Immunocytochemical Localization of Progesterone Receptors in Breast Cancer with Anti-Human Receptor Monoclonal Antibodies

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ABSTRACT

Monoclonal antibodies (MAbs), recently produced against human progesterone receptors (PR), were used for immunocytochemical localization of PR. The specificity of the immunocytochemical assay for PR was demonstrated by incubation with control MAbs, preabsorption of MAbs with highly purified human PR, and by the cell and tissue distribution of the immunostaining reaction. With human breast cancer cell lines, immunoreactivity was confined to cells that contain PR by steroid-binding assay. Moreover, immunostaining was induced by estradiol in estrogen-responsive cells, MCF-7 and ZR-75-1. In a preliminary study with 33 breast carcinomas, a good correspondence was obtained between immunocytochemical staining and PR content assessed by conventional steroid-binding assay. Immunoperoxidase localization was also obtained with other human target tissues. In normal breast and benign breast disease, immunoreactivity was observed with nuclei of ductal epithelial cells and hyperplastic epithelium. In uterus, immunostaining of endometrium was localized to nuclei of stromal and glandular epithelial cells and in myometrium to nuclei of smooth muscle cells.

The effect of the progesterin agonist, R5020, and antagonist, RU 486, on PR localization was investigated with the PR-rich T47D human breast cancer cell line. In the absence of hormone, immunostaining was exclusively nuclear. This was true under a number of cell culture conditions designed to eliminate endogenous progestins from the culture medium. Exclusive nuclear localization of PR was not due to a failure of the MAbs to recognize unoccupied PR, since each MAb bound equally well in vitro with different receptor forms. These included liganded and unliganded cytosol PR, molybdate stabilized PR, and nuclear-transformed receptors. Nor was failure to detect cytoplasmatic staining due to a selective destruction or loss of unoccupied PR from the cytoplasmic compartment as a result of cell fixation. This was assessed by dot blot immunoadassay of PR antigen distribution in subcellular fractions of fixed and unfixed cells. Continuous exposure of cells to R5020 resulted in a transient (30–60 min) increase in nuclear staining intensity (without change in cytoplasmic reactivity), followed by a progressive decline in immunoreactivity. By 24 h of R5020 treatment, the vast majority of cells displayed no immunostaining reaction. These immunocytochemical data are consistent with progestins down regulating their own receptors due to a loss in cellular PR content and not to inactivation of receptors. Continuous exposure of cells to RU 486 also resulted in a transient increase in nuclear PR staining, but immunoreactivity remained relatively unchanged after 24 h of treatment. Thus, receptors bound with RU 486 are not down regulated to the same extent as they are by progestins.

INTRODUCTION

PR have been studied extensively for their fundamental properties in eliciting the biological effects of progestins in endocrine target tissues (see References 1–3 for reviews). PR have also been studied in human breast cancer and are well established as an important clinical marker. Patients with PR-positive tumors have a higher probability of responding to endocrine therapy, better prognosis, and longer disease-free survival than women with PR-negative tumors (4–9). Most basic and clinical studies have employed radioligand-binding assays with cell or tissue extracts as the method of receptor detection. The recent development of antibodies to PR provides an important advancement in our ability to detect and study receptors. As probes which recognize receptor protein directly, antibodies can be used to detect receptors in both liganded and unliganded forms, under the denaturing conditions of many high resolution protein separation techniques, and in histological tissue sections by immunocytochemical staining methods. Immunocytochemistry provides the opportunity to identify the specific cells in target tissues that contain PR, and explore the dynamics of intracellular PR localization in response to changes in the hormonal environment. In breast carcinomas, immunocytochemical detection of PR is of potential clinical value since this assay has the advantage of not requiring large tissue samples, and will provide information on cellular heterogeneity and PR content in specific cell types.

The physicochemical properties of PR have been most extensively characterized in chicken oviduct (1, 2, 10, 11), rabbit uterus (12, 13), and human breast cancer cell lines (14, 15). Chick and human PR have been found to consist of A and B hormone binding proteins that differ in molecular mass. Chick A and B receptors have been reported to be Mr, 79,000 and 110,000, respectively. Human A and B proteins are larger, with apparent molecular weights of 94,000 and 120,000. The rabbit uterine system, however, appears to contain a single steroid binding subunit comparable in size to the B protein of human PR (see review in Reference 16). MAbs have now been produced to isolate each of these systems. Toft and coworkers (17) produced five MAbs to chick oviduct PR, one of which (PR-6) cross-reacts with human B receptors. Logeat et al. (18) prepared several MAbs against rabbit uterine PR. Two are cross-reactive with human PR, but also appear to react with human B receptors only (19). More recently, Clarke et al. (20) produced MAbs against A and B forms of PR from human uterus, and Estes et al. (21) reported production and characterization of MAbs to PR purified from human breast cancer cells.

The present study describes the first use of these anti-human PR MAbs for immunocytochemical localization of PR in breast carcinomas and normal human target tissues. Using breast cancer cell lines as a model system, we have also explored the influence of progestin agonists and antagonists on PR localization.

MATERIALS AND METHODS

Materials. [3H]R5020 (promegestone; 17,21-dimethyl-19-norpregna-4,9-diene-3,20-dione-1[7α-methyl-3H]; 87 Ci/mmol) and unlabeled R5020 were from New England Nuclear Corp. (Boston, MA). [14C]-labeled molecular weight standards for SDS-polyacrylamide gels were...
from Bethesda Research Laboratories. Nitrocellulose filters (pore size, 0.45 μm) were obtained from Schleicher and Schuell (Keene, NH). Protein A-Sepharose CL-4B, bovine serum albumin, fraction V (96–99% purity), dextran (clinical grade), and activated charcoal (250–350 mesh) were obtained from Sigma Chemical. Diaminobenzidine (3,3'-diaminobenzidine tetrahydrochloride dihydrate, 97%) was obtained from Aldrich Chemical Co., Inc. In addition to the above anti-progestin antibodies (light chain specific) and affinity purified rabbit-anti-mouse IgG (heavy and light chain specific) were obtained from Cappel Cooper Biomedical (Malvern, PA). Biotinylated sheep-anti-mouse IgG, streptavidin-biotinhorseradish peroxidase complex, [35S]protein A, and [125I]protein A were from Amersham Corp.

