A NUCLEAR ANTIGEN ASSOCIATED WITH CELL
PROLIFERATION AND BLAST TRANSFORMATION
Its Distribution in Synchronized Cells*

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In a small percentage of patients with systemic lupus erythematosus (SLE),\(^1\) an
autoantibody has been demonstrated that is reactive with a nuclear antigen present
in proliferating cells (PCNA) \((1)\). The nuclear antigen was not detected by immuno-
fluorescence in renal tubular or glomerular cells nor in hepatic parenchymal cells, but
was detected in certain unidentified cells in the interstitial tissues of these organs.
Normal peripheral blood lymphocytes did not contain detectable amounts of PCNA,
but it was present in some lymphocytes in lymph node follicles, spleen, and thymus.
After mitogen-induced blast transformation, peripheral blood lymphocytes became
positive for PCNA, demonstrable as nuclear staining in the blast-transformed cells.
Continuous tissue culture cell lines of different species such as WiL-2, Hep-2, baby
hamster kidney walls, and Ehrlich ascites tumor cells were positive for PCNA.

The reactive nuclear antigen was extractable from tissue culture cells with physio-
logical saline, and the solubilized PCNA formed precipitin lines with autoantibody
that were immunologically distinct from previously identified autoantibodies, such as
Sm antigen, nuclear RNP, and the SS-A/Ro and SS-B/La antigen-antibody systems
\((2)\). In previous studies, immunofluorescence demonstrated a speckled pattern of
nucleoplasmic staining.

Because of the apparent association of PCNA with proliferation and blast transfor-
mation, we have attempted to determine more precisely the time relationship of
PCNA to DNA, RNA, and protein synthetic events in the cell cycle. In both
continuously proliferating and blast-transformed cells, there appears to be a close
relationship with a phase preceding active DNA synthesis. In addition, PCNA appears
in the nucleolus at this time, a phenomenon not previously recognized.

Materials and Methods

Preparation of Specific Anti-PCNA Sera. Two anti-PCNA sera (MN and EB) obtained from
patients with SLE were used. In addition to anti-PCNA, these sera contained autoantibodies to
other nuclear antigens, including double-stranded DNA, DNA-histone, and Sm (Table I). In
serum MN, antibody to PCNA was present in highest titer, and at the serum dilution of

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\(^1\) Abbreviations used in this paper: FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; PHA,
phytohemagglutinin; PCNA, proliferating cell nuclear antigen; RNP, ribonucleoprotein; Scl-70, sclero-
derma-70 antigen; SLE, systemic lupus erythematosus; SS-A, Sjögren's syndrome-A antigen; SS-B, Sjögren's
syndrome-B antigen.

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Characteristics of Sera Containing Antibody to PCNA

| Serum | PCNA | DS-DNA* | DNA-Histone | Sm |
|-------|------|---------|-------------|----|
| MN    | +    | +       | +           | -  |
| EB    | +    | -       | -           | +  |

* Double-strand DNA.

1:1,024, this serum was monospecific for PCNA as demonstrated by immunofluorescence. However, insufficient quantities of this serum were available, and it was used to confirm the observations made with serum EB, which was available in larger quantities. Serum EB was rendered operationally monospecific for PCNA in the following manner.

