Antibodies to Proliferating Cell Nuclear Antigen as S-Phase Probes in Flow Cytometric Cell Cycle Analysis

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ABSTRACT

The usefulness of different anti-proliferating cell nuclear antigen monoclonal antibodies as S-phase probes in flow cytometric analysis was evaluated after various fixation procedures on human cell lines. With a newly developed detergent extraction/fixation method the monoclonal antibody PC10 acted as a selective S-phase marker. A total of 27 human hematopoietic tumors were analyzed using PC10, and all exhibited the characteristic S-phase recognition pattern. The percentage of PC10-positive cells was easily calculated and showed strong correlation to the fraction of S-phase cells determined from DNA histograms. Using alternative fixation procedures PC10, on the other hand, could recognize all actively cycling cells, a feature also observed for the monoclonal antibodies, 19A2 and TOB7.

INTRODUCTION

PCNA is involved in DNA replication and necessary for adequate leading strand synthesis, acting as the auxiliary protein of DNA polymerase δ (1, 2). There are at least two different forms of PCNA (3). One extractable-form is present in significant amounts in proliferating cells but is almost undetectable in resting cells. Another form of PCNA is detergent resistant and closely related to DNA synthesis. The staining pattern of the S-phase-related PCNA resembles the topographical patterns of DNA replication sites (4). Thus, PCNA could theoretically serve as a marker for cells actively replicating DNA. This feature is, from a diagnostic point of view, an important aspect, since the fraction of S-phase cells is an independent prognostic parameter for human tumors, such as malignant lymphoma and breast cancer (5-7). The possibility of quantitating S-phase cells using an antigen-antibody reaction would be of great value.

Several MoAbs to PCNA have been produced, reacting with the protein in Western blots (8-10). After special fixation procedures two of these MoAbs (19F4 and 19A2) could detect S-phase cells in the MOLT-4 cell line using FCM (11). However, in another FCM study no significant S-phase staining was achieved using the 19F4 MoAb (12). The MoAb PC10 showed by FCM strong reactivity with Hela cells without any obvious cell cycle phase variation (13). To clarify the usefulness of PCNA MoAbs as S-phase markers in FCM analysis, we have tested four MoAbs (19F4, 19A2, TOB7, and PC10) and evaluated the cell cycle phase-specific reactivity in human cell lines after various fixations. With a specific extraction/fixation method, PC10 was shown to be an excellent S-phase marker in all tested cell lines as well as in lymphoma cells.

Cells. Thirteen human cell lines were used in the present study: Daudi (14); MN-60 (15); RPMI-8226 (16); Jurkat (17); CCRF-CEM (19); KG-1 (20); HL-60 (21); U937 (22); Hela (23); HT29 (24); 158-B4; and UM-42. The cell lines are described in the references given, except for the 158-B4 and UM-42 lines which are diploid lymphoblastoid B-cell lines not previously described. Cells were grown in Ham’s F10 or RPMI 1640 medium (Gibco, Scotland) supplemented with 10% fetal calf serum and antibiotics. KG-1 cells were grown in 20% fetal calf serum.

Cells growing as monolayers (Hela, HT29) were detached with trypsin treatment or by adding a lysing buffer (see below, Fixation D). Cell suspensions from 24 fresh lymph nodes and three bone marrow samples were prepared. Eighteen cases were non-Hodgkin’s lymphomas classified according to the Kiel classification (25), 10 were of low-grade malignancy, and 8 were of high-grade malignancy. Two cases were Hodgkin’s lymphomas, and 4 were diagnosed as benign lymphadenitis. Also, 3 acute leukemias (2 acute myeloblastic, one acute lymphocytic) were included in the study.

PCNA Antibodies. A human anti-PCNA serum was obtained from a patient (A. K.) with systemic lupus erythematosus. This serum has been used in previous studies (26-29) and is well defined. 19F4 (IgG1) and 19A2 (IgM) are MoAbs generated from mice immunized with purified PCNA from rabbit thymus (8). 19F4 was obtained as purified antibody and 19A2 as ascites fluid. The human anti-PCNA serum and 19F4 and 19A2 antibodies were kindly provided by Dr. E. M. Tan.

TOB7 (IgG1) was a kind gift from Dr. Y. Takasaki, who established it using purified calf thymus PCNA as immunogen (9).

