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Cell-cycle–Related Staining Patterns of Anti-proliferating Cell Nuclear Antigen Monoclonal Antibodies

Comparison with BrdUrd Labeling and Ki-67 Staining

Jan Hein van Dierendonck,* Jan H. Wijsman,* Rob Keijzer,* Cornelis J. H. van de Velde,* and Cees J. Cornelisse†

From the Departments of Surgery and Pathology,† State University of Leiden, Leiden, The Netherlands*

Monoclonal antibodies (MAbs) to nuclear antigens are increasingly used as tools to obtain valuable information concerning the proliferative characteristics of various types of cancer. Prerequisite for the application of these MAbs in surgical pathology is establishment of the level of expression and/or cellular distribution of the antigens in relation to distinct cell-cycle compartments. In this study the topologic distribution of proliferating cell nuclear antigen (PCNA), an auxiliary protein of DNA polymerase δ, as recognized by human autoantiserum (AK) and two recently developed MAbs (19A2 and 19F4), was evaluated. Using cultured human cancer cells as a model system, and providing optimal fixation/permeation procedures are applied, these antibodies display a high affinity for PCNA bound to nuclear replicon clusters, resulting in distinct granular staining patterns. A more diffuse nucleoplasmic PCNA staining was mainly restricted to non–S-phase cells; in methanol-fixed cells, staining intensity of this form relative to the replicon-bound form appeared higher after staining with 19A2 than with 19F4 or AK. Comparing PCNA expression (detected with 19A2) with the expression of the Ki-67 antigen, PCNA-negative cells are also Ki-67 negative. In MCF-7 human breast cancer cells treated with 10^{-6} mol/l (molar) tamoxifen, the fraction of nuclei showing replication patterns decreased from 42% to 8% within 8 days, but PCNA and Ki-67 antigens remained detectable in most cells during this interval, indicating a relatively slow decrease of antigen expression in cells that have entered a quiescent state. Treatment of MCF-7 cells with 10^{-6} mol/l methotrexate resulted in a rapid accumulation of cells with an early S-phase DNA content; PCNA replication patterns showing a frequency distribution reflecting this DNA content were observed up to 48 hours after treatment. This indicates that the presence of replication patterns as visualized with anti-PCNAs is not a measure of replicative activity per se. It is concluded that, providing nuclear non–S-phase PCNA staining is faint relative to staining of replicon clusters, anti-PCNA antibodies may be excellent markers to detect* in situ *cells with S-phase DNA contents. (Am J Pathol 1991, 138:1165–1172)

Cell kinetic data are among the most important indicators of treatment response and relapse in many types of cancer.[1] However conventional methods to estimate proliferation rates are complex, time-consuming, and not easily applicable to routine clinical material.

In this context, the advent of monoclonal antibodies (MAbs) to cell-cycle–related antigens is promising because these MAbs allow an easy and rapid evaluation of immunostained tissue sections.[2–8] Many proteins involved in DNA replication, chromosome condensation, or mitosis can be useful markers, provided that variations in total expression and/or topographic distribution are specifically related to processes involving cellular growth and division.

In MCF-7 human breast cancer cells, we recently studied the nuclear distribution and kinetics of the antigen recognized by the Ki-67 MAb, which is increasingly applied as a proliferation marker in histopathology. Comparing staining patterns of Ki-67 with immunocytochemical detection of DNA-incorporated 5′-bromodeoxyuridine

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Address reprint requests to Dr. J. H. van Dierendonck, Department of Pathology, University of Leiden, P.O. Box 9603, 2300 RC Leiden, The Netherlands.

(BrdUrd), we observed that no unambiguous determination of S-phase or growth fractions could be obtained with this Ki-67 MAb.[9]

In the present study, we evaluated immunocytochemical staining patterns of the 36-kd proliferating cell nuclear antigen (PCNA),[10,11] identified as an auxiliary protein of DNA polymerase-δ.[12,13] We compared staining results of two commercially available mouse MAbs (19A2 and 19F4) and autoantibodies obtained from a patient with systemic lupus erythematosis (SLE) to staining patterns observed after immunocytochemical detection of DNA-incorporated BrdUrd. Recently Garcia et al[14] described the application of the 19A2 MAb to paraffin-embedded tissues and showed that PCNA-positive fractions were consistently higher than S-phase fractions as calculated from flow cytometric DNA histograms. Similar results recently were obtained by Galand and Degraef,[15] who compared 19A2 staining to (^{3}H)-thymidine incorporation.

