Immunocytochemical Study with Monoclonal Antibodies to Progesterone Receptor in Human Breast Tumors

Martine Perrot-Appalan, Marie-Thérèse Groyer-Picard, Frédéric Lorenzo, André Jolivet, Mai Thu Vu Hai, Claude Pallud, Frédérique Syparatos, and Edwin Milgrom


ABSTRACT

Mouse hybridomas secreting monoclonal antibodies against rabbit uterine progesterone receptor (PR) have been prepared. Several of these immunoglobulins exhibited high affinity towards human progesterone receptor and two (LET 126 and LET 64) were selected as giving the best immunoperoxidase staining of human progesterone target organs.

Using the indirect peroxidase-antiperoxidase method of Sternberger, optimal conditions for demonstrating PR involved brief fixation of frozen sections with formaldehyde-containing fixatives, among them picric acid-parafomaldehyde. This method allowed us to detect the receptor in breast carcinoma epithelial cells, T47D cell line, and uterine endometrium and myometrium. No staining was observed in intestine and muscle. Specific staining for PR was confined to the nucleus, irrespective of the concentration of progesterone in the blood of the patient.

In a preliminary study of 27 human breast cancers by the immunocytochemical method, the presence or absence of nuclear staining for PR correlated well with the concentration of cytosolic progesterone receptor determined by a steroid-binding assay on tumor extracts. Differences in the intensity and distribution of staining within a section were observed, suggesting heterogeneity of the PR content of breast cancer cells. In 19 tumors, the immunocytochemical method for PR localization was also used in combination with a slightly modified Abbott ER-ICA staining for estrogen receptor to compare the distribution of both receptors within the same biopsy on adjacent frozen sections. Various combinations of estrogen receptor and PR contents that have been determined by steroid-binding assay have also been detected by the double immunocytochemical assay.

INTRODUCTION

Estrogen and progesterone regulate the growth of several hormone-dependent cancers. Receptors for these hormones have been detected in tumors from breast (1, 2), endometrium (3), ovary (4), kidney (5), brain (6, 7), and several other tissues. Extensive studies have been performed on breast cancers showing that the presence of estrogen and progesterone receptors was correlated with (a) high probability of response to endocrine therapy (8, 9); and (b) a more favorable prognosis (10-16).

Determination of steroid hormone receptors has thus become widely used in the management of this disease. The methods which are used most often involve incubation of the cytosol with radioactive hormones (17) or more recently, in the case of estrogen receptor, with monoclonal antibodies (18, 19). These biochemical or immunological methods do not measure, however, receptors tightly bound to nuclei and not solubilized, and do not take into account cellular heterogeneity in receptor content. It thus appeared important to develop immunohistochecmical methods for receptor detection.

Using monoclonal antibodies, King and Greene (20) and King et al. (21) followed by McClellan et al. (22), Shimada et al. (23), and Skovgaard Poulsen et al. (24) have devised immunocytochemical methods for studying ERs in normal and neoplastic tissues (20-26). We have prepared a number of monoclonal antibodies against rabbit PR (27), and we have studied the localization of PR at the cellular (28) and subcellular (29) level in rabbit and guinea pig using immunohistochemical methods.

We have now selected monoclonal antibodies which strongly cross-react with human progesterone receptor. We describe here an immunocytochemical method that is suitable for detecting progesterone receptor in human tissues, with special emphasis in breast cancers. We also report on (a) the preliminary correlation between PR immunostaining and the concentration of cytosolic PR determined by a steroid-binding assay and (b) the possibility of detecting both PR and ER immunostaining on adjacent sections of the same tumor sample.