Cell Cultures. Human breast cancer cells, T47D, ZR-75-1, MCF-7, MDA-231, and MDA-330, were cultured as previously described (22). T47D cells have little or no estrogen receptors and high constitutive PR levels which are not affected by estrogens (23). ZR-75-1 and MCF-7 contain a functional ER, and PR is inducible by estrogen. MDA-231 and MDA-330 cells are ER- and PR-negative as are HBL-100 cells which were derived from normal human milk epithelium (24). Cells were plated in either Corning 150-cm² T flasks at a density of 5 × 10⁶ cells/flask or in 8-well chamber slides (Miles Tissue Tek) at a density of 5 × 10⁶ cells/well, and grown in a forced air humidified incubator at an atmosphere of 5% CO₂ at 37°C. In some experiments fetal bovine serum was treated with dextran-coated charcoal to remove endogenous steroid hormones.

Cell Fractionation. Cell cultures were harvested by a 10-min incubation in 1 mM EDTA, and were washed once in serum-free minimum essential medium and once in homogenization buffer. Cell pellets were homogenized at 0°C in a Teflon-glass Potter-Elvehjem homogenizer at a cell/buffer ratio of 1/2 (v/v) in either of two buffers: TEDG (10 mM Tris-OH, pH 7.4, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol), or TEG (10 mM Tris-OH, pH 7.4, 1 mM EDTA, 10% glycerol). A cocktail of proteolysis inhibitors originally described by Loosfelt et al. (12) was included in homogenization buffers. Homogenates were centrifuged (4°C) at 800 × g for 20 min to yield crude nuclear pellets. Nuclear pellets were washed twice in TEDG by resuspension and centrifugation at 800 × g for 20 min. The supernatant and washes were combined and centrifuged at 105,000 × g for 30 min in a Beckman 50Ti rotor to yield cytosol supernatant and microsomal pellets. The pelleted crude nuclear fraction was extracted for 1 h at 4°C with homogenization buffer containing 0.5 M NaCl and centrifuged at 105,000 × g for 30 min. The supernatant was then precipitated with 1.5 volumes of cold saturated ammonium sulfate (pH 7.0, buffered in 10 mM Tris-OH, 1 mM EDTA) for 30 min at 4°C. The precipitate was pelleted by centrifugation at 10,000 rpm for 10 min and the pellet resuspended in TEDG. Salt residual nuclei were solubilized as described by Garrels (25) by their resuspension at 4°C in 50 mM Tris-OH, pH 7.0, containing 0.5% SDS, 1 mM β-mercaptoethanol, 0.1 mg/ml DNase, 0.05 mg/ml RNase, and 5 mM MgCl₂.

Receptor Steroid-Binding Assays. Cytosol or salt nuclear extracts were incubated for 16 h at 4°C with 20 nM [3H]R5020 in the presence or absence of a 100-fold excess (2 μM) of unlabeled R5020. R5020 binding was measured by DCC assay as previously described (26). Briefly, free hormone was absorbed with DCC, the DCC pelleted by centrifugation, and the supernatant containing receptor-bound R5020 was counted for radioactivity. Radioactivity was counted in 5.0 ml of 3A70B complete liquid scintillation cocktail (Research Products International Corp.) in a Beckman LS-230 liquid scintillation counter at a counting efficiency of 33% for tritium.

Gel Electrophoresis and Western Immunoblot Assay. Proteins were separated by discontinuous SDS-polyacrylamide gel electrophoresis on a vertical slab gel apparatus as described previously (21). The separating gel consisted of 7.5% acrylamide/0.1% bisacrylamide and the stacking gel, 3% acrylamide/0.25% bisacrylamide. SDS (0.1%) was included in the gel buffers and in the upper and lower reservoir buffers. [35S]-labeled protein A was used as the detection probe in place of goat anti-mouse peroxidase and colorimetric visualization of immunoreactive bands. After the blocking step and incubation of nitrocellulose filters (Schleicher and Schuell, 0.45-μm pore) overnight at 4°C with purified MAbs, filters were washed and incubated for 3 h at rt with affinity purified rabbit-anti-mouse IgG (Cappel) diluted 1:100 in dilution buffer. Filters were then washed to remove unbound IgG, and were incubated with 3,3′-diaminobenzidine tetrahydrochloride (DAB) (0.1% in 10 mL 0.05 M Tris, pH 7.4, 1 mM EDTA, 30 mM NaCl, 0.05% Triton X-100, 0.25% gelatin) and for 5 min with DAB. After air drying, filters were exposed for 3 days at —70°C to Kodak X-Omat XAR-5 film in a cassette with DuPont Cronex Quanta III intensifying screens.

Dot Blot Immunoblot. Subcellular fractions prepared from T47D cells (cytosols, salt nuclear extracts, microsomes, and solubilized salt residual nuclei prepared as described above) were measured for protein concentration by the method of Bradford (27). T47D cytosol containing a known amount of PR (measured by steroid-binding assay) was used for generation of standard curves by serial dilution over a PR range of 50 to 5 ng spotted per well (the nanograms of PR were estimated from the picomoles of hormone-binding activity and an average molecular weight of 100,000 for human receptors). A dot blot manifold (Schleicher and Schuell) was set up with two sheets of Whatman 3-mm filter paper, and one sheet of nitrocellulose (0.45-μm pore size, Schleicher and Schuell) pretreated with TEDG. Samples were applied under vacuum as 300 μl aliquots per well, the wells washed with 300 μL of TEDG, and the nitrocellulose removed from the manifold and incubated in block buffer (same buffers for blocking and washing as for Western immunoblots) for 1 h at rt. Replicate nitrocellulose sheets were spotted and one sheet was incubated with anti-PR MAbs (AB-52 and B-30) at a concentration of 10 μg/mL, and the other with control unrelated mouse MAb IgG. After 2 h incubation at rt, filters were washed three times with wash buffer for 5 min each, and then incubated for 2 h at rt with affinity purified rabbit-anti-mouse IgG (Cappel) diluted 1:100 in dilution buffer. The filters were washed to remove unbound IgG and were then incubated for 1 h at rt with [125I]protein A (Amersham) diluted to 5.0 × 10⁵ cpm/mL in dilution buffer. The filters were then washed five times for 10 min each with wash buffer, once for 5 min with DHO₂ air dried, and exposed for 24 h at —70°C to Kodak X-Omat XAR-5 film in a cassette with Dupont Cronex Quanta III intensifying screens. Gel scanning of the autoradiogram to determine relative spot densities was performed using an LKB model 2022 laser densitometer interfaced to a Varian CD5401 high-performance liquid chromatography integrator.