It was previously observed that differentiated tissues such as rabbit kidney contained negligible amounts of PCNA (1). The small amounts present were probably in the unidentified cells scattered in the interstitium. This tissue was therefore used as a source of antigen(s) to remove all autoantibodies except anti-PCNA from serum EB (1). Rabbit kidney was homogenized with a Potter homogenizer in 0.15 M NaCl (1 g rabbit kidney/4 ml 0.15 M NaCl), and the suspension was sonicated for 6 × 15 second cycles at maximum output with a model W185 sonicator (Heat Systems-Ultrasonics, Plainview, N. Y.). The sonicate was stirred for 4 h at 4°C and centrifuged at 3,500 g for 30 min. The supernatant contained ~60 mg protein/ml and was the source material for making solid phase immunoadsorbant after the method of Avrameas and Ternynck (3). 4 ml rabbit kidney extract was mixed with 4 ml of bovine serum albumin (60 mg/ml), the latter being used as nonspecific protein carrier. To this mixture, 0.8 ml of 2 M acetate buffer, pH 5, was added, after which 2 ml of 2.5% glutaraldehyde was added dropwise to this mixture. The reaction mixture was allowed to stand for 3 h at room temperature without stirring. After the gel formed, it was homogenized with a Potter homogenizer and dispersed in 50 ml of 0.1 M phosphate buffer, pH 7.4. After centrifugation, the immunoadsorbent gel was washed repeatedly with phosphate buffer until the OD 280 nm of the supernatant was <0.050. The gel was then rehomogenized, dispersed in 50 ml of 0.2 M HCl-glycine, pH 2.8, and stirred for 15 min at room temperature. The gel was subsequently neutralized with 1 M K2HPO4, centrifuged and dispersed in 10 ml of 0.1 M lysine, and incubated overnight at 4°C. For use as immunoadsorbant, the gel was washed in phosphate-buffered saline (PBS) (0.15 M NaCl, 0.01 M phosphate, pH 7.4) and resuspended in PBS containing 0.01% sodium azide. 1 ml of anti-PCNA serum (EB) was diluted 1:10 with PBS, added to 30 ml of packed gel, and incubated for 1 h at room temperature and for 24 h at 4°C. After this incubation, the mixture was centrifuged at 3,500 g for 30 min, the supernatant recovered, and concentrated to the starting volume of anti-PCNA serum with a Minicon B125 concentrator (Amicon Co., Bedford, Mass.). In the absorbed serum (EBa), autoantibodies to Sm antigen were removed, but anti-PCNA was still present (see Results).

An extract of WiL-2 cells, a line of continuously growing human B diploid lymphocytes, were used as the source for PCNA antigen (4). The extraction of antigens from WiL-2 cells has been described (5). Briefly, cells in the log phase of growth were harvested and sonicated in a buffer containing 0.25 M sucrose, 4 mM CaCl2, and 10 mM PO4, pH 6.2. The extract obtained from this sonication contained PCNA, as well as other soluble nuclear antigens. The WiL-2 extract was used as the source of antigen in double diffusion to identify precipitating anti-PCNA antibody.

**Immunodiffusion and Immunofluorescent Methods.** A double diffusion method was used to demonstrate precipitating antibodies to PCNA and other soluble nuclear antigens (6). In our laboratory, reference sera have been available for the identification of precipitating antibodies to double-strand DNA, DNA-histone, Sm antigen, and other nonhistone nuclear antigens. These reference sera were placed in wells next to anti-PCNA sera to determine identity or nonidentity of precipitin lines. The reactions were allowed to proceed at room temperature for 72 h, and precipitin lines were observed daily.
Indirect immunofluorescence was used with WiL-2 cell or human peripheral blood lymphocyte preparations. Cytocentrifugation spreads of these cells were prepared from a 0.1-ml suspension containing $1.5 \times 10^6$ cells/ml. The cell spreads were fixed in acetone for 10 min at room temperature. It was found that cytocentrifuged cells were superior to cell smears because cytocentrifugation produced flat preparations of cells with more even distribution of cell number. The conjugate used was fluorescein isothiocyanate (FITC)-labeled goat anti-Cohn fraction II and had the following characteristics: FITC-132 µg/ml; protein-14 mg/ml; unitage-8 U/ml; plateau endpoint-1:64 dilution (7). This conjugate was diluted 1:20 in PBS for use in the indirect immunofluorescence technique. Stained slides were observed at a magnification of ×500 with an incident light microscope equipped with a 200-W Mercury light source, KPF 90 exciter filter, and K530 barrier filter.

