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**Yoshinari Takasaki · Soichiro Ando · Keiji Matsumoto
Hirofumi Yamada · Keigo Ikeda · Masuyuki Nawata
Masakazu Matsushita · Ran Matsudaira
Kazuhiko Kaneda · Ken Takeuchi · Yoshiaki Tokano
Hiroshi Hashimoto**

Activated peripheral blood mononuclear cells detected in lupus patients using cDNA coding for proliferating cell nuclear antigen

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Abstract The expression of proliferating cell nuclear antigen (PCNA) mRNA in peripheral blood mononuclear cells (PBMCs) from patients with systemic lupus erythematosus (SLE) was measured by dot blot hybridization using a PCNA cDNA, and correlated with the percentage of PCNA-positive cells detected immunohistochemically using a monoclonal anti-PCNA antibody. PCNA-positive PBMCs were detected in 72.2% of SLE patients ($n = 36$), which is significantly more than among healthy controls. In addition, among those in whom PCNA expression was detected, the percentage of PBMCs expressing PCNA was significantly higher in SLE patients (mean 2.5% vs. 0.15%). The level of PCNA mRNA was increased in PBMCs from 83.3% of SLE patients, and was significantly correlated with the percentage of PCNA-positive cells ($r = 0.54$, $P < 0.01$) and with the disease activity score ($r = 0.56$, $P < 0.01$). A longitudinal study of two SLE patients confirmed that PCNA mRNA expression and the percentages of PCNA-positive cells varied in parallel with disease activity. Thus, an analysis of activated PBMCs from SLE patients using PCNA cDNA may be a useful method by which to estimate SLE disease activity.

Key words Anti-PCNA monoclonal antibodies · Disease activity · PCNA cDNA· Proliferating cell nuclear antigen (PCNA) · Systemic lupus erythematosus (SLE)

Y. Takasaki (✉) · S. Ando · K. Matsumoto · H. Yamada · K. Ikeda · M. Nawata · M. Matsushita · R. Matsudaira · K. Kaneda · K. Takeuchi · Y. Tokano · H. Hashimoto
Department of Internal Medicine and Rheumatology, Juntendo University School of Medicine, 2-1-1 Hongo, Bunkyo-ku, Tokyo 113-8421, Japan
Tel. +81-3-3813-3111 (ext. 3314); Fax +81-3-5800-4893
e-mail: tyoshi@med.juntendo.ac.jp

Introduction

Proliferating cell nuclear antigen (PCNA) was first identified as an autoantigen that reacted with autoantibodies in patients with systemic lupus erythematosus (SLE).[1] Subsequent studies have shown that expression of this 34-kDa intranuclear protein increases in the late G1/S phase of the cell cycle, immediately before DNA synthesis.[2–5] These observations imply an association between PCNA and the DNA replication apparatus, and indeed PCNA is essential for DNA replication since it acts as an auxiliary protein with DNA polymerase-delta.[6,7] In addition, recent reports indicate that PCNA interacts with many other proteins involved in the mechanics of DNA replication,[6–8] repair,[8,9] and methylation,[10] chromatin assembly,[11] cell cycle regulation,[8,12] and ribosomal DNA transcription.[13]

These characteristics enabled PCNA autoantibodies from SLE patients to be used as a reagent for detecting blastoid cells as well as crisis-phase cells of patients with chronic myeloid and acute leukemia.[14,15] More recently, monoclonal anti-PCNA antibodies[16] were used in conjunction with immunofluorescence microscopy and flow cytometry to detect proliferating and blastoid cells, and they have proven to be highly useful for diagnosing and evaluating the prognoses of patients with various malignant diseases.[17–21] In addition, these anti-PCNA antibodies have also been used to detect activated peripheral blood mononuclear cells (PBMCs) in SLE patients, the numbers of which are significantly correlated with disease activity.[22] However, it remains unclear whether the increased detection of PCNA in PBMCs from SLE patients was due to its increased synthesis of the protein in peripheral blood, an abnormal alteration of its turnover, or increased antigenicity due to a conformational change in the molecule.