Photoaffinity Labeling of PR. Cytosol PR prepared from T47D cells was prebound at 0°C with 20 nM [3H]R5020 or [3H]R5020 plus a 100-fold excess of cold hormone. Samples were then photoirradiated for 2 min at 4°C directly on the surface of a 300-nm UV transilluminator as described by Horwitz et al. (15). Photolabeled receptors were then submitted to denaturing SDS-polyacrylamide gel electrophoresis, and electrotransferred to nitrocellulose as described above. The nitrocellulose was spray En'Hanced (New England Nuclear Products) and exposed for 7 days to Kodak X-Omat XAR-5 film at —70°C in a cassette with Dupont Cronex Quanta III intensifying screens.

Immunoblotting of PR. Protein A-Sepharose (Sigma) was precoated for 4 h at 4°C with secondary rabbit-anti-mouse IgG (1 mg/ml of packed beads) and unbound IgG was removed by three washes of the beads in PBS. IgG-coated protein A-Sepharose was then incubated with purified preparations of anti-receptor MAbs (at a final concentration of 20 μg/100 μl of beads) for 4 h at 4°C and then washed with PBS and twice with TEG to remove any free MAb. Aliquots of cytosol or nuclear receptor-[3H]R5020 complexes were then incubated on an end-over-end rotator for 4 h at 4°C with the MAb-coated protein A-Sepharose beads. After incubation with receptors, protein A-Sepharose beads were washed three times with TEG and extracted with 100% alcohol for 1 h at 37°C. Beads were centrifuged and aliquots of the supernatant were counted for radioactivity. Immunoblotting of unoccupied receptors was measured by postincubation of protein A-Sepharose beads with 20 nM [3H]R5020 for 4 h at 4°C. Beads were then washed and counted for bound [3H]R5020 as above.
Receptor Assays in Breast Carcinomas. 28 breast tumor biopsies were obtained from the John Muir Cancer and Aging Research Institute, and five from Surgical Pathology at the University of Colorado Health Sciences Center (UCHSC). Surgical specimens were cut into several small pieces and rapidly frozen at −70°C. One piece (=100 mg) was used for steroid-binding assay and another for immunocytochemistry. Samples were stored in 2-cc cryovials at −70°C until analysis. Tumors used for immunocytochemistry were frozen for periods ranging from several days to more than 1 year. Steroid-binding assay for ER and PR on tumors obtained from John Muir Cancer and Aging Research Institute were assayed by DCC as previously described (28). Tumors obtained from UCHSC were sent to Roche Biomedical, Columbus, OH, for steroid-binding assay.

Immunocytochemical Assay of Cell Lines. Human breast cancer cell lines, grown as monolayers in eight-well chamber slides (Miles Scientific), were fixed by incubation for 15 min at rt with 3.7% formaldehyde-PBS (0.06 M NaH2PO4, 0.15 M NaCl, pH 7.3), followed by cold acetone for 15 s. Fixed cells were then incubated for 1 h at rt with 1% BSA in PBS to block nonspecific binding sites. Cells were next permeabilized by a 5-min treatment with 0.1% Triton X-100, washed and then incubated overnight at rt with receptor MAbs. Control incubations included a mouse monoclonal IgG against an unrelated antigen (29), nonspecific mouse ascites fluid produced from NS-1 myelomas, and substitution of PBS for the MAb. Binding of MAbs was detected by the indirect streptavidin-biotin-immunoperoxidase method. After incubation with primary MAb, cells were incubated for 4 h at 4°C with biotinylated sheep-anti-mouse IgG (Amersham), diluted to 33 µg/ml in PBS with 1% BSA, followed by incubation for 1 h at 4°C with a streptavidin-biotin-horseradish peroxidase complex (Amersham) diluted 1:50. Binding was visualized by incubation for 4 min at rt with 0.04% H2O2 (0.04%) in 50 mM Tris-OH, pH 7.2. Stained cells were then dehydrated through a graded series of ethanol and mounted in cytosel for light microscopy. All incubations were followed by three washes of 30 s each with PBS. No counterstain was used.

Immunocytochemical Assay of Breast Tumors and Other Tissues. Frozen tumors and tissues were infiltrated with OCT embedding medium (Miles) at −20°C. Cryostat (Dynatech) sections (6 µm) were cut at −20°C and thaw-mounted on poly-L-lysine-coated glass microscope slides. Seven sections were routinely cut from each tumor. One section was stained with hematoxylin & eosin, two were incubated with 1% BSA-PBS as negative controls, a third and fourth control included incubation with nonspecific mouse ascites produced from NS-1 myelomas, and substitution of PBS for the MAb. Binding of MAbs was detected by the indirect streptavidin-biotin-immunoperoxidase method. After incubation with primary MAb, cells were incubated for 4 h at 4°C with biotinylated sheep-anti-mouse IgG (Amersham), diluted to 33 µg/ml in PBS with 1% BSA, followed by incubation for 1 h at 4°C with a streptavidin-biotin-horseradish peroxidase complex (Amersham) diluted 1:50. Binding was visualized by incubation for 4 min at rt with 0.04% H2O2 (0.04%) in 50 mM Tris-OH, pH 7.2. Stained cells were then dehydrated through a graded series of ethanol and mounted in cytosel for light microscopy. All incubations were followed by three washes of 30 s each with PBS. No counterstain was used.

RESULTS

Properties of MAbs to Human PR. Three MAbs were recently produced against human PR isolated from T47D human breast cancer cells. Further details regarding receptor purification, immunization of mice, hybridoma procedures, and specificity of MAbs for human PR have been previously described (21). These are mouse IgG1,8 and have been designated as AB-52, B-30, and B-64. AB-52 recognizes both A and B forms of PR while B-30 and B-64 detect B receptors only.

