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Monoclonal antibody to chicken oviduct progesterone receptor

(steroid receptor/iodinated receptor/hybridoma/immunoglobulin G/breast cancer)

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ABSTRACT Antibodies to molybdate-stabilized chicken oviduct progesterone receptor were raised in a Wistar rat and detected by interaction with homogeneous radioiodinated progesterone receptor. Spleen cells of this rat were then fused with mouse Sp2/0-Ag14 myeloma cells and the antibodies produced by the hybrid cells were detected by double immunoprecipitation using the ^{125}I-labeled receptor. Cells of one of the positive cultures were then cloned by limiting dilution and one hybridoma cell line was studied. The monoclonal antibody produced was an IgG2b, and it reacted with the molybdate-stabilized "8–9S" form of the chicken oviduct progesterone receptor, labeled with either [^{3}H]progesterone or [^{3}H]ORG 2058 (a high-affinity synthetic progestin). K_d for the 8–9S progesterone receptor was ≈1 nM. Progesterone receptor–monoclonal antibody complexes were labeled with radioactive progesterone, suggesting that antibody does not prevent hormone binding. By using a [^{35}S]methionine-labeled antibody, we were able to detect the progesterone receptor independently of its characteristic function of binding radioactive hormone. No crossreaction with human progesterone receptor was detected.

Although the chicken oviduct progesterone receptor (PR) has been extensively studied (for review, see ref. 1), initial attempts to obtain PR antibodies were unsuccessful (2). Now, however, a homogeneous molybdate-stabilized "8–9S" form of PR has become available (3) and we have obtained anti-PR antibodies in a rabbit and a goat (4). The goat antiserum crossreacted not only with mammalian PRs (4) but, more surprisingly, with chicken glucocorticosteroid receptor (5). The latter unexpected result prompted us to obtain monoclonal antibodies (6) to chicken PR.

We now report on the monoclonal antibody BF4 with high affinity for the molybdate-stabilized 8–9S form of chicken oviduct PR. This monoclonal antibody was detected with [^{125}I]-PR, in addition to the conventional use of radioactive hormone receptor complexes. It did not react with human PR.

MATERIALS AND METHODS

Immunization. PR was isolated from estrogen-treated chicken oviduct cytosol and purified as before (3). An adult male Wistar rat received an intradermal injection of 30 μg of purified receptor emulsified with an equal volume of complete Freund's adjuvant (Difco); seven booster injections of 15–80 μg of receptor in incomplete adjuvant were given over a period of 1 yr. For the first two injections, 5–10% pure receptor (7) was used. For the six subsequent injections, 50–100% pure receptor, as described in ref. 3, was used. The rat was sacrificed 3 days after the last injection.

Cell Culture and Fusion Procedure. Mutant myeloma cells Sp2/0-Ag14 (8) were provided by Gérard Buttin (Institut Pasteur) and grown in reinforced Eagle's minimal essential medium (Eurobio, Paris, France) (9).

Exponentially growing mouse myeloma cells were fused with spleen cells by using 50% (wt/vol) polyethylene glycol 4000 and 5% dimethyl sulfoxide according to a published procedure (10) with minor modifications: 5×10^7 myeloma cells were exposed to 4×10^8 spleen cells and plated in selective medium (50 μM hypoxanthine/10 μM azaserine). The next day, surviving cells were counted and 0.1-ml aliquots containing 10^4 cells were plated in the presence of 4×10^5 rat macrophages. The medium was changed once a week. Two to 3 wk later, when the cell concentration had reached 10^5/ml, growing cells were tested for antibody production.

The antibody-secreting hybrids were cloned by limiting dilution in the presence of 2×10^6 mouse thymocytes per ml. Wells containing a single cell cluster were assayed for anti-PR antibody production.

Labeling of Chicken Oviduct Progesterone Receptor. *Iodination.* Highly purified (>90% pure) molybdate-stabilized PR (6 μg) was labeled with 1 mCi of ^{125}I (specific activity, ≈2,200 Ci/mmol; 1 Ci = 37 GBq; New England Nuclear) by using the Bolton and Hunter reagent (11).

Radioactive steroid. The PR was labeled with 20 nM [^{3}H]-Progestin ([^{3}H]progesterone; specific activity, ≈96 Ci/mmol) or [^{3}H]ORG 2058 (16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]-pregn-4-ene-3,20-dione; specific activity, ≈42 Ci/mmol), both from the Radiochemical Centre, in the presence of 1 μM cortisol for 2 hr at 0–4°C and then treated for 10 min with dextran-coated charcoal [0.5–5% (wt/vol)]. The concentration of the receptor–ligand complexes was ≈10 pmol/ml.

