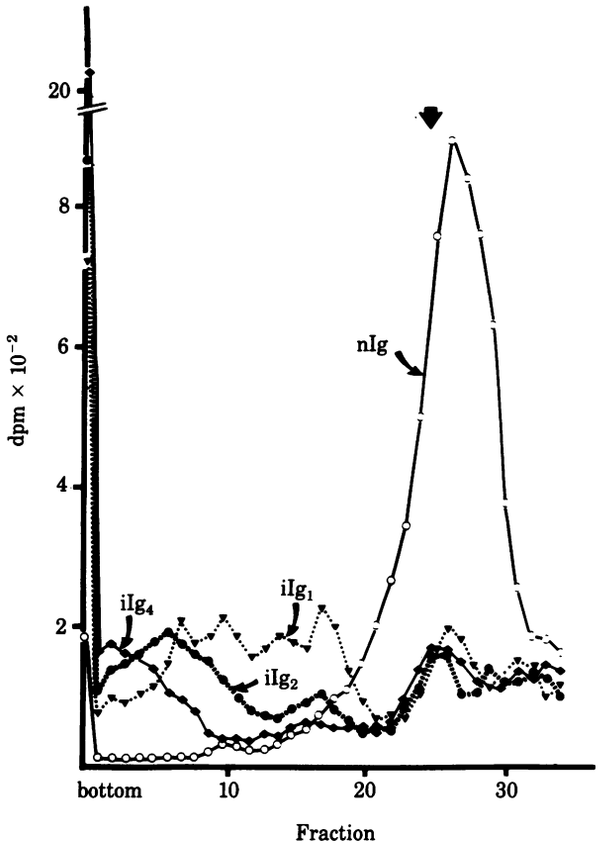

FIG. 1. Detection of antibodies against the cytosolic progesterone receptor from rabbit uterus. Progesterone receptor was purified by Polymin P and ammonium sulfate precipitations in the absence of hormone. The preparation contained 14 pmol of hormone binding sites per mg of protein (1.3 mg of protein per ml). It was incubated for 2 hr at 0°C with 5 nM ^{3}H-labeled R5020. Phosphate-buffered saline containing either 720 μg of nIg or various amounts of iIg (iIg_1, 148 μg; iIg_2, 295 μg; iIg_4, 590 μg) was added to an aliquot (0.1 ml) of the incubation. After 4 hr at 0°C, 150 μl of the incubation was centrifuged for 16 hr on a sucrose gradient. Receptor–steroid complexes in the absence of immunoglobulins gave a pattern identical to that obtained with nIg. Nonspecific binding in this partially purified preparation was extremely low (100 dpm at fraction 26) (not shown). Large arrow, position of bovine serum albumin.

or absence of preimmune immunoglobulins (nIg). For this reason, all further experiments were performed at 0.3 M KCl.

Immunoglobulins interacting with the progesterone receptor were obtained after the second immunization and are designated iIg. Fig. 1 shows the sedimentation at 4 S of the progesterone receptor in the absence of immunoglobulins or in presence of nIg. Incubation with iIg suppressed almost completely the 4S peak. Sedimentation of the receptor varied according to the concentration of immunoglobulins. At a relatively low concentration of iIg, part of the bound hormone was precipitated to the bottom of the tube; some of the complexes migrating between 8.5 and 13.5 S. With increasing concentrations of iIg the proportion of complexes precipitated to the bottom of the tube was increased and practically all the complexes were in this situation after incubation with the highest concentration of iIg.

Tissue Specificity of the Antireceptor Antibodies. Progesterone receptors from vagina and pituitary cytosol were allowed to bind ^{3}H-labeled R5020, incubated with antireceptor immunoglobulins (iIg), and centrifuged through a density gradient. In both cases a major part of the saturable binding in the 4S