The reactivity of the MAbs with human PR was previously demonstrated based on three criteria (21). Each antibody (a) immunoabsorbed (by use of protein A-Sepharose) receptor-hormone complexes, (b) increased the sedimentation rate of 4S receptors on sucrose density gradients and (c) reacted with the correct receptor band(s) by Western immunoblot assay. The previous immunoblot assays were performed by an enzyme-linked immunosorbent assay method using anti-mouse IgG-peroxidase as the detection probe. To examine specificity of the MAbs more rigorously, immunoabsolts of cytosol prepared from PR-rich T47D human breast cancer cells were performed by a more sensitive method using a bridging anti-mouse IgG followed by [35S]protein A as the detection mode. An autoradiogram of the Western immunoblot assay (Fig. 1) shows that AB-52 reacts with bands at M, 120,000 and 94,000, and has little or no cross-reactivity with other proteins. B-30 and B-64 react with a single band at M, 120,000 and also show little or no cross-reaction with other proteins. No reaction was observed with any of these MAbs by immunoblout assay of cell extracts prepared from the PR-negative breast cancer cell line, MDA-231 (not shown). For reference, photoaffinity labeling of T47D cytosol PR with [3H]R5020 is shown in Fig. 1. Radiolabeled R5020 was cross-linked in a specific compatible manner with bands at M, 120,000 and 94,000 which comigrate on SDS-gels with the immunoreactive bands. We typically observed, by immunoblotting and photoaffinity labeling, that B receptors migrate on SDS-gels as doublets or triplets. Clarke et al. (20) also found that B receptors from human uterus migrate on SDS-gels as multiple closely spaced bands. Based on this sensitive protein A Western blot assay, all three MAbs appear to be monospecific for human PR.

Since transformed hormone-bound cytosol receptors were used as antigen to produce these MAbs (21), it was important for subsequent immunocytochemical localization studies to define whether each antibody reacts with other forms of PR. This is of particular importance if cells or tissues to be assayed are exposed to different levels of endogenous steroid hormones. To examine this, immunoblot assays were performed with unoccupied cytosol PR, cytosol PR bound with [3H]R5020, molybdate-stabilized PR-[3H]R5020 complexes, and nuclear receptor-hormone complexes extracted by salt from nuclei of T47D cells after their treatment in culture with [3H]R5020. Results shown in Fig. 2 illustrate that each MAb is capable of recognizing and absorbing these different receptor forms with approximately equal efficiency. Since AB-52 recognizes both A and B proteins, it absorbs, as expected, twice the number of receptors as the two B-specific MAbs, B-30 and B-64.

Optimal Conditions for Immunocytochemistry and Specificity for PR. Optimum fixation conditions for immunocytochemistry were tested with the PR-rich T47D cell line. Best results were achieved by fixation for 15 min at rt with 3.7% buffered PBS, pH 7.4, formaldehyde. This gave a strong staining reaction, minimal nonspecific background, and good preservation of morphology. Positive reactivity was also observed with 15% picric acid/2% paraformaldehyde and 4% paraformaldehyde, but staining was weaker, more heterogeneous, and background was higher than with 3.7% formaldehyde. Cold methanol:acetone fixation (70:30 v/v) resulted in a strong staining reaction but with high background and poor morphology. No specific staining was observed with ethanol:acetic acid (95:5 v/v) or with 2% glutaraldehyde.

The optimal range of MAb concentration was determined by incubating formalin-fixed T47D cells with serial dilutions of purified MAbs over the range of 50 to 0.5 µg/ml. The lower end for optimal staining was chosen as the concentration below which a decrease was obtained in a proportion of immuno-
Fig. 1. Western immunoblot assay of T47D cytosol with receptor monoclonal antibodies. **Left panel,** cytosols from PR-rich T47D cells were electrophoresed on 7.5% SDS-polyacrylamide gels, transferred to nitrocellulose, and immunoblotted with each MAb, AB-52, B-30, or B-64, as described in "Materials and Methods." Approximately 50 ng of PR (estimated by steroid-binding assay) and a total of ≈220 μg of protein were loaded onto each gel lane. **Right panel** photoaffinity labeling of T47D cytosol PR with [3H]R5020 (A) or [3H]R5020 plus a 100-fold excess of cold hormone (A+c). Samples were also transferred to nitrocellulose, the filters spray En3Hanced, and fluorographed. [3H]-labeled molecular weight standards were also transferred and fluorographed.

A comparison was next made of the immunostaining of T47D cells by each MAB alone and by a mixture of AB-52 and one B-specific MAB. The staining pattern (i.e., percentage of cells and location of stain) with the B-specific MAbs alone (B-30 and B-64) or with AB-52 alone were similar. However, a mixture of AB-52 and one B-specific MAB produced a stronger staining signal than did either antibody alone (not shown). This result was likely due to recognition of separate epitopes by AB-52 and the B-specific MAbs, allowing their simultaneous binding to receptors. There seemed to be no advantage in using one B-specific antibody over the other, so in all of our immunocytochemistry studies a mixture of all three MAbs was used to assure optimal staining intensity and sensitivity.

Immunostaining of formaldehyde-fixed T47D cells with anti-PR MAbs is shown in Fig. 3A. A predominantly nuclear staining pattern was observed which was not evident with a control antibody (Fig. 3B). Nucleoli do not stain, and some heterogeneity in intensity between cells was observed. A faint cytoplasmic reaction was typically observed which appears to be nonspecific since it occurs to the same extent with a control MAB. To further examine specificity of the immunocytochemical reaction, receptor MAbs were preabsorbed with highly purified human PR. A and B receptors were purified by immunoaffinity chromatography from cytosols of T47D cells to a specific activity of ≈2000 pmol/mg protein. An immunomatrix of AB-52 coupled to affigel-10 was utilized, and purifications were performed as previously described (21). As shown in Fig. 4, preabsorption of MAbs with purified PR blocked staining of nuclei. With preabsorbed MAbs, we still observed a weak cytoplasmic reaction which further illustrates this staining as being nonspecific background. Immunocytochemistry was next performed with the different human breast cancer cell lines listed in Table 1. With each cell line, immunoreactivity correlated with PR content measured by steroid-binding assay. T47D cells, which constitutively produce high levels of PR in the absence of estrogen (23), gave a strong nuclear reactivity whether they were treated or not with estradiol (not shown).
Immunocytochemical detection of progesterone receptors

Fig. 3. Immunocytochemical staining of formaldehyde-fixed T47D human breast cancer cells. Cell monolayers grown on coverslips in medium containing 5% fetal calf serum were fixed in 3.7% formaldehyde-PBS and stained by an indirect streptavidin-biotin immunoperoxidase procedure. A, staining with anti-PR MAbs at 10 µg/ml (AB-52, B-30, & B-64); B, control MAb. Original magnification, × 250.