Human Mammary Cancer Cytosol. PR-containing cytosol was prepared from MCF-7 cells (12) and total tumor, as described (4).

Ultracentrifugation Study of Immunocomplexes. Samples of antibody–receptor complexes in (final vol, 200–250 μl) TEK-M buffer [0.01 M Tris·HCl/1.5 mM EDTA/10% (vol/vol) glycerol/12 mM 1-thioglycerol/0.15 M KCl/0.02 M Na_2MoO_4, pH 7.0, at 25°C] containing 0.3 mM phenylmethylsulfonyl fluoride, 50 μg of horseradish peroxidase (Sigma, type VI) (3.6 S), and 50 μg of fungal glucose oxidase (Boehringer Mannheim) (7.9 S) as internal standards were ultracentrifuged as described (4).

Double Antibody Precipitation of Immunocomplexes. Mixtures of pure ^{125}I-labeled PR (≈5,000 cpm), hybridoma medium (100–150 μl), and 50 μg of pure rat IgG (Miles) in (final vol, 200–250 μl) TEK-M buffer were incubated overnight at 0–4°C. Crude Igs obtained after injection of rat IgG in a goat were added to precipitate rat IgGs. Two hundred to 250 μl of the incubation mixture was layered on 100 μl of 0.25 M sucrose in TEK-M buffer in a 400-μl Beckman centrifuge tube, and the tubes were centrifuged and then frozen in liquid nitrogen and

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Abbreviations: PR, progesterone receptor; IgG-G0, nonimmune goat IgG; IgG-G3, immune goat IgG; [^{3}H]ORG 2058, 16α-ethyl-21-hydroxy-19-nor[6,7-^{3}H]-pregn-4-ene-3,20-dione.

cut above the pellet. The pellet and an aliquot of the supernatant were assayed in a Minigamma counter (LKB).

For affinity measurement, [^{3}H]ORG 2058–PR complexes were incubated overnight at 0–4°C with monoclonal antibody. The binding of the ^{3}H-labeled ligand to the receptor (whether or not complexed to the antibody) was measured by adsorption to hydroxylapatite (13). Specific immunocomplexes were measured by the double-immunoprecipitation technique described above. After immunoprecipitation, the hydroxylapatite adsorbent and pellets were assayed in 10 ml of Ready-solv MP solution (Beckman) with ^{3}H assay efficiencies of 40% and 50%, respectively.

Partial Purification of Monoclonal Antibody. The monoclonal antibody was precipitated from the medium by two treatments with ammonium sulfate (50% saturation). Crude Ig fractions were dialyzed against 5 mM potassium phosphate (pH 8) and fractionated by DEAE-cellulose chromatography (14). The class and the subclass of the monoclonal Ig were determined by Ouchterlony analysis.

[^{35}S]Methionine-Labeled Antibody. Exponentially growing cells (7 × 10^6) were centrifuged, suspended in 5 ml of methionine-free reinforced Eagle's minimal essential medium (reconstituted from GIBCO minimal essential medium kit) containing 250 μCi of [^{35}S]methionine (specific activity, ≈1,245 Ci/mmol; Radiochemical Centre), and incubated at 37°C for 5 to 6 hr. The medium was dialyzed against phosphate-buffered saline to remove free [^{35}S]methionine.

Production of Ascites. Hybridoma cells (10^7) were injected intraperitoneally into *nude* mice primed 8–10 days earlier with 0.5 ml of pristane. Ten to 15 days later, ascitic fluid was collected.

RESULTS

Iodinated Progesterone Receptor as a Probe for Detecting Antibodies. To detect small amounts of anti-PR antibodies, highly purified 8–9S PR was labeled with ^{125}I. After the iodination process, the sedimentation coefficient was determined to be 6 S. This difference between S values of the two receptors is discussed below. Because the native form and the iodinated PR had different sedimentation coefficients and no other radioiodinated steroid receptor has yet been found to be immunoreactive, we carried out experiments using goat anti-PR IgG "IgG-G3" (4). The sedimentation coefficient of the radioactive receptor shifted from ≈6 to ≈16 S in 10–35% glycerol gradient ultracentrifugation, as shown in Fig. 1*A*. No such effect was observed when the ^{125}I-labeled PR was incubated with nonimmune goat IgG (IgG-G0). There was an increase of radioactivity at the bottom of the tube in the presence of IgG-G3, as described previously for the native noniodinated receptor (4).

