ABSTRACT

Progestosterone receptors (PR) are the strongest predictors of response to hormone therapy in metastatic breast cancer, while PR and the DNA indices of cell ploidy and percentage of S phase are useful prognostic indicators in early-stage breast cancer. We have developed a flow cytometry method to measure PR and DNA indices simultaneously using two aneuploid breast cancer cell lines—PR-positive T47D cells and PR-negative MDA-231 cells. Cytostats were pretreated with the progesterin 17,21-dimethyl-19-nor-4,9-pregnadiene-3,20-dione, harvested, counted, fixed with paraformaldehyde, and permeabilized with Triton X-100. To measure total PR, cells were first exposed to a mixture of the mouse anti-PR monoclonal antibody AB-52, which binds both Protein A and Protein B of human PR, and to monoclonal antibody B-30 or B-64, which bind only Protein B. Then the cells were treated with fluorescein isothiocyanate-conjugated goat anti-mouse second antibody to produce a green fluorescence signal corresponding to PR. To measure nonspecific binding, cells were treated with mouse IgG1 as the first antibody in a parallel incubation. Specific immunoassayable PR is the difference between total and nonspecific binding. Following the antibodies, the cells were treated with RNase A and propidium iodide to give a red fluorescence signal corresponding to DNA content. Red and green fluorescence per cell was then quantified by flow cytometry. This method gives a strong specific signal for PR in several T47D cell sublines but no specific binding in MDA-231 cells. Progesterin treatment led to apparent increases in PR. The proportion of cells in the G0-G1, S, and G2-M phases of the cell cycle was determined from DNA histograms and showed that both cell lines were hyperdiploid. The simultaneous flow cytometry method allowed assignment of relative PR levels in subsets of cells segregated by their DNA content. In T47D cells, PR were present throughout the cell cycle, and levels doubled in G2 and mitosis.

INTRODUCTION

ER+ and PR are important biological markers in primary breast cancers localized to the breast and axilla and in metastatic tumors (1-10). PR are a product of estrogen action, and in breast cancer their presence is a marker of hormone responsiveness (5). Patients with PR-positive tumors have longer relapse-free survival in Stage I disease (6) and longer relapse-free and overall survival in Stage II disease (2). In metastatic breast cancer, the presence of PR has the strongest positive predictive value that hormone treatment will be successful (4, 7). PR may also play a role as predictors of response to adjuvant chemotherapy or hormone therapy. The presence of PR in the primary tumor helps to predict which patients will benefit from adjuvant chemotherapy (8, 9) or from adjuvant hormone therapy following adjuvant chemotherapy (10). In a corollary fashion, patients with PR-negative tumors may not benefit from chemotherapy (8, 9), or may do less well when adjuvant hormone treatment follows adjuvant chemotherapy (10), or when adjuvant hormone therapy is used alone (2).

In the search for additional prognostic factors, attention has focused on DNA parameters: the proliferative capacity or %S or labeling index; and the amount of DNA per tumor cell relative to the normal diploid content (DNA index or ploidy). By measuring the %S using labeled DNA precursors, three groups have shown that the proliferative capacity is an important prognostic indicator independent of lymph node or ER status (11-14). Lymph node-negative patients with high %S may be at greater risk of relapse than those with low %S (12-14). In lymph node-positive patients, both relapse-free and overall survival are lower in patients with a high labeling index, even if chemotherapy is given in an adjuvant setting (12, 14).

As an expression of aneuploidy, the DNA index may also be an important prognostic indicator (15-19). Although some groups have found aneuploidy to have no prognostic significance (20, 21) or to have value only in subgroups of patients (22, 23), in a large series (17) it was the most significant factor predicting early relapse of lymph node-negative patients. Long-term follow-up on patients whose DNA content was studied from archival material indicates that nearly all 15-yr survivors had diploid or tetraploid tumors, while patients surviving less than 2 yr had predominantly aneuploid tumors (16).

Determining the proliferative index using labeled precursors requires fresh tissue, 1 to 7 days of assay time, and a skilled pathologist to interpret the results (11-14). Due to these technical limitations and because aneuploidy can be determined simultaneously, measurement of DNA indices by FCM has gained favor (24-26). In experienced hands, FCM has given S-phase estimates that correlate well with S phase by labeling indices (25), and further refinement and standardization of FCM techniques should lead to reliable intra- and interlaboratory measurements (24, 27).

At present, techniques to measure ER and PR by radioligand assays or immunocytochemical methods require separate tissue preparation from that needed for FCM analysis of DNA indices (25, 26). Radioligand assays also require relatively large tumor samples (25, 26). Because of the importance of measuring all of these prognostic indicators in breast tumors, we have set out to develop a method that will allow measurement of steroid receptors, the %S, and aneuploidy simultaneously in a single small tissue sample by FCM.

