Autoimmune Response to Proteins of Proliferating Cell Nuclear Antigen Multiprotein Complexes in Patients with Connective Tissue Diseases

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ABSTRACT. ***Objective.*** To analyze the autoimmune response to the proliferating cell nuclear antigen (PCNA) multiprotein complex in patients with connective tissue diseases (CTD).

Methods. The PCNA complex was purified by affinity chromatography using anti-PCNA monoclonal antibodies. Then 196 serum samples from patients with systemic lupus erythematosus (SLE) and 82 from patients with other CTD were tested for reactivity with the complex by immunoblotting.

Results. Of 196 SLE sera, 61 (31%) reacted with at least one component of the PCNA complex, and most reactive sera contained autoantibodies to several components of the complex. Autoantibodies to PCNA complex were less common in patients with other CTD, and most of their sera reacted only with one or a few proteins in the complex. Two out of 20 scleroderma sera reactive with 100, 85, and 70 kDa proteins in the PCNA complex also had autoantibodies to topoisomerase I (topo I) antibodies, which is an element of the complex. These findings suggest that the autoimmune response to the PCNA complex was specific for SLE. Anti-PCNA complex antibodies were associated with an increased serum level of PCNA detected by ELISA. The spreading of the autoimmune response to the elements of the complex was observed in parallel with the increased serum PCNA level when a series of sera from a lupus patient were tested longitudinally. In addition, anti-PCNA complex antibodies were significantly correlated with lupus erythematosus cells.

Conclusion. The "antigen-driven" system may play a crucial role in inducing the autoimmune response to the PCNA complex in patients with SLE. (J Rheumatol 2004;31:2142–50)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS
AUTOANTIBODY
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PROLIFERATING CELL NUCLEAR ANTIGEN

Proliferating cell nuclear antigen (PCNA) was first identified as an autoantigen that reacts with autoantibodies in patients with systemic lupus erythematosus (SLE)[1]. Our group subsequently found that expression of this 34 kDa intranuclear protein is increased in the late G1 to S phase of the cell cycle, immediately before DNA synthesis[2-5]. These observations implied an association between PCNA and DNA replication, and PCNA was later identified as an auxiliary protein of DNA polymerase (Pol)-δ, which plays an essential role in DNA replication and repair[6-10]. Further analysis of the structure and function of PCNA has revealed that PCNA interacts not only with enzymes involved in the mechanics of DNA replication and repair[6-10], but also with many other proteins involved in DNA methylation[11], chromatin assembly[12], cell cycle regulation[4,13], and ribosomal DNA transcription[14].

We recently found that PCNA did not react with autoantibodies in the serum of patients with SLE when it is bound to other proteins associated with cell proliferation (PCNA complex), although it still reacted with a group of anti-PCNA monoclonal antibodies (mAb) raised in our laboratory[15,16]. We also demonstrated that PCNA complexes making up a substantial part of the "protein machinery" for DNA replication and cell cycle regulation could be purified from rabbit thymus extract (RTE) by affinity chromatography using anti-PCNA mAb[16]. Using the purified PCNA complex, the reactivity of lupus sera (including anti-PCNA-positive sera) to elements of the complex was studied, and it was found that many proteins in the PCNA complex are targeted as autoantigens, suggesting that "intermolecular and/or intrastructural help" leads to the spread of autoim-

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mune response from PCNA to other proteins that are functionally associated with it in patients with SLE[17].

We investigated the reactivity of serum samples obtained from patients with various connective tissue diseases (CTD) to further analyze the mechanisms underlying the autoimmune response to proteins interacting with PCNA. We also studied the clinical significance of antibodies to elements of the PCNA complex in patients with SLE.