The present report describes our use of an anti-PCNA monoclonal antibody and a PCNA cDNA (PCR-1) cloned in our group[5] to detect activated PBMCs in SLE patients, and to characterize PCNA expression at the transcriptional and posttranscriptional levels. We also examined the clinical significance of PCNA expression by PBMCs.

Material and methods

Patients

Thirty-six SLE patients were randomly selected from among the patients coming to Juntendo University Hospital. All fulfilled the 1982 American Rheumatism Association revised criteria for SLE,[23] and their disease activities were assessed using the method of Lahita et al.[24] In addition, 11 healthy blood donors served as control subjects.

Cell lines

Molt-4, a T-lymphoblastoid line (ATCC CRL-1582), and HL-60, a myeloblastic leukemia line (ATCC CCL-240), were grown in RPMI 1640 supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, and antibiotics (streptomycin/penicillin G). These continuously growing cultured cells served as positive controls expressing PCNA protein and mRNA.

Monoclonal antibodies to PCNA

TOB7 (IgG1κ),[16] a murine monoclonal anti-PCNA antibody, was used to detect PCNA-positive PBMCs in SLE patients and controls.

Immunohistochemical detection of PCNA-expressing PBMCs

PBMCs from SLE patients and healthy controls were separated from heparinized blood on a Ficoll–Hypaque gradient.[25] They were then washed twice in cold phosphate-buffered saline (PBS: 0.01 M phosphate buffer, pH 7.4, 0.15 M NaCl) and resuspended to a concentration of 5×10^5 cells/ml in cold PBS. Aliquots (80 μl) of the cell suspension were deposited onto slides by cytocentrifugation and fixed in methanol for 5 min at −20°C, followed by 0.5% Nonidet P40 (NP 40) in PBS for 5 min at room temperature. Thereafter, 70-ml aliquots of TOB7 solution were applied to the cell smears, and the reaction was detected using fluorescein isothiocyanate (FITC)-conjugated goat antimouse γ-globulin (Capplel, Cochranville, PA, USA) according to the method of Murashima et al.[22] The percentage of PCNA-positive cells was determined by microscopic examination of 200 cells by an independent investigator.

PCNA cDNA

The PCNA cDNA probe was isolated from a rat thymus cDNA library, after which we confirmed that it showed no cross-reactivity with human ribosomal RNA.[5] When subjected to gel electrophoresis, the pst I-digested PCR-1 insert yielded two bands in addition to the plasmid DNA. The larger fragment (0.75 kb) was used as a hybridization probe. Since levels of β-actin mRNA are known to remain constant during cell activation, a β-actin cDNA probe was used to obtain the reference mRNA content of each PBMC sample. Both cDNA probes were labeled to a specific activity of 10^9cpm/μg of DNA with ^{32}P deoxynucleotides (New England Nuclear, Boston, MA, USA).

RNA isolation and hybridization

RNA was isolated from PBMCs using the guanidine thiocyanate method,[26] after which the RNA was separated from the remaining DNA and protein by ultra-centrifugation through cesium chloride. The resultant concentration of total RNA was determined spectrophotometrically at 260 nm, and the purity of the RNA preparation was assessed by measuring the ratio of the optical densities at 260 and 280 nm. Samples (10 μg) of the RNA were then denatured and electrophoresed through 0.7% agarose gels containing 2.2 M formaldehyde, and transferred onto nitrocellulose filters by capillary blotting. Before transfer, however, the ethidium-stained gels were visualized under ultraviolet illumination to determine the positions of the 28S and 18S ribosomal RNA bands in order to assess the integrity of the RNA and to verity that equal amounts of RNA were loaded. The RNA was fixed to the filter by air-drying and baking at 80°C for 2 h. To quantify steady-state mRNA levels, serial two-fold dilutions of RNA were applied to nitrocellulose filters using a 96-well vacuum manifold. The filters were then air-dried, baked at 80°C for 2 h, hybridized with random primed cDNA probes (Random Primed cDNA Labeling Kit, Boehringer–Manheim–Yamanouchi, Tokyo, Japan), and washed twice in 2 × SSPE (1 × SSPE = 180 mM NaCl, 10 mM sodium phosphate, and 1 mM EDTA) and 0.5% sodium dodecyl sulphate (SDS) at 65°C prior to autoradiography. The filters were exposed overnight to Kodak X-OMAT-AR film at −70°C with the aid of intensifying screens. Multiple exposures were then scanned with a Shimadzu CS-9000 densitometer (Shimadzu, Kyoto, Japan) to obtain the linear range of exposure. Each grade corresponded to a two-fold increase in expression (grade 0, neat; grade 1, 1:2; grade 2, 1:4; grade 3, 1:8; and so on).