Our initial attempts to develop such an assay focused on PR. PR purified from human breast cancer cells were used to generate MAbs (28). We have now used these anti-PR MAbs with secondary fluoresceinated antibodies as PR markers, and
propidium iodide as a DNA marker, to measure intranuclear PR along with the DNA indices in PR-rich T47D (29) and PR-negative MDA-231 (30) human breast cancer cell lines. The cell lines simplify quality control and data analyses by avoiding mixed cell populations (20, 26, 27). The accompanying paper (31) addresses the value of the assay in mixed cell populations and as a tool to study tumor cell biology.

MATERIALS AND METHODS

Cells and Cell Culture. T47D cells were established from the pleural effusion of a patient with breast cancer. They have mammary epithelial characteristics, contain steroid receptors, and synthesize casein (32). Late passage (passage 303) T47D\(_{\text{ov}}\) cells, a strain of T47D cells that is estrogen resistant and exceptionally PR rich (29), were used for most of the experiments. Early passage T47D\(_{\text{om}}\) (passages 83 and 111), the estrogen-responsive “clone 11” subtype (33), and a new hypertetraploid variant (T47D\(_{\text{oh}}\), described in the accompanying paper) were also analyzed. The clone 11 cells were obtained from D. Zava (J. Muir Cancer and Aging Institute, Walnut Creek, CA). PR-negative MDA-231 human breast cancer cells (30) were obtained from D. Edwards (Denver, CO). Normal human lymphocytes and chicken erythrocytes were obtained from local volunteers and stored frozen.

Cells were plated in plastic flasks as previously described (29). In most studies, cells were pretreated 30 to 45 min at 37°C with medium containing 0.1 µM synthetic progesterone, R5020 (New England Nuclear, Boston, MA). Confluent cultures were harvested by a 10-min incubation at 37°C with 1 ml EDTA in Ca\(^{++}\)-free and Mg\(^{++}\)-free Hanks’ balanced salt solution, pelleted, resuspended in PBS at 4°C, triturated 20 times to disperse the cells, and counted with a hemocytometer. Cells (3 × 10\(^6\)) in PBS were placed in 1.5-ml conical clinical centrifuge tubes and pelleted at 500 × g for 2 min at 4°C. The supernatant was aspirated and discarded. All subsequent experimental steps until cells were delivered to the FCM were carried out at 4°C, except for treatment with RNase A and propidium iodide (see below), which was done at 37°C.

Fixation and Permeabilization of Cells. The pelleted cells were resuspended by vigorous pipetting in 1 ml of 0.5% (w/v) paraformaldehyde in PBS and allowed to react with the fixative for 5 min. The cells were then pelleted and resuspended in 1 ml of 0.1% (v/v) Triton X-100 in PBS for 3 to 5 min. They were repelleted and washed with 1 ml of PBS plus 1% (w/v) BSA.

Anti-PR Monoclonal Antibodies. Fig. 1 illustrates the structure of human PR. There are two natural forms: M, 120,000 Protein B and M, 94,000 Protein A. The two proteins are identical, except that the larger Protein B has additional cysteine-rich DNA-binding domain and a hormone-binding domain (34, 35), and both bind progesterins and are functional (36). The MAbs against human PR have been described (28): B-30 and B-64 recognize B receptors only; AB-52 recognizes both Proteins B and A. All are mouse IgG1 and were purified from ascites.

Antibody Treatments. Primary antibodies were stored at 4°C. These include the three PR-specific MAbs (28) and a control nonspecific mouse monoclonal IgG1 (Coulter Immunology, Hialeah, FL). FITC-conjugated, affinity-purified goat anti-mouse secondary antibodies or FITC-conjugated F(ab')\(_2\); fragments of the same antibodies were obtained from several commercial preparers (Cappell, Inc., West Chester, PA; Boehringer-Mannheim, Indianapolis, IN; Becton-Dickinson, Mountain View, CA; and Jackson Immunoresearch, West Grove, PA). The routine assay used an affinity-purified, human absorbed FITC-F(ab')\(_2\) (Boehringer-Mannheim; No. 60529). Optimal, consistent secondary antibody signals required: antibody storage at −20°C by the manufacturer; shipment on dry ice; storage at −20°C in the dark upon receipt in the laboratory; thawing just before first use; storage at 4°C in the dark without refreezing after initial thawing; and use within 2 to 3 wk after initial thawing. In general, good quality control required that each new primary and secondary antibody lot be characterized in controlled studies. It is useful if a tested lot can be reserved from the manufacturer.

Antibodies were prepared just prior to use in PBS containing 1% BSA. The fixed and permeabilized cells were resuspended in 1 ml of the primary antibody AB-52 alone or in combination with B-30 or B-64. Optimal incubation times were 1 h at 37°C with 17.5 µg/ml of AB-52, 10.0 µg/ml of B-30, or 10.0 µg/ml of B-64. Control cells were incubated with 12.5 µg/ml of mouse IgG1. These concentrations were determined experimentally and reflect differences in the affinities of the primary antibodies for nonspecific sites and in the affinity of FITC-F(ab')\(_2\) for the primary antibodies. At these concentrations, NSB is the same in T47D and MDA-231 cells, and NSB equals TB in MDA-231 cells. At the end of the incubation, the cells were pelleted and washed twice in PBS and 1% BSA. The cells were then resuspended 30 min at 4°C in 250 µl of FITC-conjugated secondary antibody at 40 µg/ml. One ml of PBS was then added to the incubation mixture, and the cells were pelleted.