MATERIALS AND METHODS

Sera. Using anti-PCNA standard serum (denoted PT, and kindly provided by Dr. E.M. Tan, Autoimmune Research Center, Scripps Research Institute, La Jolla, CA, USA), anti-PCNA serum (denoted AK) obtained from a lupus patient in Juntendo Hospital was selected and its specificity to PCNA was confirmed by double immunodiffusion (DID) and counterimmunoelectrophoresis (CIE). Serum AK, which showed a single anti-PCNA precipitin line in these assays, served as the source of the anti-PCNA IgG used to make an affinity chromatography column, and was also employed to monitor the antigenicity of PCNA during its purification. Standard sera containing autoantibodies to other nuclear antigens, including U1-RNP, Sm, topoisomerase (topo) I, and SSA (all donated by Dr. E.M. Tan) were used to confirm the specificity of anti-topo I serum (denoted YM), anti-Sm and U1-RNP serum (denoted TY), and anti-SSA/Ro serum (denoted YK).

Serum samples from 196 patients with SLE, 20 patients with scleroderma (SSc), 22 patients with rheumatoid arthritis (RA), 20 patients with polymyositis or dermatomyositis (PM/DM), and 20 patients with Sjögren's syndrome (SS) were randomly selected from the serum bank in Juntendo Hospital and were tested to study the immunoreactivity for elements of the PCNA complex and to measure serum PCNA levels. Those sera had been collected from patients at the hospital and stored at –20°C in the serum bank soon after they were separated from the blood samples.

All the patients with SLE, SSc, and RA met the relevant American College of Rheumatology classification criteria[18-20], PM/DM was diagnosed in accord with the criteria of Bohan and Peter[21], and SS was diagnosed according to the criteria proposed by the European Community[22].

mAb to PCNA. The mAb to PCNA, TOB7, TO17, and TO30 (IgG1k, IgMk, and IgMk, respectively), were prepared as reported[15], then used to prepare affinity chromatography columns for purification of the PCNA complex and used as probes for the PCNA complex in immunoblotting and ELISA studies.

Antibodies to molecules associated with cell proliferation. Murine mAb raised against various molecules associated with cell proliferation were used to analyze the components of the PCNA complex by immunoblotting. These included mAb directed against human p21 (Biosource, Camarillo, CA, USA), replication protein A (RPA; NeoMarkers, Fremont, CA, USA), DNA helicase II (NDH II; Cosmo Bio, Tokyo, Japan), and cyclin-dependent kinase (CDK) 4 (NeoMarkers) and CDK5 (NeoMarkers).

Purification of PCNA and topo I. RTE was prepared using rabbit thymus acetone powder (Pel-Freeze Biologicals, Rogers, AR, USA) as reported[2], and was used as the source of PCNA and topo I. Next, the IgG fraction of serum AK, prepared by 33% ammonium sulfate fractionation and DE52 (Whatman, Clifton, NJ, USA) ion exchange chromatography, was coupled to CNBr activated Sepharose 4B (Pharmacia, Piscataway, NJ, USA). This was followed by anti-PCNA affinity chromatography using 2 mg of TOB7 per ml of Sepharose 4B gel to purify the PCNA[23]. Anti-PCNA gels were packed into a Bio-Rad econo-column (Bio-Rad, Richmond, CA, USA), and PCNA-containing RTE was passed over the column at a rate of 5 ml/h. After washing with more than 3 column bed volumes of phosphate buffered saline (PBS) and 1 M NaCl/0.01 M phosphate buffer (pH 7.4), the bound material was eluted with 3 M NaSCN and the eluate was dialyzed against PBS.

Topo I was purified from RTE by the same manner using IgG fraction of anti-topo I serum YM.

DID and CIE assays. DID assays were conducted on plates containing 0.4% agarose (Sea Kem, Rockland, ME, USA) and 0.01% sodium azide in PBS[2,4]. Precipitation reactions were allowed to develop for 48 h at room temperature. CIE was also performed as described[2,4].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE was performed to analyze the protein profile of the purified PCNA[2]. The slab gel consisted of 12.5% acrylamide and 0.1% SDS in Tris-HCl (pH 8.8) with 15 mm of stacking gel containing 5% acrylamide and 0.1% SDS in Tris-HCl (pH 6.8). Samples dissolved in sample buffer (3% SDS, 5% 2-mercaptoethanol, 55 mM Tris HCl, pH 6.8, 10% glycerol, bromphenol blue) and boiled for 3 min were electrophoresed (1.5 mA/cm) at 4°C, and the resultant bands were stained with Coomassie Blue R250.