Fig. 4. Preabsorption of MAbs with purified human PR. Immunostaining of fixed T47D cells was performed as in Fig. 3 with A, anti-PR MAbs alone, or B, after preabsorption of MAbs for 4 h at 4°C with highly purified PR isolated from T47D cells by monoclonal antibody affinity chromatography. Specific activity of PR was =2000 pmol/mg protein. Original magnification, × 250.

Table 1 Immunocytochemical staining of human breast cancer cell lines

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Immunoreactivity</th>
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<tbody>
<tr>
<td>T47D</td>
<td>++</td>
</tr>
<tr>
<td>ZR-75-1</td>
<td>−</td>
</tr>
<tr>
<td>MCF-7</td>
<td>−</td>
</tr>
<tr>
<td>MDA-231</td>
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<td>MDA-330</td>
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<td>HBL-100</td>
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* Cells were grown in medium without estrogen (−E).
* Cells were grown for 7 days in medium with exogenously added 17β-estradiol (10 nM) (+E).

PR levels, however, are inducible by estrogens in ZR-75-1 and MCF-7 cells (24). With either of these cells little or no immunostaining was detectable in the absence of estrogen, but staining increased dramatically after estrogen treatment (Fig. 5). It was of interest to note that estrogen induction of PR in MCF-7 and ZR-75-1 cells was highly heterogeneous. Immunostaining was detected in only a proportion of estrogen-treated cells, and many cells remained negative for PR. This contrasts with the T47D cell line which exhibits a much higher percentage of cells that stain for PR. The more homogenous expression of PR in T47D cells probably accounts for their higher PR levels compared with other cell lines. No specific immunostaining was observed in the PR-negative cell lines, MDA-231, MDA-330, and HBL-100 (Fig. 5).

The nuclear staining of T47D cells shown in Fig. 3 was obtained with cells grown in medium supplemented with whole untreated fetal calf serum. To determine whether this exclusive nuclear localization might be due to the presence of endogenous progestins in culture medium, immunocytochemistry of T47D cells was performed under a number of cell growth conditions designed to minimize the presence of endogenous steroid hormone. These included growth of cells for 7 days in charcoal-stripped fetal calf serum, serum-free medium, and in medium lacking the pH indicator dye, phenol red. Phenol red has been reported to have weak estrogenic activity at the concentration used in culture media, and to mimic the action of glucocorticoids in inducing nuclear translocation of glucocorticoid receptor (42). Under all of these conditions, immunoreactivity remained exclusively nuclear (not shown) with a staining pattern similar to that in Fig. 3.

We next examined the effect of exogenous ligand on PR immunostaining. The progestin agonist, R5020, and antagonist, RU 486, were added for various times to T47D cells grown...
Fig. 5. Immunostaining of other breast cancer cell lines. Immunocytochemical assay was the same as in Fig. 3. Cells were grown on cover slips, fixed, and Immunostained with the mix of anti-PR MAb. A. ZR-75-1 cells grown in the absence of estrogen; B. ZR-75-1 cells after addition of 10^-8 M 17β-estradiol for 7 days; C. MDA-330 cells; and D. HBL-100 cells. Original magnification, × 250.

in media supplemented with charcoal-stripped fetal calf serum. A transient increase in nuclear staining was consistently observed (N = 7 experiments) after short term (30–60 min) addition of R5020 (Fig. 6B). This R5020 effect was characterized both as a more uniform staining between nuclei compared with untreated cells, and an increase in average staining intensity. With prolonged R5020 treatment, however, a gradual decrease in nuclear immunoreactivity was observed which began at about 4 h of treatment (intermediate time points not shown), and by 24 h a dramatic loss in nuclear immunoreactivity was observed (Fig. 6C). The effect of continuous exposure of T47D cells to RU 486 (30) is shown in Fig. 6, D–F. As with R5020, short term incubation (30–60 min) resulted in a transient increase (N = 3) in nuclear staining (compare Fig. 6D and Fig. 6E). Prolonged (24 h) RU 486 incubation, however, did not produce the same decrease in PR immunostaining as R5020. Intermediate times of RU 486 treatment (between 4 and 12 h) also caused little change in nuclear immunoreactivity (not shown). This loss in nuclear immunoreactivity that resulted from continuous exposure to R5020 is indicative that progestins induce down regulation of their own receptors.

Comparison of PR Subcellular Fractionation in Fixed and Unfixed Cells. In most target systems, homogenization of cells and tissues in hypotonic buffer results in the majority of unliganded steroid receptors partitioning in the soluble cytosol fraction. Because of this it has long been held that unliganded receptors are cytoplasmic and translocate to nuclei upon binding hormone. More recent immunocytochemical data (including PR immunostaining in this study) and results from cell enucleation techniques that do not require cell lysis have, for the most part, detected exclusively nuclear localization of unoccupied steroid receptors. These data suggest that hormone-free receptors are nuclear but loosely bound, resulting in their fractionation in the soluble cytosol upon cell lysis (see Reference 31 for review). One could argue, however, that immunocytochemistry fails to stain cytoplasmic receptors due to selective destruction of receptors in the cytoplasmic compartment or to their loss from the cell (leakage) as a result of cell fixation. To further explore these possibilities, biochemical subcellular fractionation of PR in formalin-fixed and unfixed T47D cells was compared, using a dot blot immunoassay to quantitate PR antigen. The amount of PR present in different subcellular fractions was estimated from comparison of dot blot densities with that of a standard curve generated by serial dilution of known amounts of PR from T47D cytosol (estimated from hormone binding assays). An autoradiogram and densitometric tracings of the PR standards are shown in Fig. 7A. Spot densities were a linear function of PR over the range of 50 to 5 ng of receptors spotted per well. The subcellular fractions analyzed by this dot blot immunoassay were prepared from unfixed and formalin-fixed T47D cells that had been pretreated for 1 h at 37°C with progestin (+R5020) or left untreated (−R5020). The dot blot data were expressed as percentage of total cellular PR antigen contained in each subcellular fraction (Fig. 7B).