RNase and Propidium Iodide Treatment. Following the antibody steps, the cells were exposed to a mixture of 15,000 units/ml of RNase and 50 µg/ml of propidium iodide prepared in PBS. Incubation was for 15 min at 37°C in a 500-µl volume. To stop the reaction, 750 µl of ice-cold PBS were then added, and the cells were pelleted. Finally, the pellet was brought up in 500 µl of PBS at 4°C and passed through a 75- by 75-µm nylon mesh filter. Diploid DNA was determined from normal human lymphocytes or chicken erythrocytes (0.38 × human diploid) assayed in parallel incubations.

Flow Cytometry. Cells (10,000 or 20,000) were analyzed on a Coulter Epics 752 flow cytometer (Coulter Electronics, Hialeah, FL), using an incident beam at 488 nm, 500 mW. The cells were gated on forward angle versus 90° light scatter to eliminate cellular debris and doublets. Green and red fluorences were optically separated using a 550-nm dichroic filter. Green fluorescence, corresponding to PR, was collected through a 525-nm bandpass filter, and red fluorescence, corresponding to DNA, was collected through a 590-nm longpass filter.

Data Analyses. The data were analyzed with modified Coulter Easy 2 software. Because of the wide range of PR green fluorescence, the FITC data were collected in a log mode and converted to a linear mode using the following formula: y(linear) = 10^x(log-channel number/100). The green fluorescence was detected on 256 channels designated 0 through 255. By the above formula, Channel 0 has a fluorescence value of 1.0; Channel 85, a value of 10.0; Channel 170, a value of 100.0; and Channel 255, a value of 1000.0. Fig. 2 shows how linear quantitative values are assigned: mean channel number for TB was 130, which converts to 10^12 (30/45) or 33.57. Mean channel number for NSB was 35, which converts to 10^12/45 or 2.56. The PR-SB is the difference between TB and NSB: 33.57 − 2.56 = 31.01. PR are expressed either in direct fluorescence units, or the highest level is set at 100%.

Two-dimensional and Three-dimensional Contour Plots. Data generated by the simultaneous univariate analyses of DNA and PR were accumulated to form a bivariate 64- × 64-channel distribution for each
Fig. 2. Simultaneous FCM assay for PR and DNA indices in T47D\textsubscript{0n} cells. Cells were treated with paraformaldehyde and Triton, then treated with 10 \mu g/ml of AB-52 followed by 25 \mu g/ml of FITC-F(ab')\textsubscript{2} (TB); or with nonspecific mouse IgG1 followed by FITC-F(ab')\textsubscript{2} (NSB). Cells were then treated with RNase and propidium iodide and analyzed by FCM. Panels 1 and 2 show the log green PR fluorescence using AB-52 (Panel 1) or the control antibody (Panel 2). Panels 3 and 4 show the linear red fluorescent DNA content of the same T47D\textsubscript{0n} cells shown in Panels 1 and 2. DNA levels in chicken erythrocytes (cRBC) and human lymphocytes (H\textsubscript{Lym}) are shown by the arrows. Panels 5 and 6 show the combined data by 2-parameter contour plots with simultaneous analysis of PR (log y axis) and DNA (linear x axis) of total AB-52 binding in Panel 5 and of nonspecific IgG1 binding in Panel 6.

Fig. 3. Three-dimensional contour plots of the DNA/PR distribution measured in T47D\textsubscript{0n} cells and MDA-231 cells. Hormone-untreated T47D\textsubscript{0n} cells (top) or MDA-231 cells (bottom) were fixed, permeabilized, and then treated with 10 \mu g/ml of AB-52 (TB) or a nonspecific mouse IgG1 (NSB). Cells were then incubated with 25 \mu g/ml of FITC-F(ab')\textsubscript{2}, followed by RNase and propidium iodide, and analyzed by FCM. The upper left panel used T47D\textsubscript{0n} cells and shows the log green fluorescence with AB-52 on the y axis, the linear red DNA fluorescence on the x axis, and the number of cells containing a set amount of PR and DNA on the z axis. The upper right panel is similar but shows the nonspecific green fluorescence with the control IgG1. The bottom panels used MDA-231 cells with AB-52 (left) or control IgG1 (right).