In an additional experiment, [^{3}H]progesterone-labeled receptor from crude cytosol was used as a competitor for binding ^{125}I-labeled PR to IgG-G3. Gradient ultracentrifugation analysis showed that the 6S radioactive receptor was shifted to ≈12 S after incubation with IgG-G3. Addition of [^{3}H]progesterone cytosol receptor to the incubation medium completely abolished the ^{125}I peak of the immunocomplexes. Concomitantly, ^{3}H-labeled immunocomplexes were observed in the 12- to 15-S region and at the bottom of the tube (Fig. 1*B*), instead of the iodinated receptor which, as predicted, again sedimented at the 6-S position.

These results show that the ^{125}I-labeled protein is PR and that it is immunoreactive and can be used as a probe for detecting antibodies.

Rat Antiserum. After incubation of the ^{125}I-labeled PR with rat antiserum, a shift of the 6S radioactive receptor to ≈14 S was shown by gradient ultracentrifugation analysis. No such interaction was observed with nonimmune rat serum, as shown in Fig. 1*C*.

The complex of immune rat serum antibodies and ^{125}I-labeled PR was quantitatively precipitated by anti-rat IgGs (data not shown).

Antibody Production by 41G7 Hybrid Cells. The double-immunoprecipitation technique was also used to detect anti-PR antibodies secreted by the hybrid cells. Microtiter wells con-

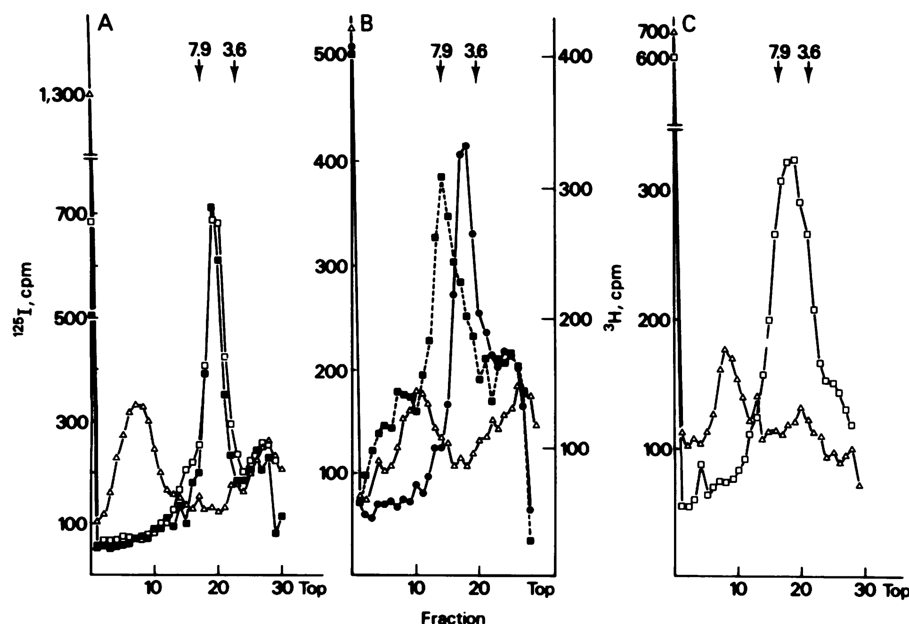

FIG. 1. Interaction of ^{125}I-labeled PR with goat IgGs and rat serum. Sedimentation profiles in 10–35% glycerol/TEK-M buffer gradients of ^{125}I-labeled PR (*A*) alone (■) or in the presence of 50 μg of IgG-G0 (□) or 50 μg of IgG-G3 (△), ^{125}I-labeled PR that had been incubated with 10 μg of Ig-G3 (*B*) in the absence (△) or presence (●, ^{125}I; ■, ^{3}H) of ^{3}H-labeled progesterone-PR (0.05 pmol) or ^{125}I-labeled PR in the presence (*C*) of 50 μl of nonimmune (□) or immune (△) rat serum. After 6 hr of incubation at 4°C, samples were centrifuged for 16.5 hr at 220,000 × *g*. ——, ^{125}I; ----, ^{3}H.

taining proliferative hybrids (143/504) were scored positive for anti-PR antibody production if they secreted enough antibody to complex at least twice the amount of ^{125}I-labeled PR measured when control medium or medium from negative wells was used. Culture 41G7 was positive for anti-PR antibody production.