PC10 (IgG2) is a MoAb established after immunization with a recombinant PCNA protein, and it was a kind gift from Dakopatts A/S, Denmark (10).

The PCNA serum was diluted 1:400; PC10, 1:50 to 1:100; 19A2, 1:200; and the MoAbs 19F4 and TOB7 were used at a concentration of 50 µg/ml. As a negative control for the PCNA autoantibody a serum from a healthy laboratory worker was used. Controls for the MoAbs were: anti-IgE MoAb (IgG1), Dakopatts A/S (19F4 and TOB7); an irrelevant IgG2 control MoAb, Becton-Dickinson (PC10); and anti-Leu M1 MoAb (IgM), Becton-Dickinson (19A2).

Fixations. Six different fixation procedures (A to F), which have been shown to be useful in detecting nuclear antigens with FCM or with immunohistochemistry, were evaluated. These procedures are: A, 70% ethanol for 10 min at −20°C; B, 100% methanol for 10 min at −20°C followed by 0.5% NP-40 (in PBS) on ice for 5 min; C, 1% PF in PBS for 2 min and then, after a PBS wash, fixation in 100% methanol for 10 min at −20°C followed by 1% bovine serum albumin in PBS for 30 min on ice followed by addition of 3 ml of 10% formaldehyde for 10 min at −20°C; D, 0.1% Triton X-100; 0.2 µg/ml of EDTA:1% bovine serum albumin in PBS for 15 min on ice followed by addition of 2% paraformaldehyde for 15 min; and F, 1% PF for 15 min followed by permeabilization in 0.1% Triton X-100.

The initial cell number in each sample was 2 × 10⁶ cells, and after fixation the cells were washed twice in PBS before immunostaining.

Staining. Fixed cells were incubated with the primary antibody for 30 min at room temperature. After two PBS washes, FITC-conjugated anti-human antibody (1:50) or FITC-conjugated anti-mouse antibody (1:20) was added, and the samples were kept in the dark for 30 min (all FITC antibodies were from Dakopatts A/S). After two additional PBS washes and two PBS washes, the samples were incubated with second antibody for 30 min in the dark.

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2 To whom requests for reprints should be addressed.

3 The abbreviations used are: PCNA, proliferating cell nuclear antigen; FCM, flow cytometry; FITC, fluorescein isothiocyanate; PF, paraformaldehyde; MoAb, monoclonal antibody; PI, propidium iodide; IgG, immunoglobulin G (other immunoglobulins defined similarly); NP-40, Nonidet P-40; PBS, phosphate-buffered saline; BrdUrd, bromodeoxyuridine.
washes, the cells were resuspended in a DNA-staining solution containing PI (10 μg/ml) and RNase (1.8 Kunits/ml) and kept cold and dark for at least 30 min until FCM analysis.

Standard DNA histograms were obtained from the clinical samples after PI staining according to the method of Vindelöv et al. (30). The percentage of S-phase cells were calculated using a modified peak reflect method (31).

In order to evaluate the number of cells actively replicating DNA, Jurkat cells were incubated with 10 μM BrdUrd (Sigma Chemical Co., St. Louis, MO) for 20 min, whereafter the cells were ethanol fixed and treated with 2 M HCl containing 0.2 mg/ml of pepsin for 20 min (32). After treatment with 0.1 M Borax and two PBS washes, 10 μl of anti-BrdUrd MoAb (Becton-Dickinson) were added for 30 min in room temperature, followed by an FITC-labeled anti-mouse MoAb. In the last step the cells were resuspended in a PI/RNase solution.

Flow Cytometry. Cells were analyzed in a FACScan flow cytometer equipped with an argon laser (488 nm) (Becton-Dickinson Immunocytometry Systems, Mountain View, CA), and data were registered and stored in list mode. Debris and damaged cells were excluded by gating on a forward and side scatter dot plot or on the DNA histogram. FITC fluorescence was detected in the FL1 channel (530 ± 15 nm) and stored using logarithmic or linear amplification. DNA was recorded in the FL2 channel (585 ± 22 nm) using linear amplification. Data obtained were evaluated with the FACScan software (Becton-Dickinson).