MATERIALS AND METHODS

Antibodies. Details about PR purification, immunization of mice, cell fusion, hybridoma cloning, and screening procedures have been described elsewhere (27, 30, 31). Briefly, rabbit uteri were used to prepare cytosol from which receptor was purified by immunoaffinity chromatography (31). This material was used to immunize BALB/c mice whose spleen cells were fused with Sp2/OAg myeloma cells to obtain hybridomas lines making antibody to the receptor protein according to a procedure previously described (27). In two such experiments 14 hybridomas were found to react in both immunoenzymatic and double immunoprecipitation tests with the human progesterone receptor. Two monoclonal antibodies (LET 64 and LET 126, IgG1 class) obtained from ascitic fluids were selected after specificity tests with human receptor (see "Results"). Immunoglobulins were prepared by precipitation with ammonium sulfate; they were stored at —20°C at 5 mg protein/ml and diluted just before use. In some cases immunoglobulins were purified with the Bio-Rad Affi-Gel Protein A kit (Bio-Rad Laboratories, Richmond, CA).

Rabbit (or goat) antisera against mouse IgG were obtained from Dakopatts (Copenhagen, Denmark) and Nordic (Tilburg, The Netherlands). Mouse monoclonal PAP was obtained from Sternberger-Meyer Immunocytotechnics (Jarretsville, MD).

Mouse monoclonal antibodies (IDA, a gift from Dr. P. Legrain, Institut Pasteur, Paris, France), unrelated to the receptor under study, served as controls. They were antidiotype antibodies raised against mouse myeloma antilevan antibodies (32).

A polyclonal antiserum against the M, 110,000 form of rabbit PR was also produced in goat. PR was purified by chromatography on immunoaffinity column (31), electrophoresis, and electroelution of the M, 110,000 band.

The specificity of this antibody was tested by density gradient experiments and immunoblotting analysis of crude human breast cancer and uterine cytosols.

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2 To whom requests for reprints should be addressed.
Fixatives. The following fixatives were used: picric acid-paraformaldehyde, pH 7.4 (33); 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4; 1% glutaraldehyde in Sorensen buffer, pH 7.4; Boulin’s (saturated picric acid-formalin-acetic acid (75:25:5)); periodate-lysine-paraformaldehyde, pH 7.4 (34); ethanol; and acetone.

Tumors. Twenty-seven surgical samples from primary breast carcinomas were obtained at the Centre René Huguenin (Saint-Cloud, France). In each case, one fragment was used for routine histological examination. It was graded according to the classification of Bloom and Richardson (35). Two other fragments were trimmed of adhering fat and necrotic tissue, quickly frozen in liquid nitrogen, and stored until analyzed. Both biochemical analysis and immunocytochemical staining for PR were performed on these tumor samples.

Among the 27 biopsy samples examined, 21 were diagnosed as being predominantly infiltrating ductal carcinoma (with or without accompanying intraductal carcinoma), 3 were infiltrating lobular carcinoma, one was medullary carcinoma, and one mucinous carcinoma.

Immunocytochemical Staining for PR. Tumor samples were sectioned at 4-μm thickness at -20°C, and the sections were thaw-mounted onto gelatin-coated glass slides. Sections were then fixed immediately without drying in picric acid-paraformaldehyde for 15 min at -10 to -20°C. Slides were transferred to PBS for 30-45 min at 4°C before proceeding. Sections were first treated with a 0.5% solution of hydrogen peroxide in PBS for 15 min at room temperature in order to inactivate the endogenous peroxidase activity of erythrocytes, rinsed with PBS, and incubated for 10 min with a 1:40-diluted rabbit serum to reduce the nonspecific binding of subsequent reagents. After gentle rinsing in PBS, the sections were incubated successively with monoclonal mouse anti-PR antibodies overnight at 4°C in a moist incubation chamber, rabbit anti-mouse IgG (1:80 dilution) for 45 min at room temperature, and mouse horseradish peroxidase: antiperoxidase (PAP) complex (1:100 dilution) for 45 min at room temperature. Each incubation was followed by three 10-min washings in PBS containing 0.05% Tween. After the final wash, sections were incubated in the dark for 10 min with the DAB solution (0.05 M Tris-HCl buffer, pH 7.6, containing 0.5 mg DAB/ml and 0.01% H2O2) for to min at room temperature (36). Duplicate sections were counterstained lightly with hematoxylin to facilitate the identification of cellular elements within the tumor. Sections were then dehydrated and mounted for examination by light microscopy.