We now demonstrate that, depending on fixation/permeation conditions, both mouse MAbs and human autoantibodies can be used to detect subphases of DNA replication, similar as observed by immunostaining of DNA-incorporated BrdUrd.[16,17] To elucidate whether the presence of replisome-bound PCNA depends on replicative activity, we examined PCNA staining in MCF-7 cells treated with the cytostatic compound methotrexate (MTX).

Apart from S-phase PCNA, anti-PCNA antibodies recognize a more diffusely distributed nuclear form, present in all other phases of the cell cycle. The kinetics of non–S-phase PCNA was compared with that of the Ki-67 antigen in MCF-7 cells blocked in G1/G0-phase.

Materials and Methods

Antibodies

19A2 and 19F4 (murine IgM and IgG MAbs, respectively), originally developed by Ogata et al,[18] were provided by American Biotech (Plantation, FL).

Polyclonal anti-PCNA antibodies (designated AK), isolated from an SLE patient, were provided by Dr. E. M. Tan (Scripps Clinic, La Jolla, CA).

IU-4 anti-BrdUrd/IdUrd MAb and Ki-67 were gifts from Dr. F. Dolbeare (Lawrence Livermore National Laboratory, Livermore, CA) and Dakopatts (Glostrup, Denmark), respectively. Br-3 MAb, which prefers binding to DNA-incorporated BrdUrd over IdUrd by a factor 50–100,[19] was purchased from Caltag Laboratories (San Francisco, CA). Combined application of IU-4 and Br-3 in cell populations labeled with both IdUrd and BrdUrd for different time intervals allow an estimation of the S-phase duration.[19]

Cell Lines and Culture Conditions

The hormone responsive MCF-7 human breast carcinoma line (a gift from Dr. M. E. Lippman, National Cancer Institute, Bethesda, MD) and the IGROV ovary carcinoma line (a gift from V.T.H.B.M. Smit, Department of Pathology, Leiden, The Netherlands) were grown at 37°C as monolayer cultures on glass slides in Roswell Park Memorial Institute (RPMI) 1640 medium, supplemented with 10% fetal calf serum, 0.02 mol/l (molar) HEPES buffer, 15 mmol/l (millimolar) sodium bicarbonate, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate.

Unless stated otherwise, culture medium was replaced by fresh medium twice weekly.

To accumulate cells in the $G_{1,0}$ phase of the cell cycle, the antiestrogenic compound tamoxifen (TAM, ICI-Pharma, Macclesfield, UK) was used in a final concentration of 10^{-6} mol/l. Tamoxifen was added in fresh culture medium to MCF-7 cultures 3 days after plating (when confluency was about 25%). Methotrexate (MTX, Pharmachemie B.V., Haarlem, The Netherlands) was used to accumulate cells in early S phase (also in a final concentration of 10^{-6} mol/l).

BrdUrd Labeling and Immunocytochemistry

Cells were labeled by adding BrdUrd (Sigma, St. Louis, MO) to the culture medium in a final concentration of 20 μmol/l (micromolar) and were fixed after 30 minutes. To optimize PCNA detection, different fixation protocols were tested, as summarized in Table 1.

To test various immunocytochemical stainings on a

Table 1. *Effect of Various Fixatives on Anti-PCNA and Ki-67 Immunoreactivity in MCF-7 Monolayers*

	Immunoreactivity*		
Fixation procedure	19A2	AK	Ki-67
Formaldehyde (4%, 10 minutes, RT)	−	++	−
Methanol/acetic acid 3:1 (10 minutes, 4°C)	−	−	+
Ethanol (70%, 10 minutes, −20°C)	−	−	+
M: Methanol (100%, 10 minutes, −20°C)	++	+++	+
Acetone (100%, 10 minutes, −20°C)	+	+++	+++
PF-I: Paraformaldehyde (2%, 10 minutes, RT)	−	++	++
PF-II: Paraformaldehyde (1%, 2 minutes, RT)	+	ND	++
PF-II followed by M	+++	+++	+++

PCNA, proliferating cell nuclear antigen; RT, room temperature; M, methanol; ND, not done.