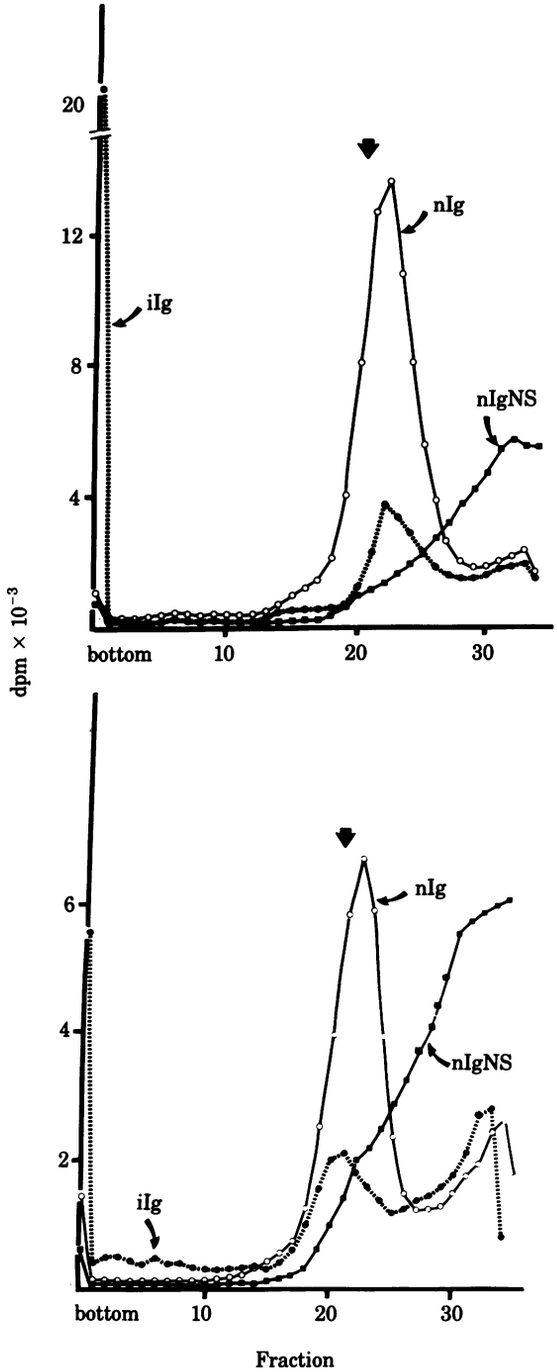

FIG. 2. Effect of anti-uterine-receptor immunoglobulins on progesterone receptor from vagina (*Left*) and pituitary (*Right*) cytosol. Cytosol was prepared from the vagina (1.9 ml of Tris/EDTA buffer per g of tissue) and pituitary gland (0.5 ml of buffer per g of tissue) of rabbits primed with diethylstilbestrol. It was incubated for 2 hr at 0°C with 5 nM ^{3}H-labeled R5020 without or with 1 μM unlabeled R5020 (NS, nonspecific binding). In all cases, 1 μM unlabeled cortisol was added to prevent binding to glucocorticoid receptor. An aliquot of each incubation (0.2 ml) was incubated for 4 hr at 0°C with 0.1 ml of phosphate-buffered saline containing 590 μg of either iIg or nIg; 0.2 ml of each incubation mixture was centrifuged on sucrose gradients. Large arrow, bovine serum albumin.

region was abolished and shifted to the bottom of the tube (Fig. 2).

Interaction Between Antibodies Against Cytosolic Receptor and the Nuclear Receptor. Rabbit uteri were incubated for 30