Immunoblotting assays. Purified PCNA and topo I were transferred electrophoretically onto nitrocellulose filters (Bio-Rad) as reported[2,23]. For immunochemical detection of proteins, the filters were first blocked for 24 h in 3% bovine serum albumin (BSA) in 0.1% Tween-PBS and then incubated 2 h with TOB7 in Tween-PBS (2.5 µg/ml) or with serum from patients with various CTD including anti-PCNA sera, anti-Sm, and U1-RNP serum TY, anti-topo I sera, and anti-SSA/Ro serum YK (1:200 dilution in Tween-PBS). Murine mAb were diluted according to the manufacturer's instructions. After washing with Tween-PBS, the filters were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse gammaglobulin (1:1000 dilution in Tween-PBS; Cappel, West Chester, PA, USA) or with HRP-conjugated goat anti-human IgG (1:2000 dilution; Cappel) for 2 h. After a final wash, the bound conjugate was detected by incubation with substrate solution (250 µg of 3,3-diaminobenzidine-4 HCl/ml, 0.5 µl of 30% H_2O_2/ml, 0.05 M Tris HCl buffer, pH 7.6), and the resultant bands were stained with 0.1% amido black 10B in 7% acetic acid.

ELISA to test the reactivity to PCNA complex (TOB7-PCNA). ELISA was performed to test the reactions of the PCNA complex to anti-PCNA sera and mAb, anti-topo I sera, and mAb to proteins associated with cell proliferation (NDH II, RPA, CDK4, and CDK5)[23]. Fifty microliters of the purified PCNA complex (TOB-7PCNA) solution (5.0 µg/ml) were added to the wells of Immunoplate II plates (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. These coating solutions were then removed, and the plates were washed 3 times with Tween-PBS; then 250 µl of 1% BSA in Tween-PBS was added to the wells and incubated for 24 h at 4°C. The plates were then washed 5 times with Tween-PBS, and 100 µl of anti-PCNA sera (diluted 1:1000 in Tween-PBS), mAb against PCNA (TOB7, TO17, TO30; 5.0 µg protein/ml), anti-topo I sera OS and KJ (diluted 1:1000 in Tween-PBS), and mAb to proteins associated with cell proliferation (5.0 µg protein/ml) were added and incubated at room temperature for 2 h. After washing again, 100 µl of alkaline phosphatase-labeled (ALP) goat anti-human IgG (1:1000 dilution in Tween-PBS; KPL, Gaithersburg, MD, USA) was added for the detection of autoantibodies to PCNA and topo I. Alternatively, ALP-goat anti-mouse IgG (KPL) was added to detect TOB7 and mAb to NDH II, RPA, CDK4, and CDK5, or ALP-goat anti-mouse IgM (KPL) was added to detect TO17 and TO30. After incubation for 2 h at room temperature, the plates were washed; 250 µl of the enzyme substrate (1 mg p-nitrophenylphosphate/ml, in diethanolamine buffer, pH 9.8) was added, and the optical density (OD) at 405 nm was measured.

Sandwich ELISA to measure serum level of PCNA. A sandwich-type ELISA using TOB7 and TO17 was performed to measure PCNA in sera obtained from patients with various CTD and healthy controls as reported[15,24-26]. A total of 110 sera from SLE patients, 18 sera from SSc patients, 19 sera from PM/DM patients, 22 sera from RA patients, and 18 sera from SS patients that had a sufficient volume to be tested by ELISA were selected from the serum specimens used in immunoblotting analysis of the reactivity to the PCNA complex. Fifty microliters of TO17 (protein concentration 20 µg/ml) in carbonate buffer, pH 8.5, were added to the wells of the Immunoplate II and incubated overnight at 4°C. After flicking off the coating solution, the plates were washed 3 times with Tween-PBS. The plates were drained, then 250 µl of 1% BSA Tween-PBS was added to each well and incubated overnight at 4°C. After washing as before, 100 µl solutions of PCNA puri-