Results

PCNA-positive PBMCs in patients with SLE

PCNA was detected immunofluorescently in the nuclei of PBMCs of most SLE patients, where the distribution exhibited a speckled pattern (Fig. 1). As shown in Fig. 2, the incidence of PCNA-positive cells among SLE patients was 72.2% (26 of 36 patients), and the incidence of PCNA-positive PBMCs ranged from 0% to 18% (mean 2.5%). In contrast, PCNA-positive PBMCs were detected in only 2 of 11 controls, and the percentage of PCNA-positive PBMCs ranged from 0% to 1.0% (mean 0.15%). Both the mean percentage of PCNA-positive PBMCs and the incidence of their occurrence were significantly higher in SLE patients ($P < 0.01$).

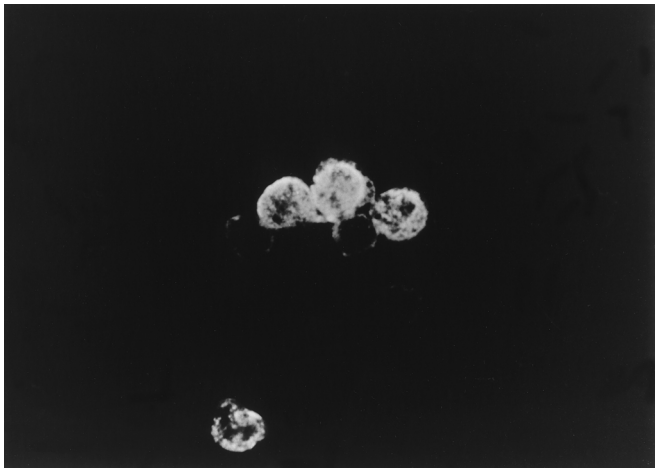

Fig. 1. Proliferating cell nuclear antigin (PCNA)-positive peripheral blood mononuclear cells (PBMCs) from a systemic lupus erythematosus (SLE) patient. The distribution of immunofluorescently labeled PCNA yielded a speckled pattern in the nucleus. ×1500