**RESULTS**

**Definition of Terms for the Flow Cytometry PR/DNA Assay.**

Fig. 2 shows the data generated from a typical experiment when T47D\textsubscript{0n} cells were treated either with AB-52 (TB), or with a nonspecific mouse IgG1 (NSB), as the first antibody, followed by the FITC-labeled second antibody and propidium iodide. The data for PR alone are on a log scale in the top panels, for DNA alone are on a linear scale in the center, and for the combined PR/DNA data are plotted in 2 dimensions in the bottom panels. Panel 1 demonstrates the heterogeneity in PR-TB levels among T47D\textsubscript{0n} cells. There is a 15- to 20-fold difference in the FITC signal intensity between cells with the lowest and highest total PR levels. Panel 2 illustrates the NSB FITC fluorescence; there is also considerable cell-to-cell heterogeneity. The source of NSB is discussed below. The overlap between TB (Panel 1) and NSB (Panel 2) receptor signals is less than 5%. Panels 3 and 4 show the DNA signal generated in the presence of the PR signal. DNA levels of diploid human lymphocytes and 0.38 \times diploid chicken erythrocytes from parallel incubations are indicated by the arrows. T47D\textsubscript{0n} cells are hyperdiploid, having a 1.60 \times diploid G\textsubscript{0}-G\textsubscript{1} DNA content. We find that the propidium iodide signal is unaffected by the intensity of the FITC fluorescence (contrast Panels 3 and 4) or by the incubations needed to label PR (not shown).

The greatest power of this FCM method is that it can assign PR levels to subsets of cells segregated by their DNA content. This can be shown either with the 2-dimensional contour plots of PR versus DNA (Fig. 2, Panels 5 and 6) where cell numbers having various PR and DNA levels are indicated by the density of the contours, or with a 3-dimensional display, which allows the variable of cell number to be added to the DNA and PR display (Fig. 3). In either case, gating allows assignment of green fluorescence values for PR-TB, NSB, and PR-SB to cells in each phase of the cell cycle. In Fig. 2 and Fig. 3, top, 57.8\% of T47D\textsubscript{0n} cells are in G\textsubscript{0}-G\textsubscript{1}, 18.2\% in S, and 24.0\% in G\textsubscript{2}-M. The highest PR-SB levels are found in G\textsubscript{2}-M. If G\textsubscript{0}-G\textsubscript{1} cells are assigned a value of 1.00, the S-phase cells have a relative PR-SB level of 1.52, and cells in G\textsubscript{2}-M have 1.92 times more PR than nondividing cells. The greatest heterogeneity in PR levels is seen in G\textsubscript{2}-G\textsubscript{1}. Fig. 3, bottom, shows the combined PR/DNA values of the PR-negative MDA-231 cells. Cells are again divided by DNA content into phases of the cell cycle, but the total and NSB curves are identical in G\textsubscript{0}-G\textsubscript{1}, S, and G\textsubscript{2}-M, demonstrating that no PR are present in MDA-231 cells at any phase.

We next analyzed each component of the assay in an effort to augment the PR signal without damaging the DNA signal. **Hormone Pretreatment.** To measure PR in their native nuclear (37-39) location and to minimize artifactual losses from soluble compartments (29, 39, 40), we tested the effect of progestin pretreatment. Fig. 4 shows a remarkable 2- to 3-fold increase in specific PR per cell following R5020 treatment, as measured with the FCM assay. The effect was seen in several different T47D sublines: T47D\textsubscript{0n} cells at passages 303, 83, or 111; the hypertetraploid T47D, (passage 39) subline (31); and the T47D clone 11 cells (33). For unknown reasons, this progestin-induced PR increase, which has also been described by Elashry-Stowers et al. (39) by immunohistochemistry, cannot be documented by ligand binding assays. In data not shown,
PR AND DNA INDICES BY FLOW CYTOMETRY

Fig. 4. Effect of R5020 pretreatment on several strains of T47D cells. T47D cells were treated with (+) or without (−) 0.1 μM R5020 for 30 min and then harvested. Cells were fixed, permeabilized, and then treated with 10 μg/ml of B-30 and 17.5 μg/ml of AB-52, or with a nonspecific mouse IgG, followed by 40 μg/ml of FITC-F(ab′)2, RNase, and propidium iodide. PR-specific binding is the difference between mean TB and NSB fluorescence signals per cell. The cells tested were T47D at passages 303, 83, and 111; a variant T47D subline (T47Dv); and T47D clone 11.

NSB changed minimally, if at all, when cells were pretreated with R5020. Therefore, the NSB/TB ratio dropped considerably, from 9.6% to 17.3% in untreated cells, to 5.1% to 8.3% after R5020 treatment. The PR content of the hormone-untreated cells ranged from 10 to 25 pmol/mg of DNA by charcoal assay.