In unfixed cells (PBS), the major portion of unoccupied PR
Fig. 6. Effects of the progestin agonist, R5020, and the anti-progestin, RU 486, on PR localization in T47D cells. Cells grown on cover slips for 5–6 days in medium containing 5% charcoal-treated fetal calf serum were incubated with or without hormone (2 × 10^{-6} M) at 37°C, and then immunostained for PR as in Fig. 3. A, no hormone; B, 60 min of R5020; C, 24 h of R5020; D, no hormone; E, 60 min of RU 486; and F, 24 h of RU 486. Original magnification, × 250.

(−R5020) partitioned between cytosol and the salt extractable nuclear fraction. After 1 h of R5020, cytosol PR antigen shifted to the nuclear fraction. The subcellular distribution of PR measured by the dot blot immunoassay, therefore, is similar to that measured by hormone-binding assay. Only a small proportion of cellular antigen was detectable in extracellular washes, salt residual nuclei, or in microsomes (Fig. 7B). After fixation of cells (formalin), the subcellular distribution of PR was different. Very little if any PR was found in cytosol or salt nuclear extracts. Nearly all cellular antigen was associated with salt residual nuclei while none appeared to leak from the cells into extracellular washes. Moreover, little difference was observed in the subcellular distribution or total amounts of antigen detected in nuclei prepared from hormone-free (−R5020) and hormone-treated cells (+R5020). Formaldehyde, therefore, appears to have cross-linked both liganded and unliganded PR to an equal extent to nuclei. In fixed cells we did observe a loss in total cellular PR antigenicity compared with unfixed cells (i.e., ≈50% reduction compared with unfixed cells). This loss, however, was equivalent in hormone-pretreated and untreated cells. These results indicate that unliganded PR is not selectively destroyed or lost from T47D cells as a result of fixation.

Immunocytochemical Assay of Breast Carcinomas. As a more rigid test of specificity, a small series of breast carcinomas was analyzed by the immunocytochemical assay. The same assay used with cell lines was employed with minor modifications. Since frozen tissues were sectioned, the detergent permeabilization step was omitted (the same staining was obtained with or without detergent), and incubation steps were shortened to reduce nonspecific background staining. Tumors were scored for nuclear staining intensity and percentage of immunoreactive tumor cells. Staining intensities of negative (−), moderate (+), and strong (+++) were assigned using PR-rich (2 pmol/mg protein) T47D cells as a visual reference for a strong (+++) staining reaction. Results of the immunocytochemical staining along with ER and PR values obtained by conventional steroid-
binding assay are given in Table 2. Eighteen tumors were PR-positive (≥20 fmol/mg) by steroid-binding assay, and of these, 14 were also positive by immunocytochemical assay. These 14 tumors had PR values ranging from 29 to 1289 fmol/mg protein. The proportion of tumor cells that stained with MAbs ranged from 5 to 100% and staining intensities varied from moderate (+) to strong (+++). Tumors with higher PR levels by steroid-binding assay tended to show a high percentage of immunoreactive tumor cells and stronger staining than tumors with lower PR levels. There were four tumors in the PR-positive group that failed to immunostain with the receptor MAbs. These had PR values in the range of 28–113 fmol/mg protein.

Lack of immunoreactivity was apparently not due to an assay sensitivity problem since definitive positive staining was obtained with nontarget tissues such as ovarian cysts, squamous cell carcinoma of the larynx, parathyroid gland, or colon carcinoma. Immunostaining was detected in nuclei of normal breast epithelium and ductal epithelium of several cases of fibroadenoma and fibrocytic breast disease. Fig. 9A shows an example of results obtained with a section of fibrocytic breast disease with hyperplasia. Immunoreactivity was localized primarily to nuclei of ductal and hyperplastic epithelial cells. Human uterus also contained cells that stained with the anti-PR MAbs. Strong immunoreactivity was localized to nuclei of stromal and epithelial cells in the endometrium (Fig. 9B) and to nuclei of smooth muscle cells in the myometrium (Fig. 9C).

Although we have examined only a limited number of different tissues, these preliminary results indicate that immunocytochemical staining with these MAbs is restricted to target tissues and cells for progesterone.

**DISCUSSION**

The MAbs used in this study were prepared against human PR purified from breast cancer cells. Their recognition of progesterin-bound receptors has been previously documented (21). All of the MAbs appear to be monospecific for human PR. By Western immunoblot assay they recognize polypeptides of the correct size for the A and B forms of human PR and do not cross-react with other proteins in crude cell extracts (Fig. 1). This study reports the first use of these MAbs for immunocytochemical localization of PR. The specificity of the immunocytochemical assay for PR was evident from control incubations and from the pattern of immunoreactivity obtained with cell lines, breast carcinomas, and other human tissues. No immunostaining was apparent when unrelated MAbs were substituted for the receptor MAbs, and preabsorption of MAbs with purified human receptors eliminated the immunocytochemical reaction. With tissue culture cells lines, immunostain-
immunocytochemical detection of progesterone receptors

### Table 2: Comparison of PR detection in breast carcinomas by immunocytochemistry and steroid-binding assay

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<th>Tumor</th>
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<th>PR (--)</th>
<th>Staining intensity</th>
<th>% Tumor cells stained</th>
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<td>28 (--)</td>
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* Tumors are coded by number with patient age in parentheses. Cases included 19 infiltrating ductal carcinomas, three intraductal carcinomas, one infiltrating lobular carcinoma, and one mucinous carcinoma. One case (136) was a male breast carcinoma, and eight were biopsies from metastatic lesions.

Steroid-binding estimate for ER and PR was by dextran-coated charcoal assay (28).