In some experiments the FITC fluorescence was expressed as a ratio between the median fluorescence channel for the anti-PCNA antibodies divided by the median fluorescence channel for the control antibody fluorescence. These ratios were calculated in specific parts of the DNA histograms for both PCNA antibodies and control antibodies. The five DNA compartments analyzed are shown in Fig. 1 and were: early G0-G1; late G0-G1; S; early G2-M; and late G2-M. For example, a ratio of 2.0 means that the PCNA fluorescence was twice as high as the control antibody fluorescence in that DNA compartment. These calculations were performed on linear fluorescence values. The ratios obtained made it possible to visualize the PCNA expression in different parts of the cell cycle in a proper way.

Correlation between PC10-positive cells and S-phase fractions was determined by linear regression analysis.

RESULTS

Three cell lines (Daudi, Jurkat, MN-60) were primarily investigated after fixation Procedures A (ethanol), B (methanol + NP-40), C (PF + methanol), and D (lysing buffer + methanol) concerning the cell cycle phase-specific reactivity of anti-PCNA antibodies. Figs. 2 to 6 show the fluorescence ratios calculated for each antibody in different DNA compartments of the Jurkat cell line. The Daudi and MN-60 cell lines gave similar results as for Jurkat.

With the human PCNA autoantibody (AK), S-phase-specific staining was obtained with Fixations A, B, and D (Fig. 2). The best S-phase separation was found for Fixation A (ethanol). With Fixation C (PF + methanol), the autoantibody yielded intermediate PCNA expression in all DNA compartments.

Low fluorescence signals were obtained for the 19F4 MoAb, but both Fixations B and C gave higher fluorescence in S-phase cells compared with the other cell cycle compartments (Fig. 3).

19A2 showed intermediate to strong fluorescence with Fixations A, B, and C without any cell cycle phase preference (Fig. 4). With Fixation D, a general weak fluorescence was observed with slightly higher levels in late G0-G1 and S.

TOB7 and PC10 yielded large differences in reactivity depending on the fixation used (Figs. 5 and 6). A weak S-phase preference with Fixations B and C was observed for TOB7, whereas PC10 preferentially stained S-phase cells after Fixation D (lysing buffer + methanol). The S-phase pattern for PC10 was very similar to that obtained with the human autoantibody (Fig. 2, Fixation D).

All four MoAbs were almost unreactive after Fixations E (Triton X-100 + PF) and F (PF + Triton X-100). The human autoantibody, however, showed S-phase-specific reactivity with Fixation E and intermediate fluorescence without cycle phase specificity after Fixation F (not shown in figures).

After the initial testing, 19F4 (methanol + NP-40) and PC10 (lysing buffer + methanol) were selected for further investigations concerning S-phase specificity. Ten human cell lines were tested with 19F4 and showed variable cell cycle phase reactivity. Some cell lines exhibited a fairly nice S-phase recognition (e.g.,
PCNA ANTIBODIES AS S-PHASE PROBES

Fig. 2. Fluorescence ratios calculated for 1A2 using the same approach as in Fig. 2.

Fig. 4. Fluorescence ratios calculated for TOB7 using the same approach as in Fig. 2.

Fig. 5. Fluorescence ratios calculated for PC10 using the same approach as in Fig. 2.

Fig. 6. Fluorescence ratios calculated for PC10 using the same approach as in Fig. 2.

Fig. 7. Jurkat cells labeled with 10 nM BrdUrd (BrdU) for 20 min and stained with PC10 and PI (lysing buffer + methanol) (A) and anti-BrdUrd and PI (B). The antibody fluorescence is presented in logarithmic scale and the DNA content in linear scale.

DISCUSSION

The different forms of PCNA existing in resting and in cycling cells have been explored in detail previously (3, 33). It has been suggested that PCNA is present in high concentrations in cycling cells in order to form a complex with DNA polymerase δ in cells entering S phase (33). The complex which is active in DNA replication is tightly bound to DNA, and this form of PCNA is not extractable with detergent. With the detergent extraction/fixation method described here, the anti-PCNA MoAb PC10 can act as an S-phase marker. The method seemed reliable and could recognize S-phase cells in all different cell lines and human hematopoietic tumors tested. When fixation with lysing buffer + methanol was used, the fluorescence intensity for PC10 diminished to approximately 25% of the values observed after methanol fixation only. This is in agreement with the finding that the S-phase-associated PCNA form constitutes about one-fourth of the total PCNA content (3). It also indicates that, after the detergent extraction/fixation procedure, only the DNA replicon-complexed PCNA was left in the cell nuclei.