Various dilutions of each monoclonal mouse anti-PR (6-100 μg/ml) were tested in preliminary experiments. Dilutions which gave maximum specific staining intensity with LET 64 and LET 126 (12.5-25 μg/ml under the incubation conditions used) were determined.

Immunocytochemical Controls and Evaluation of Immunocytochemical Staining. Negative and positive controls were run along with every tumor sample. Negative controls consisted of adjacent sections treated with a monoclonal mouse receptor-unrelated antibody (IDA), used at the same concentration, or with nonimmune mouse IgG. Positive controls consisted of sections of biochemically confirmed PR-rich breast tumors. Additional methodological controls included the use of monoclonal anti-PR antibody which has been presaturated (60 pmol receptor/μg IgG, 2 h at room temperature and 24 h at 4°C) with highly purified PR (31) before the immunostaining procedure (28). In all cases, the PR immunostaining was performed and the slides were examined without knowledge of the results of the steroid-binding assay. Two or three different parts of the tumor sample were analyzed.

Staining was considered as specific only when it was present in the tissue section that had been incubated with monoclonal anti-PR antibody and absent in the adjacent section incubated with monoclonal PR-unrelated antibody. Using this criterion, specific labeling for PR was considered as receptor positive.

Tumors were classified as PR immunocytochemical positive if they contained malignant cells showing nuclear staining regardless of the staining intensity and the proportion of epithelial cells showing such staining. The average intensity of specific staining was graded as absent (--), weak but definitely detectable (+), moderate (++) and strong (+++).

Steroid-binding Assay of PR and ER. PR was measured in human breast tumor cytosols using the dextran-coated charcoal analysis with

Fig. 1. Immunoblotting analysis of cytosol from T47D breast cancer cells containing monoclonal antibody against PR. Lanes 1 and 2, cytosol from T47D cells containing human PR (4 pmol/mg protein) was analyzed by the immunoblotting technique using either monoclonal anti-PR antibody (LET 64, 28 μg protein/ml) (lane 2) or control monoclonal antibody unrelated to PR (IDA, 28 μg protein/ml) (lane 1). Lanes 3 and 4, cytosol from rabbit uterus (3 pmol PR/mg protein) was analyzed for comparison using the same monoclonal anti-PR (LET 64) (lane 4) or monoclonal antibody unrelated to PR (IDA) (lane 3), used at the same concentrations.

RESULTS

Specificity of Monoclonal Antibodies against PR. The specificity of the two monoclonal antibodies (LET 126 and LET 64)
Fig. 2. Immunocytochemical detection of PR in human breast cancer. Adjacent 4-µm frozen sections of a PR-rich breast tumor were fixed in picric acid-paraformaldehyde and stained, using the immunoperoxidase technique, with the monoclonal anti-PR antibody LET 126 (a) (10 µg protein/ml) or with a control PR-unrelated monoclonal antibody (b) (10 µg protein/ml). This tumor was taken from a 55-year-old woman and found to contain 388 fmol of cytosolic PR/mg protein by the steroid-binding assay. Sections from two other tumors were also incubated with another monoclonal antibody to PR (c) (LET 64, 10 µg protein/ml) or with a specific polyclonal antibody directed against PR (d) (20 µg protein/ml). Note that the specific staining for PR is located exclusively in the nuclei of cancer cells. A high magnification of this specific localization is shown in the inset of Fig. 2c (original magnification, × 1000). The connective tissue is not stained. There is no counterstain. E, epithelial cells; C, connective tissue. Original magnification, × 250.

Experimental Conditions for Immunocytochemical Detection

used in the present work was tested extensively with human receptor from uterus and breast cancer using precipitation of [³H]progesterin-PR complexes with a second antibody, displacement of complexes after centrifugation on density gradients, and immunoblotting analysis (Fig. 1). Cytosol from breast cancer T47-D cells containing PR was submitted to polyacrylamide gel electrophoresis in presence of sodium dodecyl sulfate and analyzed by immunoblotting using monoclonal antibody LET 64 (Fig. 1, lane 2). A single immunogenic protein with a molecular weight of 110,000, identical to the rabbit PR (Fig. 1, lane 4), was found. No immunoactivity was observed when cytosol from T47-D cells was incubated with monoclonal receptor-unrelated antibody (Fig. 1, lane 1). Similar results were obtained with LET 126 antibody or when preparations from human uterus were analyzed by immunoblotting (not shown).