fied by anti-PCNA affinity chromatography using serum AK (dilution from 0.002 to 6 µg/ml) were added to each well and incubated for 2 h at room temperature. In the same way, 100 µl of serum samples including sera from healthy donors used as control were added to each well and incubated. After washing each well 5 times, 100 µl of biotinylated TOB7 adjusted to 5 µg/ml was added and incubated for 2 h at room temperature. The wells were washed 5 times and 100 µl of HRP-conjugated streptavidin (BRL, Bethesda, MD, USA) diluted 1/2000 were added and incubated for 1 h. After washing, 250 µl of substrate solution (1 mg/ml 2,2′-azino-di-3-ethyl-benzthiazolin-6-sulfonic acid; ABTS, Sigma) and 0.005% H_2O_2 in 0.1 M McIlvain's buffer, pH 4.6, were added and the absorbance at 405 nm was measured after 1 h incubation at room temperature. The concentration of PCNA in serum samples was calculated based on the standard curve by the concentration of the purified PCNA as reported[15,24-26].

Statistical analysis. Statistical analysis was performed using the chi-square test with Yates' correction (2-tailed test).

RESULTS

Purification of PCNA and immunoblotting analysis using antibodies against proteins associated with cell proliferation. The polypeptide components of purified PCNA were analyzed by SDS-PAGE after purification using anti-PCNA (TOB7) affinity chromatography (Figure 1A, lane 2). TOB7 reacted with PCNA complexed within multiprotein structures (PCNA complex) as well as with free PCNA polypeptide, and the purified antigen (TOB7-PCNA) showed a number of bands in addition to 34 kDa PCNA. In contrast, serum AK did not react with the PCNA complex, so antigen purified using serum AK (AK-PCNA) only yielded a single 34 kDa PCNA band (Figure 1A, lane 1). Immunoblotting analysis of the proteins making up TOB7-PCNA is shown in Figure 1B. TOB7 reacted with only the 34 kDa PCNA in TOB7-PCNA (Figure 1B, lane 4) as well as serum AK (lane 3), although many proteins were observed in SDS-PAGE analysis. In addition, anti-Sm and U1-RNP serum TY (lane 1) and anti-SSA/Ro serum YK used as control (lane 2) did not react with those proteins. TOB7-PCNA reacted with mAb to p21 (lane 5), CDK4 (lane 6) and CDK5 (lane 7), RPA (lane 8), NDH II (lane 9), and topo I (lane 10), which confirmed that multiprotein complexes associated with cell cycle regulation and DNA replication interacting with the 34 kDa PCNA were copurified with the 34 kDa PCNA as reported previously[16].

Reactivity of CTD sera with the PCNA complex. Using TOB7-PCNA as the antigen source, the reactivity of sera from patients with various CTD was evaluated by immunoblotting (Figure 2, Table 1). Of 196 serum samples from SLE patients, 61 strongly reacted with at least one component of the PCNA complex (31%, Table 1), and most of these reactive sera contained autoantibodies against several components of the complex (Figure 2). In contrast, autoantibodies to elements of the PCNA complex were less common in patients with SS (15%), PM/DM (15%), SSc (20%), and RA (9%), and these frequencies were significantly lower than in SLE (Table 1). Although it was very difficult to identify all the proteins in the PCNA complex