* Scored as negative (−), weakly positive (+), positive (++), and strongly positive (+++).

single slide, each slide was divided into 10 to 12 areas, using a Dakopen (Dakopatts). Before incubation with MAbs, which were diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (Sigma) and 0.1% Tween-20, the slides were preincubated for 5 minutes in PBS containing 0.1% Nonidet P40 to improve permeation of the antibodies.[20] The protocol for detection of DNA-incorporated BrdUrd was described previously.[9,16]

The second antibody was a peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts); diaminobenzidine (Sigma) was used as a substrate. In case of the human AK antiserum, we applied peroxidase-conjugated rabbit anti-human immunoglobulin (P212, Dakopatts) as a second step. Cells were counterstained with hematoxylin and studied with a Zeiss Axiophot microscope. To obtain BrdUrd labeling indices and fractions of PCNA and Ki-67–positive cells, respectively, 500 to 1000 cells were counted.

Flow Cytometric DNA Analysis

To compare anti-PCNA staining patterns with S-phase fractions as calculated from flow cytometric DNA histograms, and to monitor the effects of treatment with TAM or MTX on MCF-7 cells, cells were scraped from slides and prepared for flow cytometry using the detergent-trypsin method as described by Vindelov et al.[21]

After staining with propidium iodide (Sigma), samples were measured on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA).

Results

Fixation and Staining Conditions

To optimize immunostaining for simultaneous detection of PCNA and Ki-67 antigens, various fixation protocols were tested (summarized in Table 1). Excellent results for Ki-67, anti-PCNA antibodies (as judged from the preservation of replication patterns), and anti-BrdUrd were obtained by the protocol described by Kurki et al,[20] using 1% paraformaldehyde in phosphate buffer pH 7.4 for 2 minutes at room temperature, followed by methanol for 10 minutes at −20°C (Figure 1A to E). No PCNA signal was obtained when cultures were fixed in ethanol or methanol/acetic acid. In contrast to PCNA MAbs, human AK polyclonal antibodies intensely stained aceton-fixed cells, as well as cells fixed in formaldehyde.

PCNA Staining Patterns

Two types of nuclear PCNA staining were discriminated: a granular pattern probably originating from replisome-bound PCNA, and a more diffusely localized form. Cytoplasmatic staining was particularly prominent in mitotic cells.

S-phase–Specific PCNA

In a previous paper, we described characteristic patterns of DNA-incorporated BrdUrd reflecting the spatial and temporal organization of DNA synthesis and showed that it is possible to discriminate different subphases of the S phase by the different granular patterns.[16] As shown in Figure 2 for both IGROV and MCF-7 cells, the similarity between these BrdUrd labeling patterns (I, A–F) and nuclear distributions of PCNA (II, A–F and III, A–F, respectively) is striking.

Early S-phase nuclei are easily recognized as relatively small nuclei with a limited number of distinct, homogeneously distributed spots (each spot probably representing a replicon domain consisting of a cluster of multiple replisomes), which are not located at the periphery of the nucleus or in perinucleolar regions (S1; Figure 2A). A second pattern, appearing later in the S phase, is characterized by a significant increase in the number of spots (S2; Figure 2B). Subsequently staining becomes prominent at the nuclear boundary and in perinucleolar regions (S3; Figure 2C, D). In a later subphase, the number of