One question is whether PC10 MoAbs using lysing buffer
and methanol fixation exclusively recognize S-phase cells. Several authors have shown that the staining pattern obtained with anti-PCNA antibodies is similar to that obtained with thymidine labeling or anti-BrdUrd stainings (Footnote 5; Refs. 3, 4, 34, and 35). Data from PC10 stainings of cells blocked in late G1 with mimosin, in early S with aphidicolin, or in G2 with mitoxantrone also indicate that PC10 MoAbs selectively recognize cells in S phase. Furthermore, dual-parameter analysis of human anti-PCNA serum and PC10 (lysing buffer + methanol) indicates that these antibodies recognize the same cells (not shown in figures). Thus, we conclude that PC10 can be used as an S-phase marker.

One advantage in estimating S-phase cells from PC10 reactivity instead of from DNA histograms is that it might give more exact S-phase values. In a few PC10-DNA stainings of malignant lymphomas, we noticed cells with S-phase DNA content negative for PC10. This could be attributed to debris, quiescent S-phase cells, or aneuploid non-S-phase cells. These cells were, however, included in the S-phase fraction determined from a DNA histogram. This problem is often encountered in material from solid tumors and especially those with much debris giving false positive signals in the DNA histograms.

In lymphomas, the number of PC10-positive cells correlated well with the S-phase fractions determined from DNA histograms. In a previous study (36), the PCNA expression measured with the human autoantibody correlated well with S-phase percentages as well as with the number of cells positively stained for the proliferation-associated antigen Ki-67. In the same report, PC10 was shown to distinguish cycling cells from resting cells after methanol fixation and NP-40 treatment. In the present study it seemed that PC10, 19A2, and TOB7 were strongly reactive with cycling cells after methanol + NP-40 or PF + methanol treatment. Any of these MoAbs could theoretically serve as a proliferation marker using the proper fixation. Accordingly, PC10 can act as an S-phase probe or, dependent on the fixation procedure used, a marker for all proliferating cells. A similar finding has been reported for 19A2 using immunohistochemistry (37).

Human anti-PCNA autoantibodies preferentially react with S-phase cells after alcohol fixations, whereas formaldehyde treatment can reveal the non-S-phase form of PCNA probably by unmasking antibody recognition sites (3). The masked PCNA form is still present after methanol fixation, since it can be visualized with the autoantibody after postfixation in PF.

This is further substantiated in the present study with the MoAbs, especially PC10, showing strong signals in all DNA compartments after methanol fixation.

The differences in reactivity found for the autoantibody and the MoAbs can be explained by epitope differences. It has been shown that the human autoantibody identifies epitopes which are highly dependent on protein conformation, whereas the anti-PCNA MoAbs recognize linear epitopes (38). Furthermore, using synthetic peptides we have found that the 19A2, 19F4, and PC10 MoAbs recognize closely related, although not identical, epitopes.

Our results were mainly generated from studies of hematopoietic cells, but it was also possible to detect S-phase cells with PC10 in two epithelial cell lines. Thus, it might be possible to use PC10 as an S-phase marker in solid tumors. However, preliminary studies on colon, renal, and breast carcinomas have been unsuccessful so far. There might be differences between in vivo- and in vitro-grown epithelial cells concerning fixation effects and the availability of PCNA epitopes. It is obvious that

<table>
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<th>Patient</th>
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* LGM, non-Hodgkin’s lymphoma of low-grade malignancy; HGM, non-Hodgkin’s lymphoma of high-grade malignancy; An, aneuploid; ALL, acute lymphocytic leukemia; AML, acute myeloblastic leukemia.

Unpublished results.
Fig. 9. Correlation between the percentage of PC10-positive cells and S-phase cells evaluated from DNA histograms of 27 hematopoietic tumors (r = 0.936, P < 0.001).

Further studies are needed to clarify the value of PC10 as a proliferation marker for nonhematopoietic cells. This is further stressed by the reported deregulated PCNA expression detected with PC10 in normal, tumor-adjacent tissues (13).

The procedure presented here to determine S-phase cells using the PC10 MoAb and FCM is easy to perform and evaluate and can be of potential value in characterizing hematopoietic tumors. Since the S-phase fraction of non-Hodgkin’s lymphoma is an independent prognostic predictor (5, 6), it can be assumed that this property of tumors might influence the choice of therapy in the future.

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