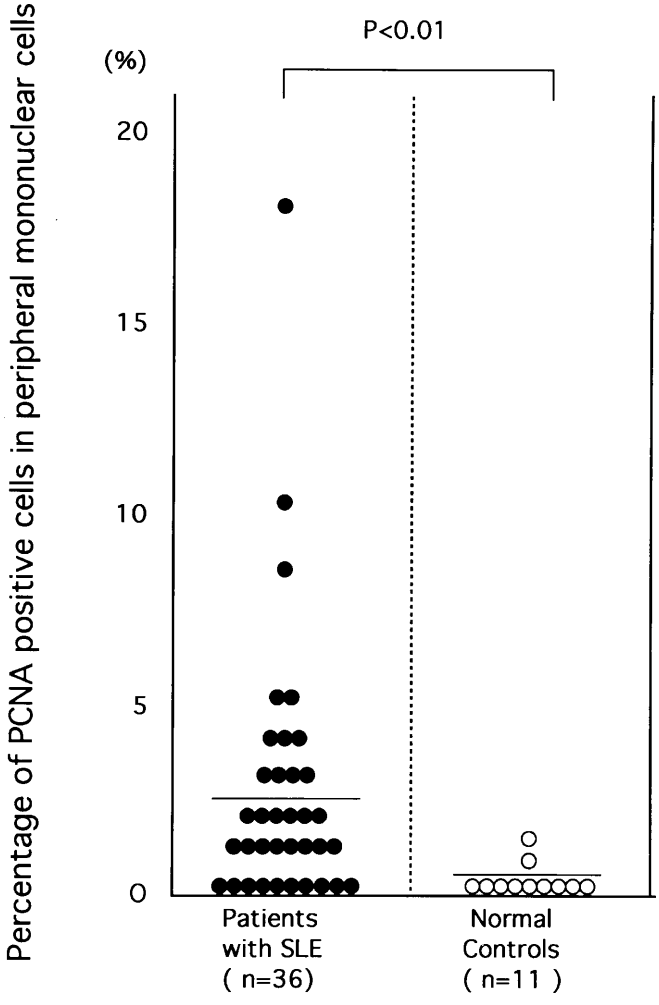

Fig. 2. Percentages of TOB7-labeled PBMCs from SLE patients (*left*) and healthy controls (*right*). PCNA-positive cells were detected in 26 of 36 SLE patients and in 2 of 11 controls ($P < 0.01$). PCNA-positive cells accounted for 0%–18% of PBMCs among SLE patients, but only from 0% to 1% among the controls. *Horizontal bars* indicate means (2.5% vs. 0.15%; $P < 0.01$)

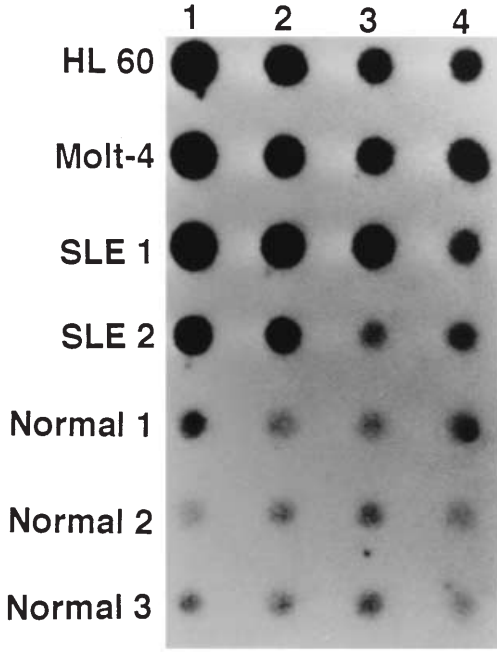

Fig. 3. Dot blot analysis of PCNA mRNA expression. The quantity of RNA in each lane varied by <10% (data not shown). Proliferating Molt-4 and HL-60 cells served as positive controls and expressed remarkably high levels of PCNA mRNA compared with fresh PBMCs from healthy subjects (normal 1, 2, and 3), as did PBMCs from two SLE patients (SLE 1 and 2)

PCNA mRNA expression in PBMCs from patients with SLE

To examine the regulation of PCNA synthesis in more detail, we evaluated the level of PCNA mRNA expression in PBMCs from SLE patients and healthy controls using dot blot analysis (Figs. 3, 4). Molt-4 and HL-60 cells, which are the continuously proliferating T cell and promyelocyte cell lines established from leukemia patients, served as positive controls. As shown in Fig. 3, these cultured cells expressed remarkably high levels of PCNA mRNA compared with fresh PBMCs from normal subjects. Likewise, expression of PCNA mRNA in PBMCs from two SLE patients was substantially higher than those from controls. When evaluated in all 36 SLE patients and 11 control subjects, levels of PCNA mRNA expression were found to be significantly ($P < 0.001$) elevated by as much as 16-fold (grade 4) in 30 of the 36 patients (Fig. 4). Also, the level of PCNA mRNA expression in the PBMCs correlated significantly with the percentage of immunohistochemically detected PCNA-positive PBMCs ($r = 0.54$, $P < 0.01$) (Fig. 5). In contrast, expression of PCNA mRNA was undetected among the controls (Fig. 4).