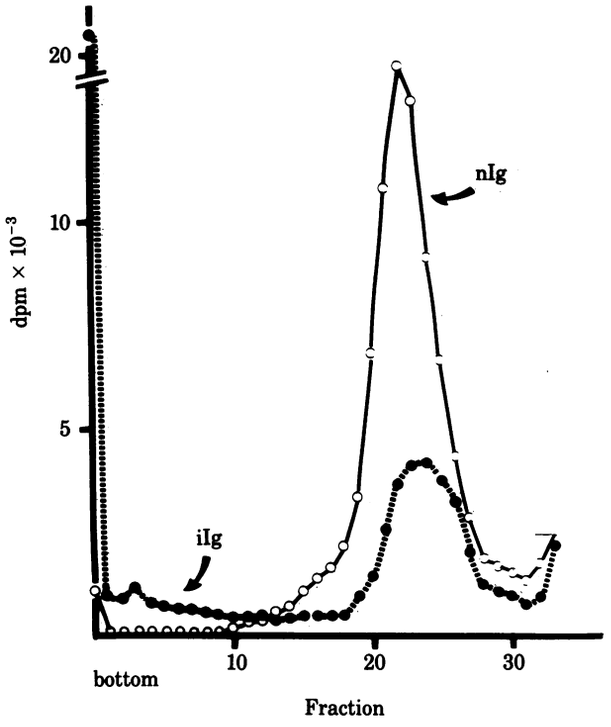

FIG. 3. Effect of anti-cytosolic-receptor antibodies on the nuclear receptor. Uteri from rabbits primed with diethylstilbestrol were cut into cubes of ≈27 mm^3 and incubated for 30 min at 37°C in Eagle's minimal essential medium containing Earle's salts and 25 mM Hepes (GIBCO) (60 ml/10 g of tissue) in the presence of 50 nM ^{3}H-labeled R5020. All further steps were carried out at 0°C. After rinsing, the tissue was homogenized in 75 ml of 10 mM Tris·HCl, pH 7.4/1 mM 2-mercaptoethanol/0.25 M sucrose/3 mM $MgCl_2$, and centrifuged for 10 min at 1000 × *g*. The pellet was resuspended in 25 ml of the same buffer but containing 2.2 M sucrose. After centrifugation for 90 min at 22,000 rpm in a Ti 50.2 rotor, the pellet was washed twice with 8.5 ml of 10 mM Tris·HCl, pH 7.4/1.5 mM EDTA/1 mM 2-mercaptoethanol/0.25 M sucrose and then extracted with 1.5 ml of the same buffer containing 0.3 M KCl but no sucrose. The soluble fraction was incubated for 4 hr at 0°C with either iIg or nIg (590 μg/0.1 ml). An aliquot (0.2 ml) of each incubation mixture was centrifuged on a sucrose gradient.

min at 37°C with ^{3}H-labeled R5020. After homogenization, the nuclei were isolated, purified by sedimentation through concentrated sucrose, and extracted with 0.3 M KCl. The nuclear extract was incubated with iIg or nIg and centrifuged through a sucrose gradient. Most of the nuclear receptor was precipitated to the bottom of the tube after incubation with iIg (Fig. 3).

Effect of Anti-Progesterone-Receptor Antibodies on the Estrogen and Glucocorticoid Receptors. Estrogen receptor from rabbit uterine cytosol was labeled with [^{3}H]estradiol; glucocorticoid receptor from rabbit liver was labeled with [^{3}H]-dexamethasone. Both were incubated with anti-progesterone-receptor immunoglobulins and sedimented through a sucrose gradient (Fig. 4). No change was observed in the sedimentation pattern of these receptors in presence of either iIg or nIg.

Species Specificity of the Anti-Progesterone-Receptor Antibodies. Cytosol was prepared from guinea pig and rat uteri, human breast cancer biopsies, and chicken oviduct magnum. In all cases, progesterone receptor was allowed to bind ^{3}H-labeled R5020 and incubated with iIg or nIg. All mammalian receptors interacted with iIg (Fig. 5). However, in the presence of an identical concentration of iIg, the majority of the human receptor migrated to the bottom of the ultracentrifuge tube whereas with the guinea pig receptor some complexes were at