Fixation and Permeabilization. We next defined the paraformaldehyde and Triton (41) treatment conditions that would give optimal simultaneous PR and DNA signals. Fig. 5 shows PR levels in T47D∞ cells exposed to paraformaldehyde alone (Panels 1 and 4), to Triton alone (Panels 2 and 5), or to the fixative and detergent in sequence (Panels 3 and 6), first in untreated cells (Panels 1 to 3) and then with R5020 pretreatment (Panels 4 to 6). Background NSB is shown in Panel 7. Paraformaldehyde alone (Panels 1 and 4) allows some PR measurement, but there is marked heterogeneity of the fluorescence signal. With or without R5020 pretreatment, many cells have NSB signal levels. Permeabilization alone without fixation (Panels 2 and 5) gave virtually no PR-SB signal relative to NSB, independent of hormone treatment. Even if PR are more tightly bound in the nucleus by R5020, the receptor proteins appear to be solubilized, degraded, or their immunological epitopes somehow masked, when cells are exposed to Triton without first being fixed. Fixation plus permeabilization gave a high and homogeneous PR signal in R5020-untreated cells (Panel 3) and even higher PR-SB after R5020 pretreatment (Panel 6). The hormone-untreated cells had a mean PR-SB of 25.9, while the R5020-pretreated cells had a mean PR-SB of 100, confirming the effect of R5020 pretreatment described in Fig. 4. Permeabilization was also required to obtain a satisfactory DNA signal (not shown).

The duration of paraformaldehyde and Triton treatment also proved to be important to obtain high PR-SB. In Fig. 6, R5020-pretreated T47D∞ cells were treated for 5 to 30 min with paraformaldehyde and 1 to 10 min with Triton. The shortest fixation time (5 min) gave the highest PR-SB signal. Longer fixation times reduced PR-SB levels 10 to 30%. This effect was independent of the Triton treatment time although, as shown above, some detergent treatment was necessary.

Several concentrations of fixative and detergent were tested, and 0.5% paraformaldehyde and 0.1% Triton were found to be optimal. While higher concentrations, longer treatment times, and detergent washes have been used by others in assays for glucocorticoid receptors (42), these conditions reduced the PR signal by more than 40% (not shown).

Antibody Treatment Conditions. After cells are pretreated with progesterin, fixed, and permeabilized, they are exposed to the PR-specific MAbs or to nonspecific mouse IgG1s. T47D∞ cells were treated with AB-52 for times ranging from 10 min to 2 h at 4°C, and 1 h yielded maximum PR-SB (not shown). To determine optimal combinations of the three MAbs, T47D∞ cells were treated with equal concentrations of the anti-PR MAbs AB-52, B-30, and B-64 alone, in pairs, or together (Fig. 7). At the subsaturating concentrations tested, AB-52 alone gave the strongest signal, while the B-specific antibodies (B-30 and B-64) gave identical lower signals. Combining one of the B-specific antibodies (B-30) with AB-52 generated signals equal to...
Conditions for the FITC-conjugated second antibody. At satu-
rated concentrations of the PR-specific first antibodies, 40 ng/ ml of FITC- second antibody, RNase, and propidium iodide. Parallel sets were treated with a non-specific first antibody. PR-SB was calculated from TB and NSB.

Fig. 6. Effect of parafomaldehyde and Triton treatment time on specific binding of PR as measured by FCM in T47Dc® cells. Cells were treated with 0.1 µM R5020 for 30 min, harvested, and then treated with 0.5% parafomaldehyde for 5 to 30 min, followed by 0.1% Triton for 1 to 10 min. Cells were then incubated with 17.5 µg/ml of AB-52 and 10 µg/ml of B-30, 40 µg/ml of FITC-
second antibody, RNase, and propidium iodide. Parallel sets were treated with a non-specific first antibody. PR-SB was calculated from TB and NSB.

Fig. 7. Contributions to PR-specific binding of the PR-specific antibodies alone and in combination. T47Dc® cells were pretreated with R5020, harvested, and treated with parafomaldehyde and Triton. The cells were then incubated with AB-52 (10 µg/ml), B-30 (10 µg/ml), or B-64 (10 µg/ml) alone, in pairs, or all three together, or to the corresponding amount of non-specific mouse IgG1. After treatment with 40 µg/ml of FITC-secondary antibody, RNase, and propidium iodide, they were analyzed by FCM. PR-SB was calculated.

to the sum of each alone, while the two B-specific antibodies together were no better than each alone. This was seen again when AB-52 was combined with both B-30 and B-64: there was little further increase with two B-specific antibodies beyond that seen with one. Our routine assay, therefore, uses AB-52 plus either one of the B-specific antibodies.

To determine concentrations of AB-52 and B-30 mixtures necessary to give maximum specific binding for PR, T47Dc® cells were incubated with equimolar concentrations of AB-52 and B-30 ranging from 0.1 to 20 µg/ml per antibody (Fig. 8). Saturation occurred between 15.0 and 20.0 µg/ml. When each antibody was used alone, AB-52 saturated at 17.5 µg/ml and B-30 at 10.0 µg/ml (not shown). These concentrations were used for subsequent experiments.

Since the PR-SB signal can be underestimated if either the PR-specific first antibody or the fluoresceinated second antibody is not at saturation, we next determined the optimum conditions for the FITC-conjugated second antibody. At saturating concentrations of the PR-specific first antibodies, 40 µg/ml of FITC-second antibody were saturating (not shown). When cells were incubated in various volumes from 75 to 300 µl of FITC-second antibody at a concentration of 40 µg/ml, 250 µl yielded maximal PR-SB (not shown).