Relationship between PCNA mRNA expression and disease activity

Murashima et al.[22] reported a significant correlation between the percentage of PCNA-positive PBMCs and dis-

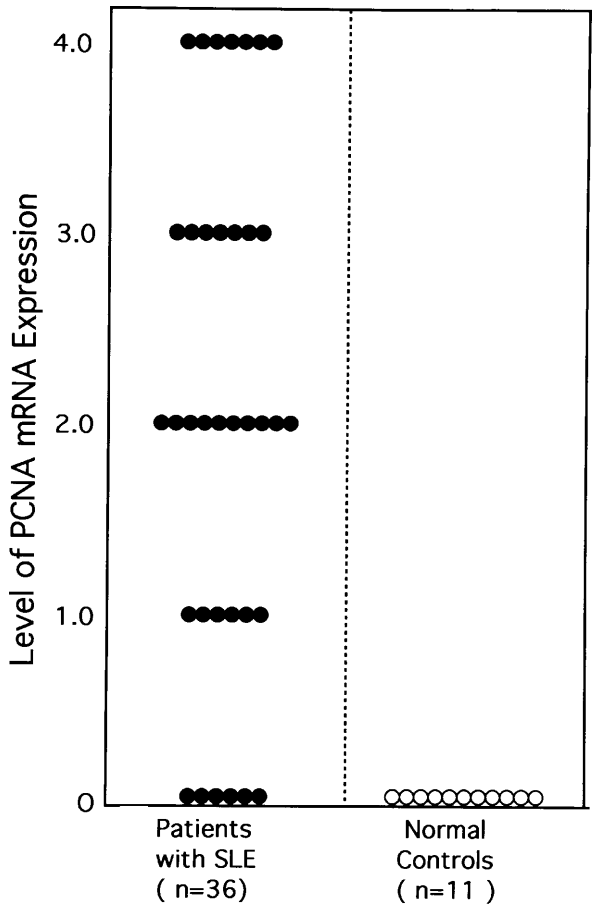

Fig. 4. Expression of PCNA mRNA in PBMCs from SLE patients (*left*) and healthy controls (*right*). Dot blot analysis was carried out to assess the levels of PCNA mRNA expression. Each grade corresponds to a two-fold increase in expression (grade 0, neat; grade 1, 2-fold; grade 2, 4-fold; grade 3, 8-fold; and so on). PCNA mRNA expression was elevated in 30 of 36 SLS patients, and was significantly ($P < 0.001$) higher than in the 11 controls subjects, where it was undetectable

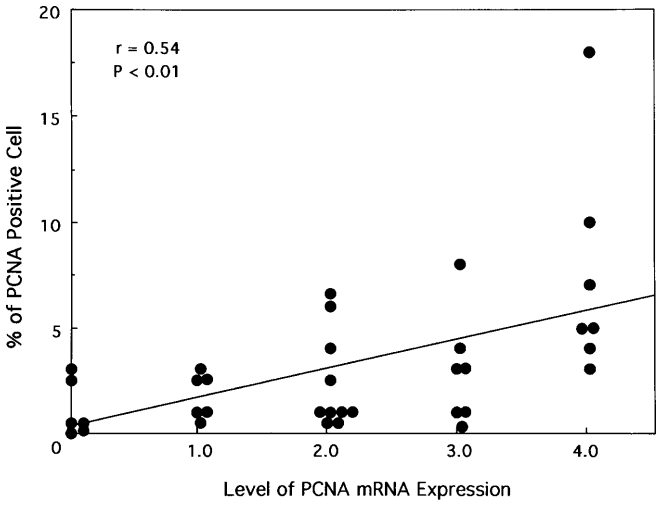

Fig. 5. Relationship between PCNA mRNA expression and the percentage of PCNA-positive cells in PBMCs from SLE patients. PCNA mRNA expression was significantly correlated with the percentage of PCNA-positive cells detected immunohistochemically using TOB7 ($r = 0.54$, $P < 0.01$)

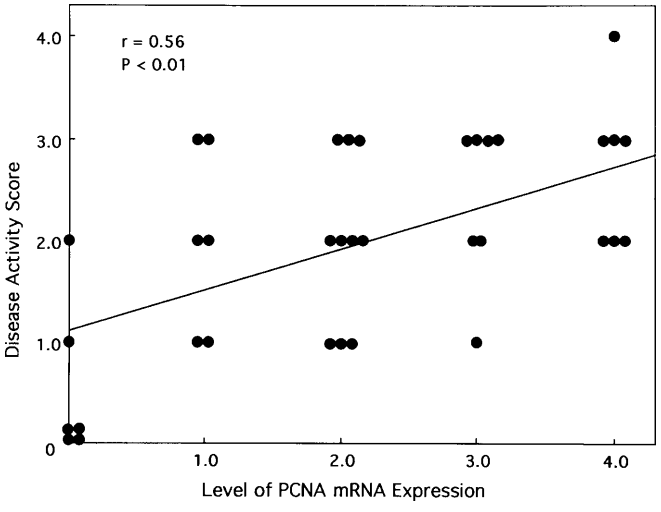

Fig. 6. Relationship between PCNA mRNA expression and SLE disease activity estimated by the method of Lahita et al.[24] A significant correlation was found between the disease activity scores and the levels of PCNA mRNA expression in PBMCs from SLE patients ($r = 0.56$, $P < 0.01$)