Synchronization of WiL-2 Cells and Determination of Phase of Cell Cycle. Two different methods of cell synchronization were used to study the distribution of PCNA in different phases of the cell cycle. In the first method, WiL-2 cells were synchronized by starvation or density-dependent arrest as described by Lerner and Hodge (8). WiL-2 cells were grown in Autopow medium (Flow Laboratories, Inglewood, Calif.), supplemented with 2 mM glutamine, vitamins, nonessential amino acids, sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% fetal bovine serum (Flow Laboratories). 6-d-old cells were used as those arrested in G₀ phase and transferred to fresh media for synchronized growth. In the second method, a modified double-thymidine block method was used as described by Galavazi and Bootsma (9). WiL-2 cells of 2-d-old cultures were treated with 2 mM thymidine (Sigma Chemical Co., St. Louis, Mo.) for 16 h. The cells were washed once in fresh medium to remove thymidine and were grown in fresh complete medium. 6 h later, 2 mM thymidine was added and incubated for an additional 16 h. After the second thymidine treatment, the cells were washed and suspended in fresh complete medium.

DNA synthesis and mitosis were studied at 2-h intervals to determine the phases of the cell cycle. For mitotic cell enumeration, cells were treated with 0.2 µg/ml colcemid (Gibco, Grand Island Biological Co., Buffalo, N. Y.) for 2 h, washed in Hanks' balanced salt solution centrifuged onto slides and stained with Giemsa. The percentage of mitotic cells was expressed as the mitotic index. For determining DNA synthesis, $[^{3}H]$thymidine incorporation of WiL-2 cells was examined. 1-ml aliquots of cell suspension containing $2 \times 10^6$ cells were pulsed with 1 µCi/ml $[^{3}H]$thymidine (6.7 Ci/mM, New England Nuclear, Boston, Mass.) for 30 min, washed twice in PBS, pH 7.4, collected on Millipore filters (0.45 µm) (Millipore Corp., Bedford, Mass.), extracted with 5% trichloroacetic acid, dried, and counted in a Beckman liquid

![Fig. 1. Immunodiffusion study showing the reaction of anti-PCNA test serum EB (neat) with WiL-2 extract before and after absorption with insolubilized rabbit kidney extract. Before absorption, EB showed two precipitin lines; a weak line was identical to anti-Sm prototype serum; the other stronger line was identical to anti-PCNA prototype serum (MN). After this serum was absorbed with rabbit kidney extract (EBa), it showed only one precipitin line identical to MN. Drawing on right is a graphic representation.](image-url)
scintillation counter (Beckman Instruments, Fullerton, Calif.). At the same time, autoradiography was used to determine the percentage of cells in DNA synthesis. 0.1-ml aliquots of cell suspension (1.5 × 10^6 cells/ml) were centrifuged onto microscope slides. The smears were fixed in methanol-acetic acid mixture (3:1) for 10 min at room temperature. Slides were dipped into Kodak NTB-2 nuclear emulsion (Eastman Kodak Co., Rochester, N. Y.), dried at room temperature for 30 min, and incubated for a week. Then, they were developed in Kodak

| Table II | Characteristics of Anti-PCNA Serum (EB) before and after Absorption with Extract of Rabbit Kidney |
|----------|--------------------------------------------------------------------------------------------------|
|          | Precipitating antibody to Mouse kidney parenchymal cells                                           |
|          | PCNA Sm WiL-2 cells                                                                               |
| Before absorption | + + 1:32 1:512                                      |
| After absorption* | + - 0 1:64                                          |
| * 1 ml of the test serum EB was diluted 1:10, then absorbed with 30 ml packed gel volume of insolubilized saline-soluble extract of rabbit kidney. The absorbed serum was concentrated back to original volume. |