Conditions for RNase A and Propidium Iodide Treatment. The timing and concurrent use of RNase and propidium iodide proved to be important to maximize PR-SB and to generate DNA histograms of high quality. We tested RNase and propidium iodide under five conditions: concurrently and sequentially either before, or after, the antibody steps; and with RNase before the antibodies, and propidium iodide after. Maximum PR-SB and the best DNA histograms resulted when RNase and propidium iodide were used concurrently after the antibody steps. The 15-min 37°C incubation after the antibodies, necessary to activate the RNase, had the added advantage of lowering propidium iodide under five conditions: concurrently and sequentially either before, or after, the antibody steps; and with RNase before the antibodies, and propidium iodide after. Maximum PR-SB and the best DNA histograms resulted when RNase and propidium iodide were used concurrently after the antibody steps. The 15-min 37°C incubation after the antibodies, necessary to activate the RNase, had the added advantage of lowering propidium iodide.

Fig. 8. Primary antibody concentrations needed to saturate PR. T47Dc® cells were pretreated with R5020, harvested, fixed, permeabilized, and then incubated with equimolar mixtures of AB-52 and B-30 ranging from 0.1 µg/ml to 20.0 µg/ ml (TB) or to the corresponding amount of non-specific mouse IgG1 (NSB). Following treatment with 40 µg/ml of FITC-secondary antibody, RNase, and propidium iodide, cells were analyzed by FCM. PR-SB is the difference between TB and NSB.

Fig. 9. Factors contributing to PR-non-specific binding. To assign background fluorescence (BG), MDA-231, T47Dc®, and T47D cells were incubated with 1 ml of PBS and 1% BSA instead of the primary antibody and with 250 µl of PBS and 1% BSA instead of the secondary antibody. To determine the contribution to NSB of the FITC-secondary antibody (2d AB), cells were treated with 1 ml of PBS and 1% BSA followed by 250 µl of the FITC-F(ab')2 at 40 µg/ml. The fluorescence signal in this set minus BG gives the contribution of the 2d AB. To determine the contribution to NSB of the primary antibody, cells were treated with the non-specific mouse IgG1 at 10 µg/ml (T10) or 27.5 µg/ml (T27.5), followed by 40 µg/ml of FITC-F(ab')2. The contribution to NSB by the primary antibody alone (1st 10 or 1st 27.5) is calculated from T10 and T27.5 as follows: T10 — (BG + 2d AB) = 1st 10; T27.5 — (BG + 2d AB) = 1st 27.5. All sets were also treated with RNase and propidium iodide.
weekend? Fig. 10 shows a study in which the biochemical assay assaying suspended cells from hypocellular or small tumors. T47D cells to speed the FCM sampling. However, as few as 1 million cells per assay tube (not shown). Additional propidium iodide is required at higher cell numbers, quantified with 1 to 4 million cells per assay tube (not shown). 

% Max. Mean PR-SB/Cell

Day of FCM Analysis

Fig. 10. Specific PR levels when FCM analysis is delayed. T47D\(_{\text{cl}}\) cells were harvested and treated with paraformaldehyde, Triton, and the primary and secondary antibodies. Parallel sets were treated for 15 min at 37°C with RNase plus propidium iodide (RNase/PI) or RNase only (RNase), then pelleted, and resuspended at 4°C in 500 µl of PBS with or without additional PI. One set of each was analyzed immediately (Day 0) on the FCM. The remainder were stored at 4°C for 1 or 2 days, before the cells were resuspended and analyzed on the FCM. PR-SB on Day 0 was set at 100%.

replacing the antibody steps), with the FITC-second antibody only, or with both the nonspecific first antibody and FITC-second antibody. All were then treated with RNase and propidium iodide. The calculations that allow assignment of the individual components of NSB are outlined in the legend to Fig. 9. MDA-231 and T47D\(_{\text{cl}}\) cells have similar total levels of NSB fluorescence, and the levels increase when the primary antibody concentration is raised from 10 to 27.5 µg/ml (Fig. 9, T10 and T27.5). T47D, cells, which are larger than the other two, have even higher total levels of NSB fluorescence at either antibody concentration. Background autofluorescence is the same in all cells and does not contribute to the differential NSB levels, but may represent 30% of the total NSB fluorescence. Autofluorescence is thought to result from endogenous fluorescent molecules, such as pyridine or flavin nucleotides (43), or alternatively from weak propidium iodide emission at the FITC-emitting wavelengths. It is higher in nuclei than in cytoplasm. The contribution to NSB fluorescence from the primary antibody increases as its concentration increases from 10 to 27.5 µg/ml, but does not differ significantly among the cell lines. It too represents approximately one-third of the total. Finally, the contribution of the secondary antibody to NSB fluorescence is also roughly one-third and is especially high in T47D, cells. Since NSB fluorescence can contribute significantly to the total apparent PR levels, the NSB fraction must be independently evaluated under appropriate assay conditions and subtracted from total binding.