Monoclonal Antibody BF4. Cells of the 41G7 culture were cloned immediately and the secretion of Igs was checked with the same immunoprecipitation assay.

One clone (BF4) was expanded. Immunocomplexes were formed by incubation of the culture medium with crude cytosol containing molybdate-stabilized PR labeled with tritiated steroid. These complexes could be detected only when the medium was concentrated more than 50-fold by ammonium sulfate precipitation. The precipitate was further fractionated by DEAE-cellulose chromatography (14), and anti-PR antibody was recovered in the 50 mM potassium phosphate eluate. In the presence of the culture medium from the myeloma cells, the [^{3}H]-steroid receptor sedimented at 8–9 S in TEK-M buffer. The peak was displaced to 11 S when BF4-purified antibody that had been incubated with the [^{3}H]steroid receptor was used (Fig. 2*A*). As in previous studies (4), cytosol-containing molybdate-stabilized [^{3}H]progesterone-labeled 8–9 S PR also displayed a tritiated 4 S peak after incubation in protein-rich medium (Fig. 2*A*). An additional experiment was conducted using hormone-free PR: unlabeled cytosol was incubated first with BF4 antibody and then with 20 nM [^{3}H]progesterone. After gradient ultracentrifugation in 10–35% glycerol, radioactive immunocomplexes were observed in the 11 S region. Again, an additional lighter peak was observed. That the 11 S fraction represented receptor-containing complexes was further indicated by the decrease of radioactivity in this fraction when nonradioactive progesterone (20 nM) was also added at the time of labeling (Fig. 2*B*). These results suggest that monoclonal antibody BF4 does not prevent binding of the hormone to the receptor. There was no direct binding of the steroid to the antibody (data not shown).

The affinity of antibody BF4 for the PR was determined by using the double immunoprecipitation technique, with [^{3}H]-ORG 2058 to label the receptor. This radioactive ligand displays high affinity for the receptor and therefore is more convenient for quantitative experiments. As determined from the Scatchard plot, K_d was $\approx 1 \times 10^{-9}$ M, based on radioactive ligand binding and using the partially purified antibody and cytosol (Fig. 3).

The elution pattern on DEAE-cellulose chromatography and Ouchterlony analysis with the appropriate sheep antiserum indicated that monoclonal antibody BF4 is an IgG2b (data not shown).

When [^{35}S]methionine was included in the culture medium of hybridoma BF4, a radioactive peak sedimenting at 7 S was observed in glycerol gradients. On incubation of the ^{35}S-labeled IgGs with cytosol containing molybdate-stabilized PR, the peak of radioactivity shifted to the 10- to 11-S region (Fig. 4). Addition of unlabeled monoclonal antibody BF4 to the incubation medium almost completely abolished this 10- to 11-S radioactive peak and [^{35}S]methionine radioactivity was recovered in the 7-S region (data not shown).

Monoclonal Antibody in the Ascitic Fluid. After injection of hybridoma cells into five *nude* mice, $\approx$10 ml of ascitic fluid was collected. Gradient ultracentrifugation analysis indicated that the antibodies shifted the 8–9S PR to $\approx$11 S (data not shown).

Lack of Interaction Between Human PR and Monoclonal Antibody. The interaction of antibody BF4 with human PR (breast cancer and MCF-7 cell cytosol) was studied by gradient ultracentrifugation analysis. When the antibody was treated with excess PR, no shift in the sedimentation coefficient of ^{35}S-labeled BF4 was observed. In another experiment, the same amount of PR–[^{3}H]progesterone complex was incubated with a large amount of antibody BF4 (from ascitic fluid) and no dis-