PR IMMUNOCYTOCHEMISTRY

of PR in Breast Cancer Tissues. All the initial experiments were carried out on human breast tumor samples known from steroid-binding determinations to contain high levels of cytosolic progesterone receptors. Four monoclonal mouse anti-PR antibodies were tested in picric acid-formaldehyde-fixed frozen sections. These antibodies were selected for their apparent high affinity towards the human progesterone receptor, as observed by an enzyme-linked immunosorbent test and the double immunoprecipitation test of [³H]steroid-receptor complexes. Picric acid-paraformaldehyde fixative was chosen, since immunoreactivity of PR in rabbit tissues was previously shown to be well preserved in this fixative (28). Two monoclonal anti-PR antibodies (LET 126 and LET 64) gave a strong nuclear staining with the immunoperoxidase technique (Fig. 2). The same nuclear localization was also observed when a polyclonal monospecific antibody against PR was used on sections from these breast tumors (Fig. 2d).

We then optimized the immunocytochemical PR detection

* F. Lorenzo, A. Jolivet, M. T. Vu Hai, and E. Milgrom, manuscript in preparation.
Frozen sections obtained from an unfixed receptor rich tumor (388 fmol of PR/mg protein) were thaw-mounted onto glass slides, then immediately immersed in fixative for the indicated time. After rinsing for 15-30 min in PBS, immunocytochemical staining was performed as described in “Materials and Methods” using antibody LET126 to localize receptor. An adjacent section was stained using a control monoclonal IgG (see text).

Table 1 Comparison of fixatives

<table>
<thead>
<tr>
<th>Fixatives</th>
<th>Duration of fixation (min)</th>
<th>Nuclear staining*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Picric acid-paraformaldehyde (Zamboni's)*</td>
<td>15</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>+++</td>
</tr>
<tr>
<td>4% paraformaldehyde</td>
<td>15</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>++</td>
</tr>
<tr>
<td>Periodic acid-lysine-paraformaldehyde</td>
<td>15</td>
<td>++</td>
</tr>
<tr>
<td>1% glutaraldehyde</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>-</td>
</tr>
<tr>
<td>Bouin's</td>
<td>15</td>
<td>+</td>
</tr>
<tr>
<td>Ethanol</td>
<td>5</td>
<td>++</td>
</tr>
<tr>
<td>Acetone (−20°C)</td>
<td>15</td>
<td>+</td>
</tr>
</tbody>
</table>

*Grading by intensity; see “Materials and Methods.”

As in Ref. 33.

As in Ref. 34.

on frozen sections of human breast tumors by use of LET 126 at two different concentrations (10 and 25 µg protein/ml) and by varying several experimental conditions (fixation procedure, nature and concentration of bridging antibody, concentration of peroxidase-antiperoxidase complexes). The influence of the nature and duration of fixation is shown in Table 1. We found that a 15-min treatment with formaldehyde-containing fixatives such as 4% paraformaldehyde, picric acid-paraformaldehyde, and periodate-lysine-paraformaldehyde were most effective. Ethanol and acetone caused weaker staining intensity while glutaraldehyde fixation left only a slight specific staining or inhibited it completely. PR detection was obtained when fixation was performed either prior to freezing or immediately after cutting cryostat sections. This latter condition was chosen to study frozen breast cancer biopsies. Treatment of fixed frozen sections with 0.5% H2O2 in order to inactivate endogenous peroxidase activity does not inhibit PR staining. In addition, with picric acid-paraformaldehyde-fixed frozen sections, the best staining using the immunoperoxidase method of Sternberger (36) was obtained by incubating slides with primary antibodies overnight at 4°C and by using bridging antibodies at a concentration of 250 µg/ml and a mouse PAP at a 1:100 dilution.