Immunocytochemical staining was scored visually according to intensity and percentage of reactive tumor cells. Staining intensity was: negative (--), moderate (+), strong (++). Adherent normal ducts gave a moderate (+) staining. Tumor cells were negative.

No tumor cells in section.

body used in this study, would be necessary for detection of total cellular PR. In the present study, an A-B-specific MAb (AB-52) was used, but mixed with MAbs specific for the B protein. This was done for the purpose of maximizing the sensitivity of the immunocytochemistry assay. The immunostaining reaction was found to be stronger with a mixture of the MAbs than with any MAb alone. This was likely due to the binding of AB-52 and the B-specific MAbs to separate epitopes. The sensitivity of Western immunoblots was also observed to increase by use of an MAb mixture (not shown). If A and B receptors are true cellular proteins, the current immunocytochemical assay would be biased toward a more sensitive measurement of the B protein (since both MAbs recognize the B protein but only one binds the A protein). A mixture of MAbs which recognized different epitopes shared by A and B receptors might provide additional sensitivity.

Immunocytochemical localization of PR, in the presence and absence of progesterins, has been studied in the chick oviduct (36, 37) using monospecific polyclonal anti-PR antibodies and in the rabbit uterus with anti-PR MAbs (38). In each of these other studies, occupied and unoccupied PR were found to be localized to nuclei of target cells. Similar findings have been reported for ER by different investigators using anti-ER MAbs. PR has been observed to be primarily nuclear in a number of target end organs including breast carcinomas and breast cancer cell lines (39, 40). The immunocytochemical data in this study are in agreement with a predominantly nuclear localization for both occupied and unoccupied PR in breast cancer cells. Under all conditions examined including use of different fixatives, growth of cells in hormone-depleted media, or after incubation of cells with exogenously added progesterins (or antiprogesterins), immunolocalization of PR in cell cultures was exclusively nuclear. We have no evidence for specific cytoplasmic staining. PR localization was also confined to nuclei of immunoreactive cells in frozen tissue sections of breast carcinomas, normal breast, fibrocystic breast disease, and human uterus.

Two biochemical experiments were performed in the present study to explore the possibility that the exclusive nuclear localization of PR observed by immunocytochemistry might be due to an inability of MAbs to recognize a cytoplasmic receptor form. (a) Results of the immunabsorption assays (Fig. 2) would seem to rule out inability of the MAbs to recognize different biochemical forms of PR as an explanation for lack of cytoplasmic staining. At least in vitro, each MAb was capable of recognizing unliganded and liganded cytosol receptors, monolysate-stabilized nontransformed receptors, and nuclear receptors transformed in vivo by addition of progesterin to cell cultures. (b) To determine if fixation of cells might lead to selective destruction of cytoplasmic PR or to unoccupied receptor loss through cell leakage, subcellular fractionation of occupied and unoccupied PR in formalin-fixed cells was examined by use of a dot blot immunoassay to quantitate PR antigen in different subcellular compartments. This kind of experiment, attempting to follow subcellular fractionation of receptors under conditions (i.e., formalin fixation) which mimic those used in processing cells for immunocytochemistry, has not been reported before. It was found that most of the cellular receptor was fixed to nuclei whether cells were grown in the absence or presence of progesterin (Fig. 7). These results would seem to rule out selective destruction of unoccupied receptors by the fixative or leakage of unoccupied PR out of the cells as explanations for the failure to detect cytoplasmic staining. If selective destruction of unoccupied cytoplasmic PR had occurred, one
would expect to have obtained lower amounts of antigen in untreated cells compared with cells treated with progestin. These data cannot exclude the possibility, however, that fixation artifactually redistributes unoccupied receptors from cytoplasm to nuclei. This seems unlikely since nuclear staining of T47D cells was observed with different fixatives, and other investigators have also reported exclusive nuclear staining of unoccupied ER and PR by a variety of different fixation procedures. These biochemical data, therefore, are supportive of a true nuclear localization for both occupied and unoccupied PR in breast cancer cells.

Although the evidence in favor of an exclusive nuclear localization for the sex steroid receptors (ER and PR) is quite strong, several immunocytochemistry studies have suggested a different distribution for GR. Both cytoplasmic and nuclear staining of unoccupied GR have been reported at the light microscopic level, and addition of glucocorticoids has been observed to decrease cytoplasmic reactivity concomitant with increased nuclear staining. These data are more consistent with the classic model of receptor translocation to nuclei upon binding hormone. Wikström et al. (41) studied intracellular localization of GR in several tissue culture cell lines using their anti-rat GR monoclonal antibody. They found that the ratio of cytoplasmic to nuclear staining of unoccupied GR was influenced by the method of processing cells for immunocytochemistry. Without a detergent permeabilization step immunostaining was mainly cytoplasmic, but shifted to nuclei with the addition of detergent. They found that nuclear staining first became evident (along with cytoplasmic staining) at a low concentration of detergent (0.05%). At higher concentrations (0.5%), cells displayed increased nuclear staining at the expense of cytoplasmic reactivity. Nuclear GR detection, therefore, appeared to require cell permeabilization for penetration of MAb into nuclei while cytoplasmic GR was diminished at high detergent concentra-
IMMUNOCYTOCHEMICAL DETECTION OF PROGESTERONE RECEPTORS

Fig. 9. Immunocytochemical localization of PR in other human target tissues. A, fibrocystic breast disease with hyperplasia. Staining is localized to nuclei of ductal epithelial cells. Surrounding stroma and blood vessels do not stain. B, proliferative phase human uterus from a 37-year-old woman with endometriosis. Staining of endometrium was localized to nuclei of ductal epithelium and stromal cells. C, staining of myometrium was localized to nuclei of smooth muscle cells. Original magnification, × 250.