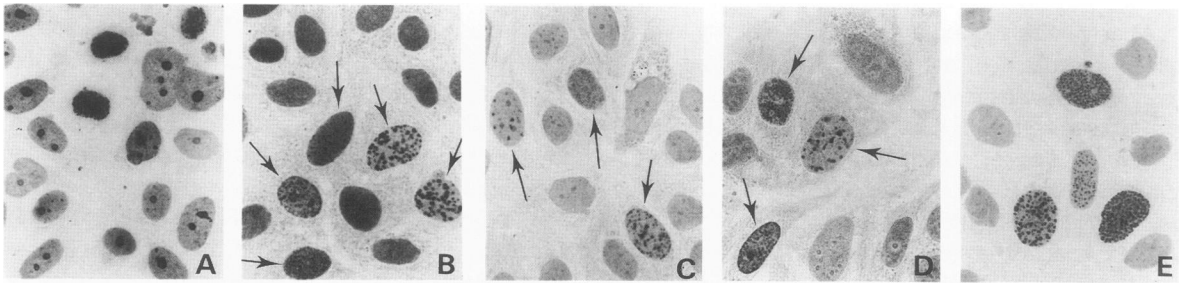

Figure 1. *Immunoperoxidase staining of Ki-67 MAbs* **(A)**, *19A2 MAbs* **(B)**, *19F4 MAbs* **(C)**, *AK antiserum* **(D)**, *and IU-4 anti-BrdUrd MAbs* **(E)** *in MCF-7 human breast cancer cells after fixation in paraformaldehyde/methanol. Arrows: anti-PCNA–stained S-phase nuclei (original magnification, ×250).*

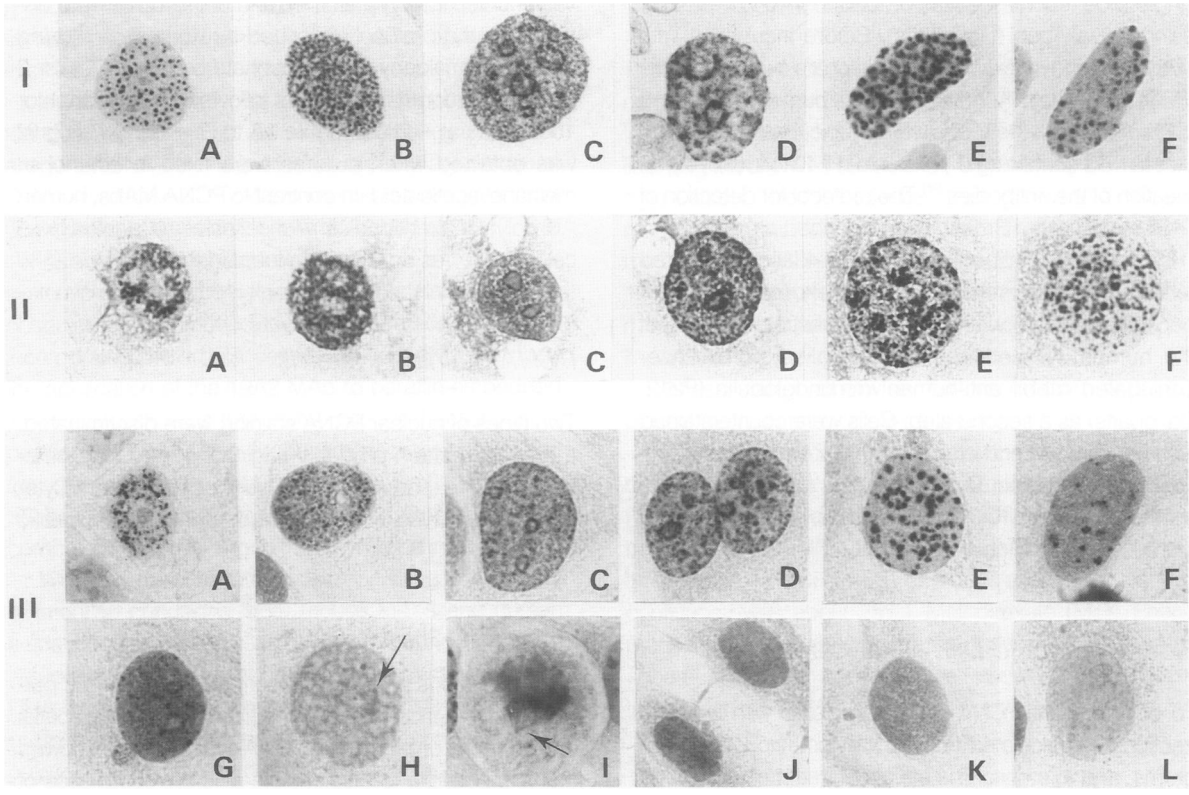

Figure 2. **IA–IF:** *Anti-BrdUrd immunoperoxidase staining patterns in MCF-7 nuclei.* **IIA–IIF:** *PCNA immunoperoxidase staining patterns in IGROV nuclei (AK autoantibodies).* **IIIA–IIIL:** *PCNA immunoperoxidase staining patterns in MCF-7 nuclei (19A2).* **A:** *Very early S phase (S1);* **B:** *early S phase (S2);* **C,D:** *mid S phase (S3);* **E:** *late S phase (S4);* **F,G:** *very late S phase (S5);* **H:** *prophase;* **I:** *mitosis;* **J:** *postmitotic cells;* **K:** *G1/0-phase;* **L:** *PCNA-negative cell. For details, see text (original magnification, ×500).*

spots is reduced, with an increase in size, presumably as a result of close spacing of late replicating heterochromatin (S4; Figure 2E).