A second area of quality control lies in the range of cell numbers over which PR and DNA indices can be reliably measured. We find that both PR and DNA are accurately quantified with 1 to 4 million cells per assay tube (not shown). Additional propidium iodide is required at higher cell numbers, but PR signals may be damaged. We routinely use 3 to 4 million T47D cells to speed the FCM sampling. However, as few as 1 million cells can be used. This may prove to be important when assaying suspended cells from hypocytoplasmic or small tumors.

An important practical question for a clinical assay is the delay that can be tolerated between the biochemical procedures and the FCM analysis. Can samples wait overnight or over a weekend? Fig. 10 shows a study in which the biochemical assay was performed in a parallel series of tubes, and the FCM analysis was done immediately (Day 0) or delayed for 1 or 2 days. The effect of propidium iodide addition with or after the RNase treatment and warming was also tested. While the PR signal was clearly stable during overnight incubation and could be analyzed on the FCM the next day, it was not stable to more prolonged storage. The highest PR levels were obtained when propidium iodide was added together with RNase at 37°C before storage in PBS at 4°C. In data not shown, DNA histograms remain satisfactory if the cells are stored overnight at 4°C in PBS.

**DISCUSSION**

We have developed a prototype FCM method that allows the simultaneous measurement of PR, DNA, and proliferation rate. These studies were conducted in the PR-positive T47D and PR-negative MDA-231 aneuploid cell lines. The technique combines cell cycle fractionation with a quantitative PR immunoassay. Pretreating cells with the synthetic progestin R5020, brief fixation with paraformaldehyde, and permeabilization with Triton permit MAbs directed against PR to penetrate and bind to exposed receptor epitopes in nuclei; the MAbs in turn are bound by fluoresceinated secondary antibodies. These conditions allow intercalation of propidium iodide into DNA without disrupting the binding of antibodies to PR. The limiting technologies to arrive at this method have been the development of MAbs against PR and the fixation and permeabilization conditions that allow PR to remain stably bound to nuclei during antibody and propidium treatments. The FITC fluorescence signal was amplified by the simultaneous use of two anti-PR MAbs which bind to nonoverlapping epitopes on the PR molecules.

As we began this project, only a few models existed for the simultaneous measurement of DNA indices and a separate intranuclear antigen by FCM (41, 44–48). None attempted to measure proteins of the steroid receptor family along with the DNA indices or to quantitate PR by immunological means. FCM measurement of ER has been carried out using fluoresceinated estrogens (49, 50). These assays demonstrated specific ER in MCF-7 cells (49, 50) and breast tumors (49), but the methods were hampered by the high dissociation constants of the fluoresceinated ligands. This required the use of high ligand concentrations and led to unsatisfactorily high levels of NSB and binding to low-affinity type II sites. Our FCM method, which is based on antibodies, circumvents these problems and includes an analysis of DNA indices at the same time.

**Fixation and Permeabilization.** Alcohols (45, 46), formalin (47), and a combination of paraformaldehyde and Triton (41, 44) are fixatives that have been used in successful efforts to measure intranuclear antigens and DNA indices simultaneously by FCM. Because of the autofluorescence of formalin and the signal losses due to cytoplasmic dispersion of steroid receptors and other nuclear proteins by alcohols (39, 41, 42), we focused on the paraformaldehyde/Triton method (41, 44) for the PR/DNA system. We find that paraformaldehyde gives minimal background autofluorescence. The detergent is necessary to permeabilize the plasma membrane and/or the nuclear membrane to allow IgG molecules access to the nuclear proteins.

In simultaneously measuring cell cycle-associated chromatin antigens and DNA by FCM, Clevenger et al. (41) described satisfactory results with prolonged treatment time (1 to 100 min) of 0.5% paraformaldehyde followed by Triton. Cells fixed in methanol or paraformaldehyde alone had sharply reduced mean fluorescence intensities compared to the cells treated with

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*Unpublished observations.*
paraformaldehyde/Triton. Our results are consistent with some of these findings. Like Cleveger et al. (41), we find that both paraformaldehyde and Triton are necessary for optimal PR and DNA determinations. However, we find that PR immunofluorescence is very sensitive to paraformaldehyde treatment time, with 10 to 30% losses of PR-SB occurring when treatment exceeds 10 min. Analogous to Cleveger et al. (41), we find that addition of Triton to the fixative significantly improves FITC signal strength and reduces heterogeneity. This step is apparently not required for immunocytochemical measurement of PR or ER in frozen or paraffin-embedded sections (51–57).