ease activity in SLE patients. Patients with PBMCs expressing PCNA mRNA tended to have a higher incidence of CNS involvement and interstitial pneumonitis compared with patients without PBMCs expressing PCNA mRNA (16.7% vs. 0% and 46.7% vs. 16.6%, respectively). There was also a significant correlation between PCNA mRNA expression in PBMCs and disease activity scores evaluated by the method of Lahita et al.[24] ($r = 0.56$, $P < 0.01$), although the disease activity scores tended to vary somewhat for each grade of PCNA mRNA expression (Fig. 6).

Clinical course and PCNA mRNA expression in SLE patients

To further clarify the clinical significance of PCNA expression in SLE patients, the relationship between the clinical features of SLE and PCNA transcription was studied longitudinally in two patients. Case NM, a 35-year-old woman, was admitted to our hospital because of persistent proteinuria (Fig. 7). During the course of prednisolone therapy, which included a regimen of methylprednisolone pulse therapy, her urinary protein gradually diminished until at discharge it was consistently within the normal range. In parallel with the improvement in her proteinuria, her titer of anti-dsDNA antibodies and the percentage of PCNA-positive cells also declined, while her serum CH50 increased. Expression of PCNA mRNA, which was high at admission, also gradually declined in parallel with the percentage of PCNA-positive PBMCs, and was undetectable at discharge.

Similarly, YM, a 28-year-old woman, was admitted our hospital because of nephrotic syndrome (Fig. 8). She was also treated with prednisolone, and as with NM, PCNA