Specificity of Staining and Nuclear Localization. In breast cancers, specific staining with anti-PR antibodies was restricted to the nucleus of tumor epithelial cells (Fig. 2, a and c). In contrast, no nuclear staining occurred in the negative controls, i.e. when nonimmunized mouse IgG or control monoclonal mouse IgG was substituted for the monoclonal anti-PR (Fig. 2b). In addition, the inhibition of immunostaining was observed when a control section was incubated with monoclonal anti-PR preincubated with highly purified receptor (see “Materials and Methods”).

No specific reaction was seen in the surrounding connective tissue, blood vessels, fat tissue, and muscle cells. Occasionally, we observed a faint brown background staining in the connective tissue, necrotic tissue, or in the cytoplasm of carcinoma cells. This was regarded as nonspecific because it was also observed in sections treated with nonimmune mouse IgG or PR-unrelated monoclonal antibodies (control sections). In a few cases, and independently of the presence of PR, nonspecific staining also occurred in the cytoplasm of some connective tissue cells (among which were mast cells but not fibroblasts). This nonspecific staining was specially observed in breast tumor samples the stroma of which was infiltrated with inflammatory cells.

In addition to being exclusively nuclear, the specific PR immunoperoxidase staining was usually heterogeneous in distribution and intensity (Fig. 3). Some carcinoma cell nuclei were intensely stained whereas others showed a weak staining or even no reaction at all (Fig. 3a). Frequently, the distribution of positive nuclear staining varied considerably, depending on the area within a section (Fig. 3b). The heterogeneity of the nuclear staining in distribution and intensity did not seem to correspond to differences in tumor histology.

The presence of nuclear immunostaining was also observed in nonmalignant hyperplastic mammary epithelium present at the periphery of tumor areas (Fig. 4b). As for carcinoma cells, PR immunostaining in the noncarcinomatous ductal cells was more or less intense, varying according to individual cells.

Fig. 3. Heterogeneous immunocytochemical staining in PR-positive breast tumors. Two tumors were taken from 55- and 60-year-old women and were found to contain 388 and 394 fmol of cytosolic PR/mg protein, respectively. Immunocytochemical staining was carried out without counterstain. a, marked heterogeneity present and evidenced by nuclei varying in staining from zero (small arrows) to intense (large arrows). Original magnification, x 400. b, PR-positive (large arrows) and -negative (small arrows) epithelial areas present within the same section. Original magnification, x 250.
Fig. 4. Immunocytochemical staining for PR in various progesterone target and non-target cells. a, human myometrium; b, hyperplastic nonmalignant mammary epithelium; c, human breast cancer cell line T47D; d, human small intestine. Frozen sections from human tissues or tumor cells were treated with monoclonal anti-PR antibodies (LET 126, 10 μg protein/ml). No counterstain (a, b, d) or light counterstain (c) were used. a, positive nuclear immunostaining present in smooth muscle cells (M) of the myometrium (M). Note in b the presence of nuclear immunostaining in the noncarcinomatous ductal cells, present at the periphery of the carcinoma. On T47D cells (c), strong immunostaining appears in black (brown), while light counterstain with hematoxylin appears in grey (blue). In d, specific staining was absent; the sparse nonspecific staining present in connective tissue cells was also observed with PR-unrelated immunoglobulins (not shown). E, intestine epithelium; C, connective tissue. Original magnification, a, b, and d, × 250; c, × 400.