Fig. 2. Incorporation of [\textsuperscript{3}H]thymidine ([\textsuperscript{3}H]thymidine uptake, ●——●), percentage of cells incorporating [\textsuperscript{3}H]thymidine by autoradiography ([\textsuperscript{3}H]thymidine labeling index, ■——■), and percentage of cells undergoing mitosis (mitotic index, △——△) in WiL-2 cells synchronized by the method of starvation are shown in A. During first 6-8 h, >90% of cells were in a phase preceding DNA synthesis. After 8 h, cells began DNA synthesis and reached maximum DNA synthesis by 16-18 h. By 22-24 h, the mitotic phase of the cell cycle had begun, preceded by a fall in thymidine uptake. From these observations, the phases of WiL-2 cell synchronization were determined. In B, cells were taken every 2 h and stained with anti-PCNA serum (EBa) to determine relationship of nucleolar staining with cell cycle. The peak time for the appearance of PCNA in the nucleolus was at 12 h, a period related to late G1 and early S.
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**Fig. 3.** Fluorescence photomicrographs of synchronized WiL-2 cells stained with anti-PCNA serum (EBa). WiL-2 cells were synchronized by the method of starvation as shown in Fig. 2 A. Fine speckled staining pattern was observed in G₀ phase (0-h cells) (A). Nucleolar staining appeared in addition to speckled nuclear staining in late G₁ and early S phase (12-h cells) (B). In late S to G₂ phase (20-h cells) (C), most cells showed only speckled staining, and its intensity was stronger than in G₀ phase. In mitosis (D, arrows), radiating strands of staining could be observed within the region of chromosome condensation in addition to staining in areas peripheral to chromosome condensation (× 1,200).

Developer 19, fixed in Kodak fixer and stained with Giemsa. The percentage of labeled cells on each cell smear was expressed as the labeling index.

**Preparation of Phytohemagglutinin-stimulated Lymphocytes.** Human peripheral blood lymphocytes were separated by Ficoll-Hypaque density gradient centrifugation as described by Böyum (10). The preparations contained >90% lymphocytes. Cells were suspended in RPMI 1640 (Flow Laboratories, Inglewood, Calif.), supplemented with 2 mM glutamine, vitamins, nonessential amino acids, sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal bovine serum (Flow Laboratories, Inglewood, Calif.) to a concentration of 1 × 10⁶/ml.

The cell suspension was divided into two groups. One was cultured in the presence of 30 µg/ml phytohemagglutinin (PHA) (Wellcome Reagents Ltd., Greenville, N. C.) and the other without PHA as control. Each was divided into four subgroups: the first used for studying DNA synthesis was incubated with 1 µCi/ml [³H]thymidine (6.7 Ci/mM New England Nuclear); the second used for studying RNA synthesis was incubated with 1 µCi/ml [³H]uridine (22.4 Ci/mM, New England Nuclear); the third used for studying protein synthesis was incubated with 1 µCi/ml [¹⁴C]-amino acids (New England Nuclear; 1276-113); the fourth was used for making cytocentrifuged spreads. The different preparations were harvested every 2 h, and radioisotope incorporation was determined by scintillation counting or autoradiography as described above.
Preparation of Monospecific Anti-PCNA Sera. Because our analysis had demonstrated that the two sera, MN and EB, both contained other antibodies besides anti-PCNA, absorption studies were performed with insolubilized immunoadsorbant of rabbit kidney extract as described above. A representative result of such studies is shown with EB serum. In double diffusion analysis (Fig. 1), EB serum reacted with WiL-2 extract to produce a strong precipitin line and a second weaker precipitin line. The strong precipitin line was anti-PCNA. In this figure, MN serum showed only one precipitin line (anti-PCNA) which identified with the strong precipitin line of EB. The characteristics of the MN precipitin line that established that this was anti-PCNA had been published previously (1). The weak precipitin line of EB serum was anti-Sm because it completely fused in immunological identity with the reference anti-Sm serum. After absorption with insolubilized rabbit kidney extract, absorbed serum (EBa) showed slightly weaker anti-PCNA precipitin, but the anti-Sm precipitin was completely removed. In immunofluorescence studies (Table II), EB serum before absorption demonstrated nuclear staining of mouse kidney parenchymal cells at 1:32 dilution (related to anti-Sm antibody) but after absorption even undiluted serum showed no nuclear staining of parenchymal cells. On WiL-2 cells, nuclear staining titers were 1:512 before absorption and 1:64 after absorption. The fall in titer after absorption that was demonstrated both by immunofluorescence and by immunodiffusion is not entirely clear. We presume that it might be related to the fact that small
amounts of PCNA antigen are present in the kidney extract because previous studies had demonstrated that a few interstitial cells in the kidney contained PCNA. The studies reported below were performed primarily with the use of absorbed EB serum that were confirmed at critical steps with MN serum.