In the final stage of DNA-replication a limited number of distinct (most often pairwise arranged) spots is observed (S5; Figure 2F). These late S-phase nuclei display a rather gradual transition to G_2 nuclei. Staining with 19A2 reveals the presence of a diffuse staining of the nucleus in cells with a relatively small number of replication spots (III, F,G).

Non–S-phase–Specific PCNA

All antibodies showed a relatively high affinity to mitotic cells. Unlike Ki-67 antigen, PCNA does not seem to be associated with metaphase chromosomes, but rather to cytoskeletal elements (Figure 2I; arrow: spindle figure). Shortly after division, methanol-fixed MCF-7 cells stained with 19A2 displayed both cytoplasmatic and nuclear staining (Figure 2J). The diffuse nucleoplasmic staining showed variable intensities, ranging from very intense to almost completely negative (Figure 2K,L).

19A2, 19F4, and AK antibodies showed clear differences with respect to their affinity to diffuse cytoplasmatic PCNA relative to S-phase PCNA, when applied to MCF-7 cultures fixed in methanol with or without previous short-term fixation in 1% paraformaldehyde (PF). Most intense non–S-phase staining was observed when 19A2 MAbs were applied, whereas application of AK antibodies resulted in lowest non–S-phase staining. In contrast to anti-PCNA MAbs, AK antiserum showed intense staining of S-phase in aceton-fixed cell populations. A significant AK staining of non–S-phase PCNA was observed after fixation in 4% formaldehyde or 2% PF.

Kinetic Analysis

The frequency distributions of DNA replication patterns as detected by BrdUrd labeling correspond well with the frequency of similar patterns obtained by anti-PCNA staining. Figure 3A shows results from a rapidly cycling cell population, whereas the cell population shown in Figure 3B were treated for 24 hours with 10^{-6} mol/l MTX. The DNA histogram depicted in Figure 3B indicates an accumulation of cells in early S phase, which also is reflected by the shift in replication pattern frequencies as compared to the unperturbed cell population. This accumulation in early S phase was also observed at 48 hours after MTX treatment (data not shown), indicating a strong

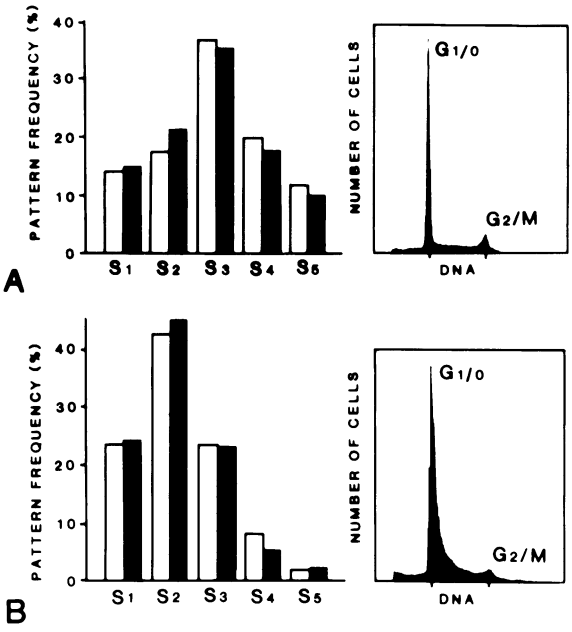

Figure 3. *Frequency distributions S-phase patterns as detected by BrdUrd labeling (open bars) and anti-PCNA staining, respectively (closed bars; AK antiserum), and flow cytometric DNA histograms from the same MCF-7 cell populations.* **A**: *Cells in log-phase growth;* **B**: *cells treated with 10^{-6} mol/l MTX for 24 hours and labeled with BrdUrd 15 minutes before fixation.*