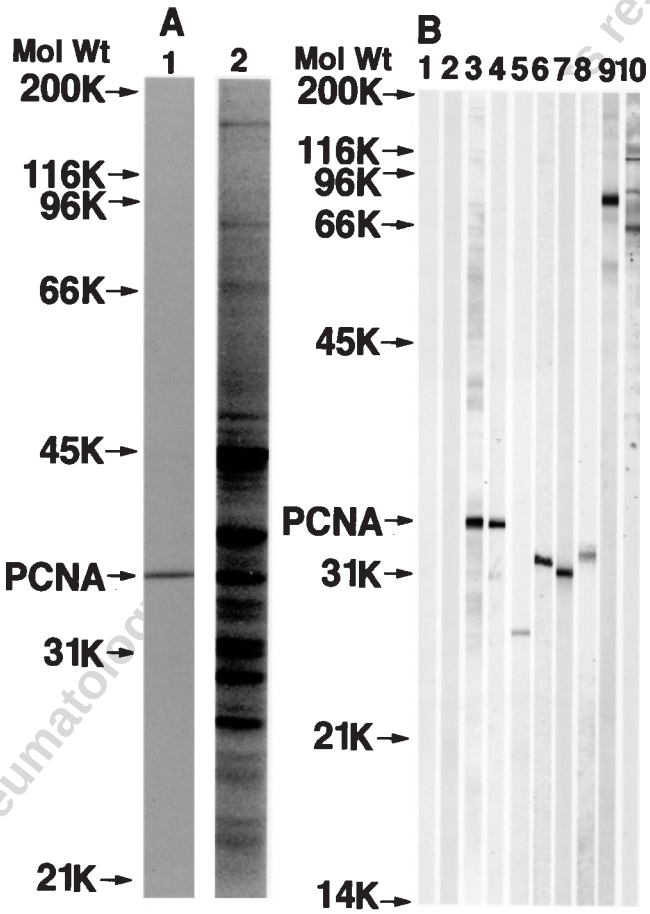

Figure 1. Purification of PCNA followed immunoblotting analysis using antibodies against proteins known to interact with PCNA. A. Purification of PCNA using TOB7 (TOB7-PCNA) yielded a number of bands in addition to a 34 kDa PCNA band (lane 2), whereas purification using serum AK (AK-PCNA) yielded only a single 34 kDa PCNA band (lane 1). B. TOB7 reacted with only the 34 kDa PCNA in TOB7-PCNA (lane 3) as well as serum AK (lane 3). Lane 1: anti-Sm and U1-RNP standard serum TY. Lane 2: Anti-SSA/Ro serum YK used as control. In addition, TOB7-PCNA also reacted with antibodies to p21 (lane 5), cyclin dependent kinase 4 (CDK4, lane 6) and CDK5 (lane 7), rereplication protein A (RPA, lane 8), DNA helicase II (NDH II, lane 9), and topoisomerase I (topo I; lane 10), which confirmed that multiprotein complexes associated with DNA replication and cell cycle regulation interacting with the 34 kDa PCNA were copurified with the 34 kDa PCNA.

reacting with lupus sera because of the reactivity of the lupus sera to many proteins in the complex, the representative reactivity to each element of the complex was summarized (Table 2). Compared with lupus sera, sera from other CTD reacted with only one or a few of the proteins in TOB7-PCNA, and most proteins in the complex specifically reacted with the lupus sera (Figure 2, Table 2).

These data suggest that many proteins of the PCNA complex associated with cell proliferation are specifically targeted as autoantigens in SLE.

Reactivity of antibodies to 70 kDa protein in the PCNA complex with topo I. Because topo I is known to be one of the elements of the PCNA complex[16,27,28], it was possible that