Table 2 Relationship of the nuclear staining of PR in frozen sections and the PR content as determined by the steroid-binding assay in 27 human breast cancers

<table>
<thead>
<tr>
<th>PR concentration (fmol/mg protein)</th>
<th>No. of tumors</th>
<th>PR immunostaining, no. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;10 (negative)</td>
<td>14</td>
<td>0</td>
</tr>
<tr>
<td>10–30 (borderline)</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>&gt;30 (positive)</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>27</td>
<td>11</td>
</tr>
</tbody>
</table>

The specificity of the immunostaining was also shown by the study of several other progesterone target or non-target cells. Nuclear staining identical to that found in human breast cancer was observed in sections of human uterus (Fig. 4a) and in the human breast cancer cell line T47D (Fig. 4c). In the uterus, staining was seen in the epithelial and stromal cells of the endometrium and in smooth muscle cells of the myometrium. In contrast, non-target tissues such as human intestine (epithelium and muscle) (Fig. 4d) and pectoral muscle (not shown) were immunocytochemically negative.

Immunocytochemical Staining of PR in Breast Cancer and Comparison with Steroid-binding Assay. Twenty-seven biopsy samples were examined with the PR immunoperoxidase staining using LET 126 at two final concentrations, 10 and 25 μg protein/ml. Eleven of the 27 biopsies (40%) examined were evaluated as PR positive and 16 (60%) as PR negative using the criteria described in “Materials and Methods.”

The immunocytochemical detection of PR in these 27 human breast tumors was compared with the steroid-binding assay. As shown in Table 2, 10 of the 11 (90%) tumor biopsies containing PR >30 fmol/mg of cytosol protein in the biochemical assay displayed a positive immunocytochemical reaction (see Fig. 5, a and b). In the two cases whose concentration of PR was borderline (between 10 and 30 fmol/mg cytosol protein), specific nuclear staining was not detected in one case, or limited to a few cells in another case (focal and strong staining) (see Fig. 5c). Also, all of the 14 tumor samples negative for PR in the biochemical assay showed no immunostaining (see Fig. 5d). Thus, the overall agreement with the biochemical assay was 92% (25 of 27). In addition, and as a preliminary result, PR-rich tumors (>200 fmol PR/mg protein) showed a high degree of staining positivity and usually a great number of positive cells (Table 3 and see Fig. 5, a and b). By contrast, tumors...
Fig. 5. Different patterns of immunocytochemical staining in PR-rich, -poor, and -negative human breast tumors. a and b, tumors taken from 48- and 55-year-old women, containing 408 and 388 fmol of cytosolic PR/mg of protein, respectively; c, receptor-poor breast tumor containing 26 fmol PR/mg protein taken from a 34-year-old woman; d, PR-negative breast tumor (taken from a 44-year-old woman). All four tumors were infiltrating ductal mammary carcinomas. Note that in a and b, tumors containing high PR content displayed strong nuclear staining. In c, lower magnification was used to visualize focal staining area (>). Light counterstain, except in d. Original magnification, a, b, and d, × 250; c, × 140.

containing 30–200 fmol PR/mg protein showed a weak to moderate number of stained cells with a usually (but not always) weaker intensity (see Table 3, cases 12, 20, 27, 31, 32, 35).

Although the number of cases was limited, the presence or absence of nuclear staining did not seem to correlate with pathological classification (among the 21 invasive ductal carcinomas, 10 were positive and 11 were negative; 2 invasive lobular carcinomas were negative and one was positive). Among the 11 positively stained tumors, one was classified as grade I (10%), 6 as grade II (60%), and 2 as grade III (20%). Among the 16 negative tumors, none was classified as grade I, 9 as grade II (56%) and 6 as grade III (37%).

Colocalization of PR and ER in Same Breast Tumors. In 19 cases, the immunocytochemical method for localizing PR was used in combination with a slightly modified Abbott ER-ICA staining to compare the distribution of both receptors within the same breast tumor biopsy on adjacent frozen sections. As shown in Fig. 6, this methodology allows the comparison of PR and ER immunostaining in the same fields of the tumor sample. Moreover, in the 11 ER and PR-positive tumors analyzed by this double ER and PR staining, preliminary observations showed that PR positive areas were also ER positive areas (see an example in Fig. 6, a and b). In addition, the ability to colocalize PR and ER by the same immunocytochemical technique allowed the detection of the various combinations of ER and PR content previously described using the steroid-binding assay (see Table 3).