inhibition of S-phase progression. BrdUrd labeling of MTX-treated cultures resulted in a strong labeling of S-phase cells within minutes. This rapid release from MTX-induced inhibition of DNA replication by BrdUrd treatment did not account for the presence of PCNA-stained replication patterns because similar PCNA staining intensities and S-phase fractions were observed in parallel cultures with and without BrdUrd; S-phase PCNA and BrdUrd labeling indices were, respectively, 71.9% and 72.6% for 24-hour MTX treatment and, respectively, 68.1% and 68.4% for 48-hour MTX treatment.

In Table 2, 'S-phase' PCNA, as detected by different antibodies, is compared with the BrdUrd-labeling index (BrdUrd-LI), and 'total PCNA positivity' (as detected by 19A2) is compared with Ki-67 positivity. Data are shown for six rapidly cycling MCF-7 cultures and an unfed plateau-phase culture.

Because BrdUrd values invariably were slightly higher than S-phase estimations obtained by anti-PCNA staining, we estimated the contribution of labeling duration: BrdUrd-LIs should be corrected for the fraction of late replicating cells entering G2 phase during the labeling period. The S-phase duration (Ts) was estimated by the following procedure: cultures were labeled during 2.5 hours with IdUrd (t1) and for 20 minutes with BrdUrd (t2). Since, in contrast to IU-4, Br-3 has a much higher affinity to BrdUrd than to IdUrd, a double staining procedure with both Br-3 and IU-4 enables discrimination of cells labeled for different time intervals.[19] Ts was calculated as follows:

$$Ts = (t1 \times LI_{IdUrd} - t2 \times LI_{BrdUrd})/(LI_{BrdUrd} - LI_{IdUrd})$$

(Footnote: it should be noted that this is a slight overestimation because the cell-cycle age distribution of the exponentially growing cell population is not taken into account). Mean S-phase duration thus determined from two 'log phase' cultures was 11.4 ± 0.5 hours, being in accordance with previously reported data on this cell line[22]; this value therefore was used to correct BrdUrd-LIs for the period of labeling.

Double staining of an MCF-7 cell population for PCNA and BrdUrd revealed that no early S-phase PCNA patterns were observed in BrdUrd-negative cells. A similar double staining for (total) PCNA and Ki-67 revealed that cells completely negative for PCNA also were negative for Ki-67; sometimes PCNA-positive cells without detectable (nucleolar) Ki-67 staining were observed (not shown).

Treatment with 10^{-6} mol/l tamoxifen, which reduces the growth fraction of MCF-7 cell populations by inducing a $G_{1,0}$ block in a majority of cells,[7] resulted in a rapid decrease of the fraction of replication-pattern–positive cells, in contrast to a slow decrease of the fraction of nuclei positive for 'total' PCNA as well as of Ki-67-positive nuclei (Figure 4).

Table 2. *PCNA Staining, BrdUrd Labeling Index (LI), and Ki-67 Positivity in Multiple MCF-7 Cultures**

PCNA S-phase patterns	Fast-growing cultures						Unfed
19A2:	43.3	39.1	35.9	29.5	33.8		3.2†
19F4:	44.2	38.9	35.1		32.2		
AK:					31.9	37.8	
BrdUrd-LIs‡		42.1	37.5	33.4	34.0	38.9	3.4
§		40.3	35.9	32.0	32.6	37.3	3.2
Total PCNA (19A2):	96.3	93.9	91.3	89.9			80.7
Ki-67 positive:	92.4	89.9	92.2	94.2			79.8

PCNA, proliferating cell nuclear antigen.
* Percentage of total cell population; 500 to 1000 cells scored.
† Plateau-phase culture left unfed for 7 days.
‡ Values without correction for duration of labeling.
§ Values corrected for duration of labeling (t = 0.5 hour), using the factor Ts/(Ts + t) (Ts = 11.4 hours; see text).