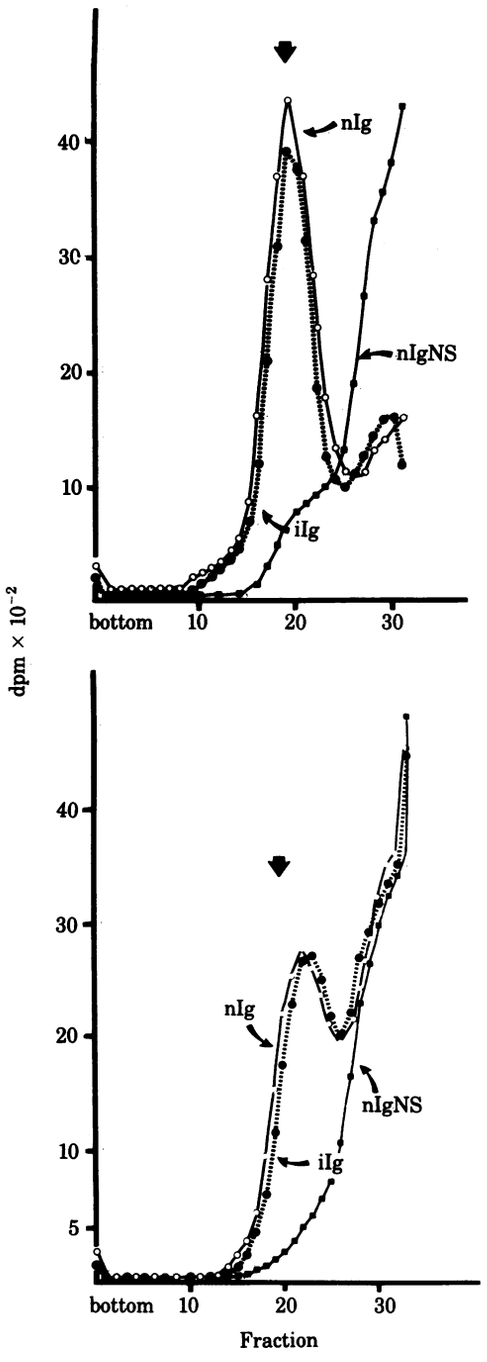

FIG. 4. Effect of anti-progesterone-receptor antibodies on the estrogen and glucocorticoid receptors. (*Left*) Rabbits (1 kg) were injected during 4 days with diethylstilbestrol (5 μg/day). On day 5, they were killed, and their uteri were dissected and homogenized in Tris/EDTA buffer (3 ml/g of tissue). The cytosol was incubated for 2 hr at 0°C with 2 nM [^{3}H]estradiol (specific activity, 91 Ci/mmol). Phosphate-buffered saline (0.2 ml) containing 1190 μg of iIg or nIg was added to an aliquot (0.2 ml) of the incubate. After 4 hr of incubation at 0°C, 200 μl was centrifuged on a sucrose gradient. nIgNS, nonspecific binding measured by incubation of cytosol was in the presence of 2nM [^{3}H]estradiol and 1 μM unlabeled diethylstilbestrol. (*Right*) As in *Left* except that liver was used and [^{3}H]dexamethasone (specific activity, 110 Ci/mmol) was the radioactive hormone. Unlabeled dexamethasone was used in the experiment to measure nonspecific binding (nIgNS).

the bottom of the tube but the majority were found at 10–11 S. In the case of rat receptor there were even less complexes sedimenting to the bottom of the tube or at very high S values.