From these results, it was concluded that unoccupied GR is both cytoplasmic and nuclear. We performed similar experiments with PR in T47D cells, and were unable to detect specific cytoplasmic staining at any concentration of detergent over the range of 0 to 1% Triton X-100. An intensification of nuclear PR staining was observed with 0.1% Triton X-100, compared with no detergent, but nuclear staining did not increase further at higher concentrations (not shown). Picard and Yamamoto (42) showed that cell culture conditions can also affect the localization of unoccupied GR. In the absence of the pH indicator dye, phenol red, or serum, immunostaining was found to be primarily cytoplasmic. After re-addition of either phenol red or serum, the majority of cells exhibited a nuclear staining pattern. The serum factor(s) responsible for nuclear localization of GR appeared from these studies not to be endogenous glucocorticoids. We have also examined the influence of phenol red and serum on the localization of PR in T47D cells. In the absence of either or both of these cell culture components, cells continued to display nuclear PR localization with no evidence of cytoplasmic staining (not shown). It appears, therefore, that GR and PR likely have a different intracellular distribution. Definitive studies to compare GR and PR localization will require that immunocytochemistry be performed with anti-PR and anti-GR MAbs by the same procedure, using a cell line which contains both receptors. This should rule out any subtle procedural differences or differences between cell lines. We have constructed a cell line, which expresses high levels of both GR and PR, by transfection of T47D cells with a GR expression vector. In preliminary immunocytochemical studies, these cells displayed both cytoplasmic and nuclear staining for GR but only nuclear localization for PR.

Although addition of ligand to cell cultures did not affect the intracellular location of PR, an effect on the intensity of nuclear PR immunostaining was observed. Short term incubation of cells with either progestin agonist (R5020) or antagonist (RU 486) resulted in a transient increase in nuclear staining intensity without an apparent decrease in cytoplasmic reactivity (since no cytoplasmic staining was detected under any conditions). Similar findings have been reported for ER. Using anti-ER MAbs for immunocytochemical assays (43), the ER-rich MCF-7 cell line was reported to display an increase in nuclear immunostaining without a decrease in cytoplasmic staining in response to short term addition of estrogen. McClellan et al. also observed an intensification of nuclear ER immunostaining in Macaque endometrial slices after their incubation for 60 min at 37°C with estradiol (44). This intensification also occurred without a change in apparent cytoplasmic staining. An explanation for these results is not readily apparent but there are a number of possibilities. First, a small pool of cytoplasmic receptor not detectable at the light microscopic level may be capable of nuclear translocation upon binding hormone. This could appear as an increase in existing nuclear staining. In chick oviduct (45) and rabbit uterus (46), some PR localization in cytoplasm over ribosomes and endoplasmic reticulum was observed by immunoelectron microscopy. Cytoplasmic PR, however, were found in just a few cells and likely represent newly synthesized PR below the level of detection at the light microscopic level. Whether progestins can induce nuclear translocation of these cytoplasmic PR was not reported. Secondly, ligand may cause an intranuclear condensation of receptors resulting in an apparent increase in immunostaining. In support of this, electron microscopic immunogold studies (45, 46) have noted changes in intranuclear localization of PR upon addition of hormone. Immunogold particles were observed to shift from condensed chromatin to areas of dispersed chromatin in response to the addition of progestins. Third, MAbs may simply be more reactive with the nuclear-transformed PR. Although each MAb in the present study was equally reactive with differ-

* Muro, Nordeen, and Edwards, unpublished data.
ent native PR forms in vitro, the situation may be different in a fixed cell.

Continuous incubation of MCF-7 and T47D breast cancer cells with progestins, has been shown previously to result in a progressive decrease in cellular PR content as measured by hormone-binding assay and by Western immunoblotting with anti-PR MAbs (47, 48). PR levels, after treatment (24-48 h) with progestins, decrease to about 10-20% that of untreated cells. The immunoblotting data which detect receptor protein directly indicate that progestins down-regulate their own receptors as a result of receptor protein loss and not inactivation of receptor-hormone binding activity. A dramatic reduction in PR content of individual cells was observed in the present study after continuous exposure of T47D cells to the progestin, R5020 (Fig. 6). Thus by an independent method of PR protein detection, immunocytochemical data are also consistent with progestins having the ability to down-regulate their own receptors through a mechanism of receptor protein loss. It was interesting to note that after 24 h of R5020 treatment, the majority of cells displayed a total loss of immunoreactivity while a few cells continued to stain strongly for PR. Thus, it appears that a small proportion of cells in the population are refractory to progestin-induced down-regulation of PR. T47D cells incubated in parallel with the progestin antagonist, RU 486, displayed little change in nuclear PR immunostaining compared with untreated cells (Fig. 6). Although RU 486 also binds to glucocorticoid receptors (GR) and has antigluocorticoid activity, the data in the present study are not affected by cross-binding of RU 486 to GR. T47D cells have little or no measurable GR and are unresponsive to glucocorticoids. PR, therefore, bound by the antagonist RU 486 do not down regulate to the same extent as receptors bound by progesterin agonists.

There have been two other reports of immunocytochemical detection of PR in breast carcinomas. Perrot-APellanat et al. (19), using anti-rabbit PR MAbs cross-reactive with human receptors, have analyzed 27 breast tumors. Greene and Press, using MAbs prepared against human PR, reported results of immunocytochemical assay of 20 breast tumors (49). Both studies obtained a good correspondence between immunocytochemical assays and conventional steroid-binding assays, results similar to those of the present study. No false-positives were detected in the present study or in these other two reports. All tumors that were PR-negative by steroid-binding were also negative by immunocytochemistry. Each study, including the present report, has also obtained immunoreactivity with a high proportion of PR-positive tumors. Each series, however did find that a few PR-positive tumors failed to react with MAbs. A number of reasons could account for this lack of immunoreactivity. Antigenic sites may have been destroyed during tissue handling and storage at -70°C, or this may be due to a tissue sampling problem. Since adjacent pieces of tissue were used for immunocytochemical and steroid-binding assays, specimens may have had a different population of tumor cells with respect to PR content. Alternatively, this could be due to a biological problem. Tumors may express mutant receptors that have retained a hormone binding domain but are truncated and missing antigenic sites.

Our preliminary results with frozen breast tumors are encouraging and illustrate the potential utility of these MAbs for the immunocytochemical detection of PR in breast carcinomas. The reliability of the immunocytochemical assay for clinical PR detection will require studies with a much larger series of tumors. The distribution of PR immunostaining obtained with other human tissues also illustrates the utility of these MAbs for study of receptor localization in normal target tissues under different hormonal states.

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Note Added in Proof


Immunocytochemical Localization of Progesterone Receptors in Breast Cancer with Anti-Human Receptor Monoclonal Antibodies


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