**PCNA in Synchronized WiL-2 Cells.** The results of synchronization of WiL-2 cells after density-dependent arrest are shown in Fig. 2 A. For ~8 h after release from density-dependent arrest, >90% of the cells were in G1, where no DNA synthesis was taking place. After this, DNA synthesis was documented by thymidine uptake and labeling index. The DNA synthetic phase (S) reached its peak by 16–18 h. Mitosis did not appear until 22–24 h of culture. WiL-2 cells were examined with EB-absorbed serum at different phases of the synchronized cells, and the results are shown in Fig. 3. At G0 (A) immediately after release from density-dependent arrest, PCNA was
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Fig. 6. Fluorescence photomicrographs of human peripheral blood lymphocytes stimulated by PHA. After stimulation, lymphocytes were stained with anti-PCNA serum (EBA) every 2 h. From 2-12 h after stimulation, positive cells showed only nucleolar staining pattern (A, 2-h cells). From 12-24 h, nuclear staining began to appear in addition to nucleolar staining (B, 24-h cells). At 60 h, speckled nuclear staining became very strong, whereas nucleolar staining had disappeared (C). Radiating strands of staining within areas of chromosome condensation could be observed in addition to staining in areas peripheral to chromosome condensation in a mitotic cell (D, arrow) (X 1,200).

The phenomenon of nucleolar staining in the 12-h cells was unexpected, and further observations were made with cells harvested every 2 h. The results are plotted on Fig. 2 B so that they could be related to the phase of the cell cycle. It is seen that a low percentage of cells with nucleolar staining could be observed in resting and early G1 cells, but its percentage started to increase from 6 h (late G1) and the peak of nucleolar staining was observed at 12 h (early S phase). After this, the number of cells showing nucleolar staining declined.

This observation of nucleolar staining in late G1 and early S was further confirmed on WiL-2 cells that were synchronized by double thymidine block. In Fig. 4 A, the
characteristics of thymidine uptake, labeling index, and mitosis of WiL-2 cells released from thymidine block are depicted. After release from block, the cells entered immediately into S phase but did not reach peak DNA synthesis until 8–10 h later. However, when cells with nucleolar staining were examined, the highest number of cells with nucleolar staining had reached maximum levels within 2 h after release from thymidine block (Fig. 4 B). Analogous to the findings shown in Fig. 2, cells in late S and G2 had a lower frequency of nucleolar staining.

**PCNA in Mitogen-stimulated Lymphocytes.** After PHA stimulation of human peripheral lymphocytes, the kinetics of amino acid, uridine, and thymidine incorporation are shown in Fig. 5 A. The percentage of cells that incorporated amino acid and uridine rapidly reached its maximum by 12 h, but thymidine incorporation did not commence until after 24 h and reached its peak at 54 h. PHA-stimulated cells were examined with anti-PCNA serum. At 2 h after addition of PHA, weak nucleolar staining could be observed in some cells (Fig. 6 A). There was probably some cytoplasmic staining also at this time. Before addition of PHA, none of the lymphocytes showed nuclear or nucleolar staining (not shown in the figure). At 24 h (Fig. 6 B), there was strong nucleolar staining in many cells, associated with speckled nuclear staining. The latter was most evident in the clump of agglutinated cells. At 60 h (Fig. 6 C), strong speckled nuclear staining was present. Nucleolar staining was not evident at this time. In Fig. 6 D, a cell that appears to be in mitosis shows the same characteristics of staining, as reported previously for synchronized WiL-2 cells. When PHA-stimulated cells were harvested every 2 h and the frequency of nucleolar staining plotted (Fig. 5 B), it was seen that nucleolar staining increased in parallel with uridine and amino acid incorporation and reached the peak at 12 h. It had already declined before initiation of thymidine uptake. In contrast, speckled nuclear staining appeared more slowly than nucleolar staining and did not reach its peak until DNA synthesis.