The PR Antibodies. Among the family of nuclear receptor proteins, including those for steroid and thyroid hormones, vitamin D₃, and retinoic acid, PR are unique in that they have two ligand-binding forms in the same cell: A-receptors and B-receptors. Recent studies with chick oviduct and human breast cancer PR suggest that both receptors are synthesized naturally in cells, that both are biologically active, but that each may subserve different functions in gene transcription (34–36, 58, 59). Both forms are found, often in unequal proportions, in human breast and endometrial cancers (28, 60). Until it is determined which form, if either, is more important physiologically or as a prognostic marker in tumors, any immunoassay that measures PR must be competent to measure both. The MABs used here (28) have this competence. It is also critical that the antibodies be specific for PR and have no cross-reactivity with other nuclear proteins so that NSB and false-positive values can be minimized. Our antibodies are highly specific for PR in nuclei (28). We find that use of AB-52 and one of the B-specific antibodies in combination gives a higher fluorescence signal than either alone, suggesting (a) that the epitopes recognized by the two MABs are sufficiently separated on B-receptors to allow both antibodies to bind simultaneously, and (b) that the FITC-secondary antibodies are able to bind two primary antibodies without steric hindrance. However, the current MAB mixture would have greater sensitivity for B- than for A-receptors.

Progestin Treatment and Immunooassays. The clinical PR immunoassays developed to date are based on immunocytochemical methods (39, 51–53). They document the nuclear location of PR (39, 51–53) and correlate well with PR in breast cancers measured by radioligand assays (39, 51) when the antibodies used are PR specific. Milgrom et al. (51) used two B-specific antibodies to measure PR in 27 breast cancers and found 92% concordance between the immunocytochemical and ligand binding assays. Using the same MABs we used here, Elashry-Stowers et al. (39) found good correlation between immunocytochemical and ligand binding assays in 33 breast tumors. On the other hand, Pertschuk et al. (53), using other antibodies directed against human PR (52, 61) and archival material, found a 24% discordance between immunocytochemical and ligand-based PR methods. These investigators also found that the immunocytochemical method more accurately predicted relapse-free survival and disease progression than the ligand binding assay.

Discrepancies between immunooassays and radioligand assays are not unexpected, however, since the former are dependent on PR epitope availability and the latter on unoccupied or exchangeable hormone binding sites. We have noted a 2- to 4-fold increase in cellular PR-SB when any of several different sublines of T47D cells were pretreated 30 min with a progestin and assayed by FCM. These increases in total cellular PR cannot be documented by ligand binding assays (see Ref. 62, for example) and may reflect increased epitope availability in hormone-transformed receptors. While most PR immunoassays (51–53) have not studied progestin pretreatment, Elashry-Stowers et al. (39) have, and they found a similar augmentation of PR in T47D cells by immunocytochemistry. By immunocytochemistry Greene et al. (63) saw little or no increase in PR after brief estradiol treatment of MCF-7 cells and several tissues, but McLellan et al. (64) showed a marked increase in nuclear ER 60 min after in vitro estrogen treatment of macaque uterine tissues. Thus, unmasking of a subset of receptor molecules that are detectable with antibodies but not with radioligands may be a generalized phenomenon in whole cell-based assays. Discrepancies between the two assays, especially in tissues from premenopausal patients, may have real biological significance.

Progestin pretreatment clearly increases the nuclear binding affinity of PR (62) and prevents their loss from soluble compartments. In other systems using estradiol pretreatment of osteoblast-like cells, it has allowed detection of ER that could not be measured by traditional ligand binding assays (65). Paradoxically, endogenous hormones or hormone pretreatment which unmarks immunoreactive sites may lead to underestimation of receptors by radioligand assays if the proteins are measured only in cytosol and not in nuclei, and if exchange assay conditions are not used (66). Furthermore, while brief progestin treatment transiently raises PR, longer exposure down-regulates PR (29, 31, 40). It is clear, therefore, that the hormonal history of a patient can critically influence tumor receptor levels, and that the interpretation of receptor data in clinical practice must rest on the endocrine background.

The Combined Assay. The simultaneous FCM assay for PR and DNA allows assignment of receptor levels to cells in various phases of the cell cycle. We find that cells in G2-M have twice the receptor levels of cells in G1-G0. This is not surprising when one considers that, in order for daughter cells to have the same receptor content as the parent cell, protein levels must double prior to cell division. However, this doubling has presumably been difficult to quantify for steroid receptors, although glucocorticoid receptor levels have been found to increase in S and G2-M in HeLa cells (67, 68) and human lymphocytes (69). Transcriptional and posttranscriptional mechanisms have been proposed to regulate these increases (68), but the cell cycle-specific factors that must control these are unknown. The combined assay also shows that there is greater heterogeneity in PR levels among cells in G2-G1, than among cells that have entered the synthetic phase of the cycle.

In sum, this paper describes a prototype FCM assay for PR and DNA indices in well-defined human breast cancer cell lines. The accompanying paper (31) describes experiments using the assay to quantitate small subsets of cells in mixed populations by their PR or DNA content, and to document the genetic instability of the T47D line.

REFERENCES


3940


Simultaneous Measurement of Progesterone Receptors and DNA Indices by Flow Cytometry: Characterization of an Assay in Breast Cancer Cell Lines