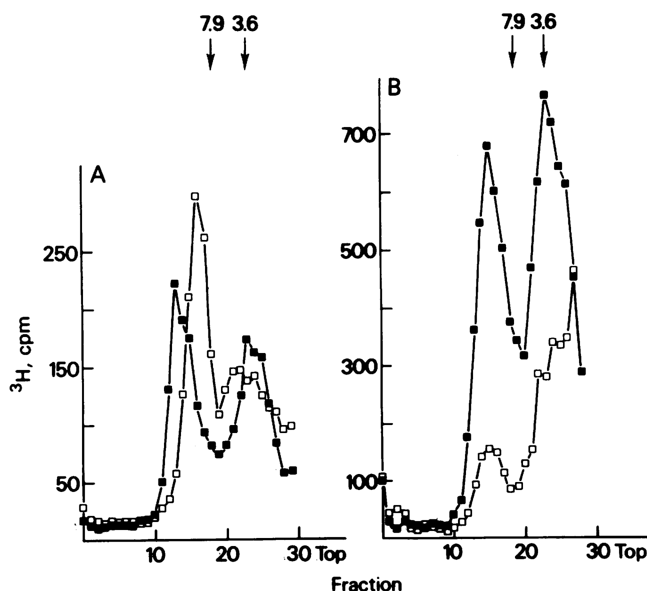

FIG. 2. Interaction of [^{3}H]Progestin–PR complexes with monoclonal antibody BF4. Sedimentation profiles in 10–35% glycerol/TEK-M buffer gradients of PR from chicken oviduct cytosol. (*A*) PR (0.05 pmol) was labeled with [^{3}H]ORG 2058 and then incubated for 24 hr with 200 μl of monoclonal antibody BF4 (total protein, 450 μg) (■) or with 200 μl of myeloma cell culture medium (□). (*B*) PR (0.15 pmol) was incubated for 24 hr with 200 μl of monoclonal antibody BF4 and then labeled with 20 nM [^{3}H]progesterone in the absence (■) or presence (□) of 20 nM progesterone for 2 hr. Mixtures were centrifuged for 17 hr at 220,000 × *g*.

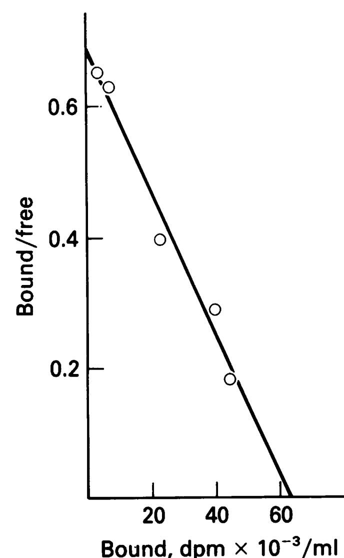

FIG. 3. Scatchard analysis of specific equilibrium binding of [^{3}H]-ORG 2058-labeled PR to partially purified monoclonal antibody BF4. BF4 (a partially purified preparation containing 400 μg of protein) was incubated with [^{3}H]ORG 2058-PR (0.05–2.5 pmol) for 20 hr at 4°C in (final vol, 500 μl) TEK-M buffer containing 50 μg of rat IgG (Miles). A 200-μl aliquot was layered on a hydroxylapatite microcolumn and 50 μl of crude goat Ig against rat IgG was incubated with another 200-μl aliquot for 5 hr at 4°C to determine, respectively, the concentration of total receptor present in the incubation mixture and the concentration of receptor bound to antibody. Specific binding was measured by subtracting nonspecific binding observed after incubation of the PR with myeloma cell culture medium.

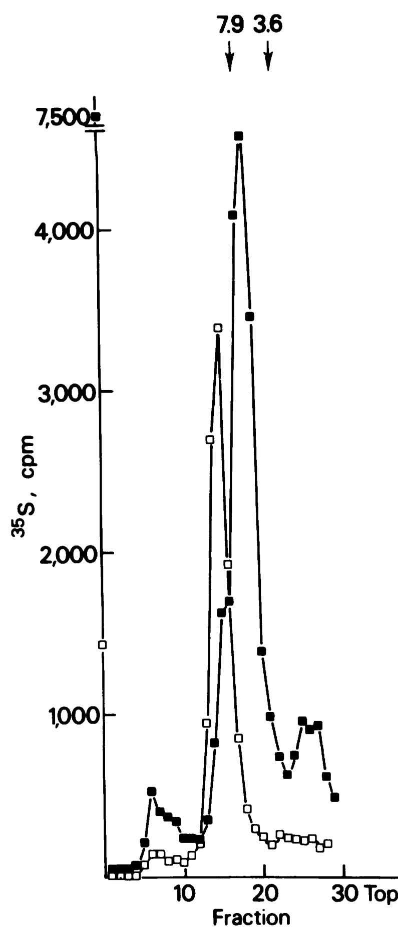

FIG. 4. Interaction of progesterone–PR complexes with ^{35}S-labeled monoclonal antibody BF4. Sedimentation profiles in 10–35% glycerol/TEK-M buffer gradients of ^{35}S-labeled monoclonal antibody BF4 (200 μl) alone (■) or after incubation of a 50-μl aliquot with excess PR (0.25 pmol) complexed to unlabeled progesterone (□) for 24 hr at 4°C. Samples were centrifuged for 17 hr at 220,000 × *g*.

placement of ^{3}H radioactivity was detected (data not shown). Control experiments using goat IgG-G3 gave the same results as those previously described (4), confirming that human PR was immunoreactive.