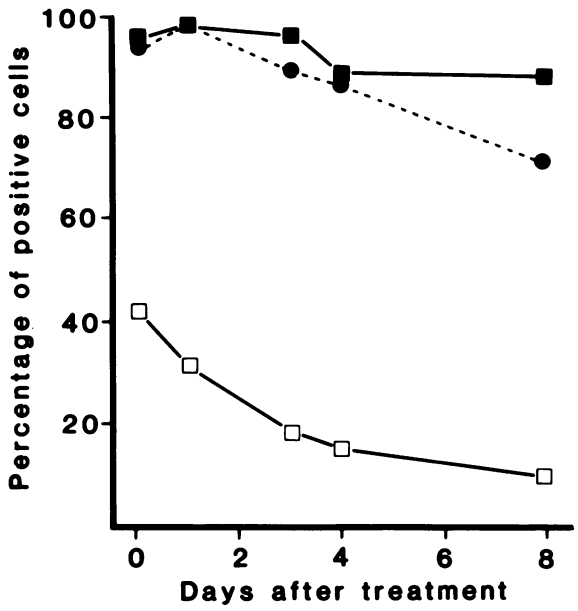

Figure 4. *Effect of tamoxifen treatment (10^{-6} mol/l) on the fractions of PCNA and Ki-67–positive MCF-7 cells. Depicted are the total fraction of positive nuclei stained with 19A2 (closed squares), the S-phase fraction as evaluated with 19F4 (open squares), and the fraction of Ki-67–positive nuclei (closed circles).*

Discussion

Proliferating cell nuclear antigen ('PCNA') was discovered by Miyachi et al[10] by the use of autoantibodies in sera of patients with SLE. Comparing two-dimensional gel electrophoresis of proteins from proliferating and quiescent cell populations, Bravo and Celis[11] described a 36-kd protein ('cyclin'), which appeared to be identical to PCNA. Because the name 'cyclin' also has been given to a 56-kd protein kinase involved in the initiation of mitosis,[23] we adopted the name PCNA to avoid confusion.

Proliferating cell nuclear antigen has been identified as an auxiliary protein of DNA polymerase-δ,[12,13] an enzyme most likely involved in the catalysis of eukaryotic leading strand synthesis and in DNA repair.[24,25] Therefore it may not be surprising that the granular nuclear staining patterns of antibodies to PCNA show a great resemblance with replicon clusters as observed after immunohistochemical detection of DNA-incorporated BrdUrd.[16,17]

To detect PCNA in paraffin sections, purified antisera from patients with SLE have been used in a number of studies.[26,27] By immunizing mice with purified PCNA from rabbit thymus, Ogata et al[18] recently generated two MAbs, IgM anti-PCNA (19A2) and IgG anti-PCNA (19F4), respectively. They observed that a larger fraction of cells was stained by the 19A2 MAb.[20] From studies on the application of human anti-PCNA autoantibodies in mouse 3T3 cells, Bravo et al[28] concluded that during S-phase, two nuclear populations of PCNA may exist, one of which is tightly associated with replicon clusters; the other form could be extracted from the cells using a detergent and could not be detected immunocytologically after methanol fixation.

In the present study, using cultured breast and ovarian cancer cells as a model system, we evaluated the reliability of the application of anti-PCNA mouse MAbs and human autoantibodies in detecting S-phase–specific staining patterns. We demonstrate that in methanol-fixed MCF-7 cells, all antibodies showed affinity to intranuclear non–S-phase PCNA, although 19F4 MAb and human AK serum to a lesser extent than 19A2 MAb. Notably this diffuse nucleoplasmic staining was present in $G_{1,0}$ and G_2/M phases, but virtually absent in S-phase nuclei, except in the final stages of DNA replication (Figure 2, IIF,G). In contrast to Bravo et al,[28] we did not succeed in reducing the non–S-phase staining relative to 'replicon cluster-associated' PCNA by treatment of cells with 0.1% Triton X-100 (in 2 mmol/l $MgCl_2$ and ethyleneglycol-bis (β-aminoethylether)-N,N,N',N'-tetraacetic acid (EGTA) containing Pipes buffer, pH 6.1 at room temperature) for various time intervals before fixation.