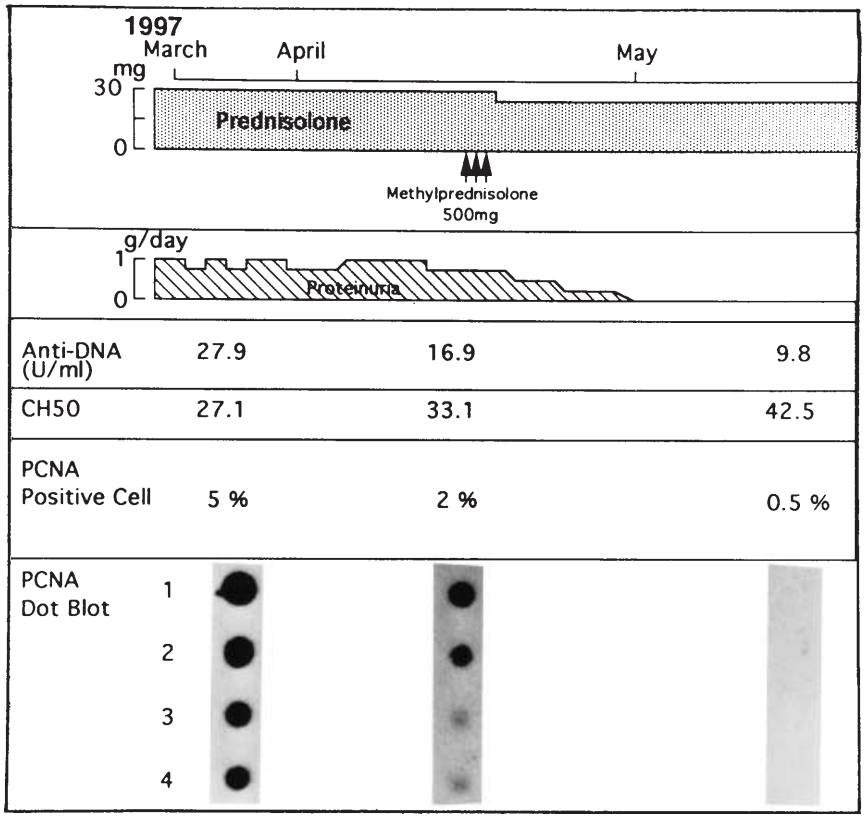

Fig. 7. Clinical course and PCNA mRNA expression in an SLE patient. Case NM, a 35-year-old women, was admitted to our hospital because of persistent proteinuria. During prednisolone therapy (*shaded area*), which included methylprednisolone pulse therapy (*arrow heads*), urinary protein gradually declined and at discharge was consistently within the normal range (*hatched area*). Also shown are the corresponding anti-dsDNA antibody titer, the percentage of PCNA-positive cells, the level of PCNA mRNA expression, and the serum CH50

mRNA expression was well correlated with disease activity and such immunological abnormalities as low serum CH50, high anti-dsDNA titer, and the presence of activated, PCNA-positive PBMCs.

Discussion

SLE is a systemic disease characterized by generalized autoimmunity consisting of hyperactive T cells and augmented synthesis of immunoglobulins by B cells. Peripheral blood lymphocytes obtained from SLE patients often contain T cells exhibiting signs of in vivo activation, including increased expression of MHC class II molecules[27–29] and interleukin (IL)-2 receptor (IL-2R),[30,31] and increased serum levels of soluble IL-2R.[32] Furthermore, using anti-PCNA monoclonal antibodies to detect activated PBMCs, Murashima et al.[22] showed that PCNA is highly expressed in activated T cells from SLE patients.

PCNA is an auxiliary protein associated with DNA polymerase-delta. It is present in small amounts in normal, resting, human T lymphocytes, but increases throughout the cell cycle, particularly in the G1/S phase, and is consistently high in proliferating cultures.[4,33] Moore et al.[34] showed that PCNA is synthesized and accumulates in the nuclei of IL-2-stimulated L2 cells during proliferation. In the same study, those investigators observed a discrepancy between the PCNA biosynthesis revealed by two-dimensional electrophoresis and the accumulation measured by immunofluorescence 8h after stimulation. They suggested that PCNA was probably often bound to another molecule, and was thus inaccessible to antibodies.[34] Consistent with that suggestion, we recently observed that PCNA autoantibodies from SLE patients do not react with the 34-kDa PCNA polypeptide when it is situated within the multiprotein complexes associated with cell proliferation.[8] It is therefore possible that the use of PCNA antibodies may result in an underestimation of PCNA synthesis in the nucleoplasm of activated or continuously growing cells.

Using RNA hybridization techniques to study the abnormal activation of PBMCs, Boumpas et al.[35] demonstrated increased transcription of the c-myc, c-myb, and c-raf protooncogenes in SLE patients. In the present study, we used a cDNA coding for PCNA, and dot blot analysis to assess PCNA transcription in PBMCs, and found that it was significantly increased in PBMCs from SLE patients. Moreover, the transcription correlated with disease activity and with the percentages of PCNA-positive cells detected immunohistochemically. It thus appears that the increased levels of PCNA protein detected in PBMCs from SLE patients reflects its increased synthesis in circulating blood, and not merely the increased antigenicity of the PCNA polypeptide. While we did not determine in which subset of PBMCs PCNA was expressed, the findings of Murashima et al.[22] showing PCNA-positive PBMCs to be T cells makes it likely that in this instance, too, the affected cells were mostly T cells.

PCNA accumulates in the nucleus of IL-2-stimulated L2 cells during proliferation, and is not detectable prior to the