**Discussion**

The two sera that were used for these studies both have precipitating anti-PCNA antibody, but each had, in addition, antibodies to other nuclear antigens. In absorption studies using insolubilized rabbit kidney extract as the immunoadsorbant, precipitating antibodies to other nuclear antigens were removed with the exception of anti-PCNA. The observations that are reported here are based on the assumption that the absorbed serum recognized only PCNA antigen under the conditions of the experiments. There is reasonable evidence to support this assumption. In our experience with many different autoantibody systems, only anti-PCNA has been shown to react with a nuclear antigen present in proliferating cells but undetectable in differentiated cells of organs such as kidney and liver. This experience includes antibodies to DNA, histones and nonhistone nuclear antigens such as Sm, RNP, SSA, SS-B, nuclear antigens associated with scleroderma such as Scl-70, and centromere antigen (5, 6, 11–14). In the final analysis, it would be necessary to use purified anti-PCNA antibody or a monoclonal anti-PCNA antibody to confirm the observations reported here. Such reagents are not presently available.

One of the striking observations concerning PCNA is its presence in the nucleolus. From the observations in an established cell line (WiL-2) and in PHA stimulated lymphocytes, the appearance of PCNA in the nucleolus appears to precede its
A nuclear antigen associated with cell proliferation in the nucleoplasm. In the case of the WiL-2 cell line, nucleolar staining was the dominant pattern in a period overlapping late G1 and early S. However, in PHA-stimulated lymphocytes, nucleolar staining was the dominant pattern well before DNA synthesis occurred. At the present time, we do not have any explanation for this apparent difference. It could be related to the fact that in the WiL-2 cell, a continuously proliferating line, PCNA was present at all times, even during interphase. In peripheral blood lymphocytes, PCNA was not detected in unstimulated cells but appeared de novo immediately after PHA stimulation. These observations raise questions concerning the possibility that PCNA may play some regulatory role in DNA replication. Further, biochemical characterization of PCNA and studies to elucidate its biological function would be of interest.

The observation that PCNA is an antigen related to blast transformation has been used in some preliminary studies to determine if anti-PCNA may be a useful reagent for examination of neoplastic cells. In studies on patients with chronic myelogenous leukemia, it has been shown that anti-PCNA can detect blast crisis in this disease by the demonstration of increased numbers of PCNA-positive blastoid cells in the peripheral blood. These studies are in progress in this laboratory and will be reported separately.

Summary

A nuclear antigen associated with cell proliferation (proliferating cell nuclear antigen-PCNA) and blast transformation is recognized by autoantibodies in the sera of some patients with systemic lupus erythematosus. This autoantibody is a precipitating antibody and also reacts in immunofluorescence, staining the nucleoplasm of proliferating and blast-transformed cells. The autoantibody was used as a reagent to determine the distribution of PCNA in a synchronized continuous B lymphoid cell line (WiL-2) and in mitogen-induced blast-transformed lymphocytes. In WiL-2 cells, PCNA was detected as speckled nucleoplasmic staining in G1, S, and G2 phases of the cell cycle. In addition, during late G1 and early S phases, PCNA was also detected in the nucleolus. During mitogen-induced blast transformation of lymphocytes, PCNA was noticed in the nucleolus before the initiation of DNA synthesis and later became nucleoplasmic with disappearance of nucleolar staining. These studies demonstrate that the relationship of PCNA to proliferation and blast transformation may be associated with events related to DNA synthesis in these cells.

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