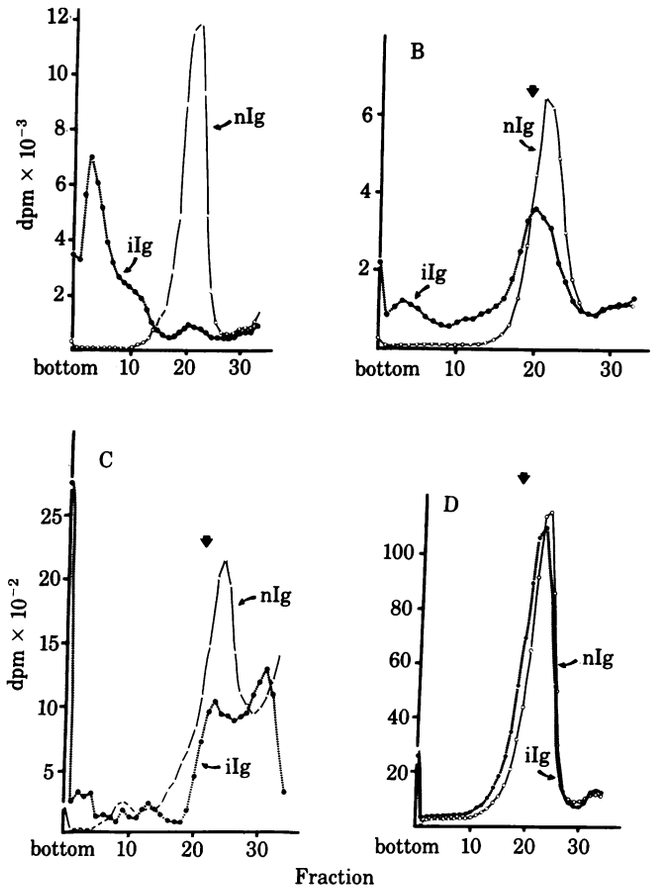

FIG. 5. Species specificity of the anti-progesterone-receptor antibodies. (*A*) Female Hartley guinea pigs (500–700 g) were castrated and 12 days later were treated with diethylstilbestrol (10 μ/day, during 3 days). Their uteri were homogenized (3 ml of Tris/EDTA buffer per g of tissue) and a cytosol was prepared. (*B*) Wistar female rats (200–250 g) were similarly treated except that the diethylstilbestrol dose was 5 μg/day. (*C*) Human breast cancer biopsy specimens were kept in liquid nitrogen. They were thawed at 0°C and homogenized in 4 ml of Tris/EDTA buffer per g of tissue. (*D*) Warren chickens (500 g) were injected with 1 mg of diethylstilbestrol per day during 15 days. Oviduct magnum was homogenized with 3 ml of Tris/EDTA buffer per g of tissue. In all cases, the cytosol was incubated first with 2 nM ^{3}H-labeled R5020 (and 1 μM unlabeled cortisol) and then with nIg or iIg as described in Fig. 1. The incubation mixtures were analyzed by sucrose gradient ultracentrifugation (0.2 ml centrifuged for 20 hr except in the case of breast cancer 0.15 ml was centrifuged for 18.5 hr).

Thus, human, guinea pig, and rat receptors all interact with the anti-rabbit-receptor antibodies but with an apparently decreasing affinity. An opposite result was obtained with the chicken; its receptor sedimentation pattern was not modified by the antibody.

Nuclei were prepared from human endometrium (8–10 weeks of pregnancy). Endogenous progesterone was exchanged with 20 nM ^{3}H-labeled R5020 for 3 hr at 0°C. A nuclear extract, obtained by treatment with 0.3 M KCl, was incubated with immunoglobulins and analyzed by sucrose gradient sedimentation. The human nuclear receptor interacted with iIg but not nIg (not shown).

Anti-Progesterone-Receptor Antibodies and Non-Receptor Progesterone-Binding Proteins. Corticosterone-binding globulin, the plasma protein that binds both natural glucocorticoids and progesterone, was labeled in rabbit plasma with [^{3}H]cortisol and incubated with iIg or nIg. Pure uteroglobin, a progesterone-binding protein from rabbit uterine fluid, was labeled with ^{125}I by lactoperoxidase and incubated with iIg and nIg. In both cases, sucrose sedimentation did not reveal any displacement of radioactivity by iIg or nIg (not shown).

DISCUSSION

Antibodies against the rabbit progesterone receptor were obtained after immunization with a purified preparation. The specific activity of this preparation was ≈2 nmol of bound hormone per mg of protein. If progesterone receptor is composed of steroid-binding subunits of ≈100,000 molecular weight (2), this preparation would be about 20% pure. However, this calculation is based on steroid-binding measurements, and some inactive (non-steroid-binding) receptor may also be present.

In previous experiments, two other goats had been repeatedly immunized without success. Because receptor preparations of lower specific activity were used, it is unknown if the production of antibodies by the third goat is due to immunological characteristics of the animal or to increased receptor purity.

From the density gradient sedimentation studies it appears that several immunoglobulin molecules were bound by the rabbit progesterone receptor. This differs from observations made with the anti-estrogen receptor antibodies. The latter induced a more limited shift in the sedimentation coefficient of the receptor [at high ionic strength the estrogen receptor was shifted from 5–5.2 S to 10–14 S (6), whereas, after interaction with immunoglobulins, the progesterone receptor sediments to the bottom of the tube] (see Figs. 1–3). The anti-rabbit progesterone-receptor antibodies crossreact with receptors from other mammals. However, the modifications of receptor sedimentation properties induced by immunoglobulins vary according to the species. A possible interpretation of this finding is that progesterone receptors of different mammals share a variable number of antigenic determinants. If this hypothesis were true, then human progesterone receptor would have more such determinants in common with rabbit receptor than the guinea pig and the rat receptor. Similarity in ligand specificity between human and rabbit progesterone receptors has been reported (8). These crossreactions with the corresponding receptor from various species are similar to observations made for estrogen receptors (5); however, a difference resides in the fact that anti-rabbit progesterone antibodies do not interact with a nonmammalian receptor (from chicken oviduct) whereas the anti-calf estrogen receptor antibodies do interact with the chicken oviduct estrogen receptor (3).

From density gradient sedimentation studies there appears to be no difference in the interaction between the antibodies and the cytosolic or nuclear receptors from rabbit uterus. There was also no difference among cytosolic receptor from uterus, vagina, and pituitary. However, it must be emphasized that density gradient sedimentation analysis would allow the detection of only major differences in the antigenic determinants. Moreover, there appears to be no common antigenic determinants between progesterone receptors and glucocorticoid and estrogen receptors from the same species. Similar observations have been made for the estrogen receptors (6).

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