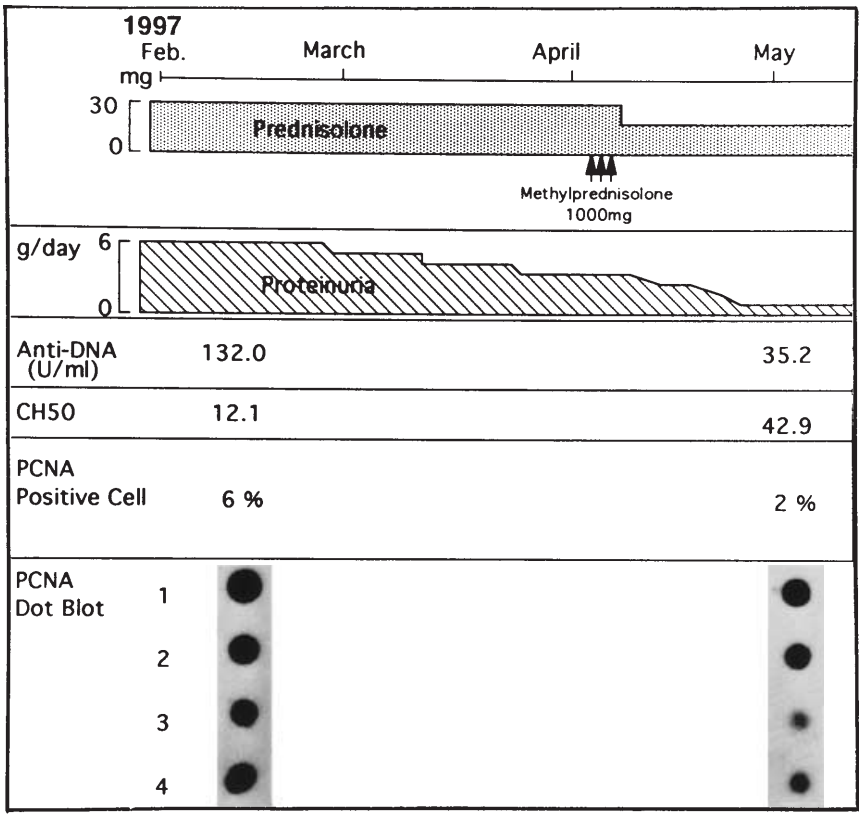

Fig. 8. The clinical course of YM, a 28-year-old woman admitted to our hospital with nephrotic syndrome. Details as in Fig. 7

initial S phase or after proliferation ceases.[34,36] Consistent with those findings, Huang et al.[37] showed increased IL-2 levels in the serum of SLE patients, and Tokano et al.[32] showed that serum levels of soluble IL-2R correlate significantly with the percentage of cells expressing PCNA. The accumulation of PCNA mRNA and synthesis of high levels of the protein are also stimulated by various growth factors, most notably platelet-derived growth factor (PDGF),[38,39] and it is thus probable that PBMCs are activated by growth factors and/or IL-2 in SLE patients.

Although the transduction pathway leading to the induction of PCNA transcription in T cells is unknown, the evaluation of PCNA mRNA nonetheless appears to be a useful tool with which to detect activated T cells. Chang et al.[40] reported that the regulation of PCNA transcription by intron 4 of the gene and posttranscriptional regulation of steady-state PCNA mRNA levels were both functioning normally in activated lymphocytes from SLE patients. In the same vein, our analysis of the PCNA cDNA sequence by hybridization of several endonuclease-generated fragments revealed no abnormalities (data not shown).

Treatment with prednisolone may induce a decreased level of expression of PCNA mRNA in PBMCs, but is not associated with the induction of PCNA transcription. This is clear because some patients with elevated levels of expression of PCNA mRNA in PBMCs had not been treated with any drugs, and many patients without PBMCs expressing PCNA mRNA as in normal controls were taking a low dose of prednisolone.

Our longitudinal study of two hospitalized patients enabled us to confirm a significant correlation between PCNA transcription and such disease activities as proteinuria, low serum levels of complement, and a high anti-dsDNA antibody titer. Indeed, as PCNA mRNA levels declined in these patients, so did the proteinuria and anti-dsDNA antibody titers. We could not study the immunological function of PBMCs expressing PCNA mRNA, but our data suggest that these cells play a key role in the immunoregulation of associated abnormalities, and in the pathogenesis of such clinical manifestations as nephritis. Further analysis of the activated T cells from SLE patients using other markers may shed light on the their aberrant biology.

In summary, we used a monoclonal PCNA antibody and a cDNA coding for PCNA to demonstrate that expression of PCNA is upregulated in the PBMCs of SLE patients. Moreover, it appears that PCNA transcription may be a useful marker with which to evaluate disease activity in SLE patients.

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