A possible explanation for the finding that the antibodies may differ with respect to their S-phase specificity is that they have different antigenic determinants.[18,20] It has been suggested that, because autoantibodies may detect conformationally dependent structures of PCNA, some of these antibodies may be highly specific for PCNA associated with the replication sites.[29]

In a preceding paper on staining patterns of Ki-67 MAb, reactive to an antigen reported to be present in cycling cells only,[2] we showed that treatment of MCF-7 cells during 3 days with 10^{-6} mol/l TAM reduces the growth fraction dramatically (from about 80% to 30%), but without a commensurate reduction in the fraction of Ki-67–positive cells.[9] Here we show a similar phenomenon for non–S-phase PCNA; whereas the fraction of S-phase cells is strongly reduced during TAM treatment, the fractions of both total PCNA and Ki-67 remain at high levels (Figure 4). Further studies are required to determine the physiologic condition of cells that have entirely lost PCNA and Ki-67 antigens.

The small fraction of PCNA-positive cells without detectable (nucleolar) Ki-67 staining probably represent late G_1 because it has been reported that Ki-67 expression may be undetectably low at the onset of DNA replication.[9,29] In contrast to Ki-67, PCNA expression shows a less pronounced variation during the cell cycle. Morris and Matthews recently reported that the fluctuating synthesis of PCNA maintains this protein at a roughly constant proportion of the total cell protein. These authors also found that in HeLa cells, only one third of total PCNA is tightly associated with S-phase cells.[30] However, in

replicating cells in properly fixed MCF-7 or IGROV cultures, we did not detect diffuse nucleoplasmic staining (except toward the end of S phase). Cytoplasmatic staining was invariably weak, except in mitotic cells (also when 19F4 or autoantibodies were applied).

The absence of clear early or late S-phase PCNA patterns in BrdUrd-negative cells suggests that the localization of PCNA at or near the origins of replication coincides with the start of DNA replication and that replicon clusters rapidly disintegrate after completion of the replication process. This is confirmed by the close accordance between 1) S-phase PCNA fractions and BrdUrd-LIs in various MCF-7 cultures, particularly when BrdUrd-LIs were corrected for the duration of labeling (Table 2) and 2) S-phase subfractions evaluated by anti-PCNA and anti-BrdUrd, respectively (Figure 3). Because in 19A2-stained cell populations it was more difficult to discriminate early S-phase patterns from strongly stained non–S-phase nuclei, this may explain why S-phase fractions estimated by 19A2 staining were consistently slightly higher than fractions estimated by 19F4 or AK staining (Table 2).

An important question is whether the binding of PCNA to replicons is affected by the rate of DNA synthesis. To answer this question, we treated MCF-7 cultures with MTX at a concentration strongly inhibiting S-phase progression. This resulted in an accumulation of cells with an early S-phase DNA content, commensurate with an increase in early S-phase PCNA staining patterns (Figure 3B). This DNA content/PCNA distribution was observed both 24 and 48 hours after MTX treatment. BrdUrd, like thymidine,[31] rapidly reversed MTX toxicity, and resulting immunocytochemical staining patterns closely resembled PCNA patterns both in morphology and frequency (Figure 3B). These results indicate that cells blocked in a particular S-subphase may retain their specific replication pattern for a considerable period of time.

In conclusion, the finding reported by others that the fraction of PCNA-positive cells in immunostained tissue sections is consistently higher than the fraction of ^{3}TdH- or BrdUrd-labeled cells may have at least two explanations. First nuclei may not be completely devoid of non–S-phase PCNA. Because in tissue sections it is far more difficult to discriminate granular S-phase patterns from the diffuse nucleoplasmic form, the presence of the latter may substantially interfere with the estimation of S-phase fractions. Important in this context is the finding that antibodies may differ with respect to their affinity to non–S-phase PCNA.

Second, even if anti-PCNA antibodies show a very high specificity to the protein structure at replication sites, this affinity may be entirely independent of the replicative activity itself. Several studies provide evidence for the presence of a substantial fraction of tumor cells with S-phase DNA contents not incorporating detectable amounts of labeled DNA-precursors,[32,33] suggesting that these cells either do not synthesize DNA or do so at a very slow rate.[34] Thus, although cells under certain conditions may fail to incorporate labeled DNA-precursors, anti-PCNA antibodies may still detect the presence of an intact replication machinery for a considerable period of time.

We conclude that replisome-bound PCNA may be an excellent marker for the *in situ* detection of cells with an S-phase DNA content but does not discriminate between cells with a normal replication rate and very slowly or nonreplicating cells in S phase.

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