DISCUSSION

We have prepared a panel of monoclonal antibodies against the rabbit uterine progesterone receptor. Several of these antibodies exhibited high affinity towards the human progesterone receptor; two (LET 126 and LET 64) were selected for the study of PR by an indirect immunoperoxidase technique in several human tissues including breast tumors and uterus.

The specificity of these anti-human PR antibodies was tested extensively by several methods including precipitation (with a second antibody) of [3H]progesterin-receptor complexes from human uterus, displacement of these complexes on sucrose density gradients, Western blot analysis of cytosols from progesterone target (uterus, breast cancer, T47D cell line) and non-
Fig. 6. Colocalization of PR and ER immunostaining in the same breast tumor sample. Adjacent frozen sections of each tumor were treated with either monoclonal anti-PR antibodies (LET 126, 10 µg protein/ml) or monoclonal anti-ER antibodies (kit from Abbott laboratories, 10 µg immunoglobulin/ml), according to the procedure described in "Materials and Methods." PR immunostaining (a, c, e) and ER immunostaining (b, d, f) are shown on two adjacent sections taken from three breast tumors. Tumor 1 (a, b) contained 469 fmol PR/mg protein and 310 fmol ER/mg protein; tumor 2 (c, d), no PR and 165 fmol ER/mg protein; tumor 3 (e, f), no PR and ER. All sections were counterstained very lightly with hematoxylin. Original magnification, × 400.
target organs. Immunocytochemical studies also gave evidence of the specificity of these antibodies, since when highly purified rabbit PR was added to the antibodies the immunostaining of human tissue sections was blocked. Moreover, staining was observed in cells or tissues known to contain PR, such as breast cancer, T47D cell line, endometrium, and myometrium, but not in PR-negative tissues, such as muscle and intestine.

The present study shows only nuclear localization of the PR, regardless of the hormonal status of the patient (pre- or post-menopausal). This was observed under a variety of experimental conditions including different fixatives and tissue processing methods: frozen sections, either fixed prior to freezing or immediately following sectioning, and sections from paraaffin-embedded tissues (data not shown). This pattern of nuclear localization of the human PR agrees with the results obtained for the rabbit and guinea pig PR using nine monoclonal antibodies. This method displays several advantages: (a) it is a sensitive technique, (b) it is specific for the PR, (c) it can be used for the measurement of the PR content, whereas they might not be detected by immunohistological examination. This explanation is supported by the examination of tumor T20 containing a similar concentration of PR (26 fmol/mg protein) and where only a small number of cells were immunostained. Two other tumors in which PR concentration was under the limit of sensitivity of the steroid-binding assay (T18 and T28) also showed extremely rare labeled cells.

In addition, we have shown that it is possible to compare ER and PR content and distribution on adjacent sections of the same tumors. Various combinations of ER and PR content that were determined by steroid-binding assay were also detected by the double immunocytochemical assay. Only one tumor biochemically classified as ER negative/PR positive was studied (case 35). It was found to be immunocytochemically ER positive/PR negative. This discrepancy might reside in a loss of PR antigenicity due to fixation or due to an unexplained in vivo "modification" of receptor. Such hypotheses were also suggested by others for ER (24). Finally, the steroid-binding assay may have detected a non-receptor binding protein; (b) in the other case (T17) where a discrepancy was observed, PR concentration was low (28 fmol/mg protein) and specific nuclear staining was absent. A likely explanation may reside in the fact that tumors containing low amounts of receptor often contain few cells that are receptor positive: such cells present in the large tumor fragment used for the steroid-binding assay would be taken into account for the measurement of the PR content, whereas they might not be detected by immunohistological examination. This explanation is supported by the examination of tumor T20 containing a similar concentration of PR (26 fmol/mg protein) and where only a small number of cells were immunostained. Two other tumors in which PR concentration was under the limit of sensitivity of the steroid-binding assay (T18 and T28) also showed extremely rare labeled cells.

In conclusion, the studies reported here show that the immunocytochemical detection of human PR may be performed with monoclonal antibodies. This method displays several ad-
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