DISCUSSION

Steroid receptors are immunogenic: antisera have been obtained after injection to rabbits and goats of calf uterus estradiol receptor (15–18), human myometrium receptor (19), rat liver glucocorticosteroid receptor (20, 21), rabbit uterus PR (22), and chicken oviduct PR (4). It is surprising that previous attempts to generate antibodies against purified chicken oviduct PR were unsuccessful (2). "Spontaneous antibodies" that bind chicken oviduct PR have, however, been observed in sheep serum (23).

Antibodies against calf uterus estradiol receptor crossreact with chicken oviduct estradiol receptor (17, 18). In the PR series, although no antibody against the rabbit uterus receptor has been found to crossreact with the chicken PR (22), we found antibodies against PR that crossreact with mammalian PR, which, however, complexed less mammalian PR than chicken PR (4).

Antibodies to chicken oviduct PR were obtained by immunizing a rat with the molybdate-stabilized 8–9S form of this receptor (3). The first two injections were made with 5–10% pure PR and the booster injections, with 50–100% pure PR. It is not known whether antibody production was due to increased receptor purity in the last injections.

Interaction between [^{3}H]Progestin–PR complexes and rat antiserum was not detectable in preliminary experiments. This was probably due to low antibody/antigen concentration ratios or to high nonspecific interactions between crude cytosol and serum. To get a more sensitive reagent for antibody detection, homogeneous 8–9S PR was labeled with ^{125}I. The 6 S sedimentation coefficient of the ^{125}I-labeled PR could result from a structural change or partial proteolysis during iodination. However, the ^{125}I-labeled PR remained immunoreactive, making it possible to detect antibodies in the rat serum and to screen hybrids after fusion. In contrast to the antibodies previously produced in the rabbit and in the goat, the monoclonal antibody provoked a more limited shift in the sedimentation coefficient of the 8–9S receptor. This suggests that only one molecule of antibody interacts with each molecule of PR.

Binding experiments showed that the monoclonal antibody had a high affinity for PR, but the titer seemed to be low. Several methodological difficulties were encountered. (*i*) Because the titer and affinity measurements were carried out with partially purified antibody and crude cytosol, the affinity of antibody for PR may be overestimated and the titer, underestimated, as the cytosol could contain some PR that had lost the ability to bind hormone but remained antigenic. (*ii*) Recently, monoclonal antibody BF4 has also been observed to interact with chicken oviduct glucocorticosteroid receptor (5). This receptor, which is present in the cytosol, probably competes with the PR for binding to the antibody and similarly could influence affinity and titer evaluations. (*iii*) In addition, we observed that the second antibody did not precipitate all the IgG present in the incubation medium and also that it provoked a partial dissociation of receptor–hormone complexes, thus leading to underestimation of the concentration of bound hormone. Technically, because BF4 is an IgG2b and does not bind to protein A, use of the protein A-Sepharose adsorption technique was excluded (14); moreover, the gradient ultracentrifugation would not have sufficient resolution to allow determination of the parameters of interaction between antibody and receptor. We have therefore concluded that the double antibody precipitation technique is actually the most accurate method to estimate affinity and titer of antibody.

We found no crossreactivity of monoclonal antibody BF4 and the human receptor extracted from MCF-7 cells and breast cancer tissue. Similar results concerning the absence of crossreactivity of monoclonal antiestradiol receptor antibodies between mammalian and nonmammalian species have been published (24). However, no general statement should be made concerning the species specificity of monoclonal antibodies against steroid receptors. We do not exclude the possibility that a monoclonal antibody might be found against chicken PR that could react with mammalian (including human) PR, which would be useful for clinical purposes.

Note Added in Proof. After this paper was submitted for publication, an abstract appeared on *in vitro* production of monoclonal antibodies to PR (25).

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