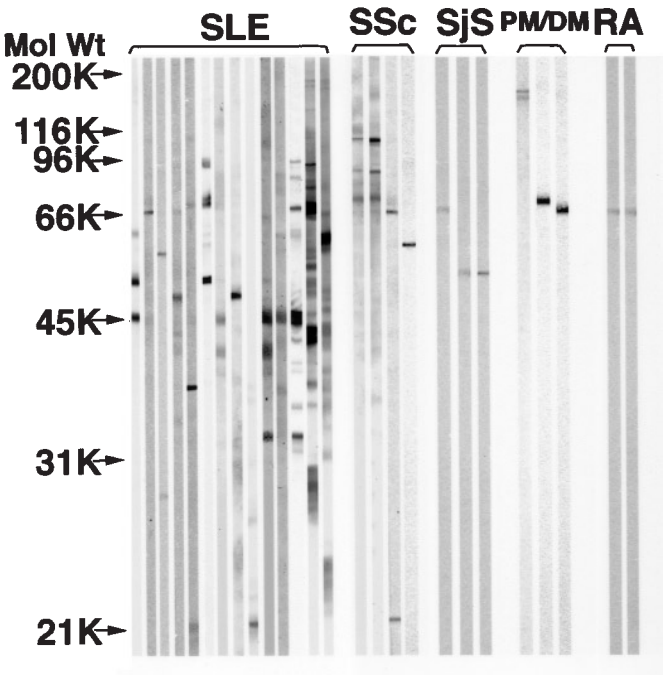

Figure 2. Immunoblots showing the reaction of sera from various CTD with the PCNA complex. Lupus sera often reacted with various polypeptides in the complex, and each serum specimen contained autoantibodies to multiple elements of the complex, whereas most sera from other CTD [SSc, RA, polymyositis or dermatomyositis, and 20 patients with Sjögren's syndrome (SjS)] reacted with one or a few polypeptides in TOB7-PCNA, as shown in Table 2.

Table 1. Reactivity to PCNA multiprotein complexes of sera from patients with various CTD in immunoblotting.

Disease	No. of Patients Tested	Frequency of Positive Test, %
SLE	196	31*
SS	20	15
PM/DM	20	15
SSc	20	20
RA	22	9
Healthy controls	15	0

* Frequencies in SLE were significantly higher than in other CTD ($p < 0.05$).

antibodies to 70 kDa, 85 kDa, and 100 kDa proteins of the PCNA complex in serum from SSc patients were anti-topo I antibodies reacting with 100 kDa topo I and its degraded products. To investigate this point, the reactivity of these sera to topo I was tested by immunoblotting using purified topo I (Figure 3A). Two SSc serum samples (serum OS and serum KJ) that reacted with 70, 85, and 100 kDa proteins in the PCNA complex also reacted with 100 kDa and degraded topo I proteins (SSc, Figure 3A, lanes 1 and 2), whereas 8 lupus sera that were reactive with 70 kDa proteins in the complex showed no reaction with the topo I polypeptides (SLE, Figure 3A, lanes 1-8). These results were expected because none of the 196 lupus sera was positive for anti-topo I antibodies in the DID and immunoblotting assay. These findings indicated that the 100, 85, and 70 kDa proteins reacting with SSc sera were topo I, and that SLE sera react with a different 70 kDa protein in the PCNA complex.

To analyze the reactivity of the anti-topo I sera with the PCNA complex in more detail, the response to TOB7-PCNA was evaluated by ELISA in comparison with that of anti-PCNA sera and mAb (Figure 3B). Because the epitopes reacting with anti-PCNA sera are hidden when the 34 kDa PCNA polypeptide is integrated into the PCNA complex, none of the sera (AK, PT, MI, YO, and EB) except serum OK reacted with TOB7-PCNA, as reported[17]. In contrast, the anti-PCNA mAb (TOB7, TO17, and TO30) and serum OK recognized the different epitopes that were not concealed in the complex, and thus reacted strongly with TOB7-PCNA in ELISA. Anti-topo I sera (OS and KJ) that reacted with topo I polypeptides of the PCNA complex on immunoblotting failed to react with the complex in ELISA, as was the case for most of the anti-PCNA sera. These data suggested that the epitopes reacting with anti-topo I antibodies were hidden when topo I was integrated into the PCNA complex, as was the case for the autoreactive epitopes on PCNA.

Antibodies to CDK4 and CDK5 (used as controls) reacted with TOB7-PCNA, but anti-NDH II and anti-RPA antibodies (reactive with the PCNA complex on immunoblotting) did not react with TOB7-PCNA in ELISA. The epitopes targeted by these antibodies may be hidden when these proteins were integrated into the complex, as is the case for the autoreactive epitopes on PCNA and topo I[17].

Table 2. Reactivity of sera from various CTD with elements of PCNA complexes.

Disease	No. of Patients Tested	No. of Patients That Reacted with Each Element of PCNA Complex (kDa)																				
		160/150	120	100	96	90	85	80	70	66	58	52	50	48	45	40	37	34	32	31	26	21
SLE	196	3	3	3	4	4	2	2	8	21	5	5	2	4	18	4	13	3	5	3	3	10
SS	20									1			2									
PM/DM	20	1							1	1												
SSc	20			2			2		2	1												1
RA	22									2												

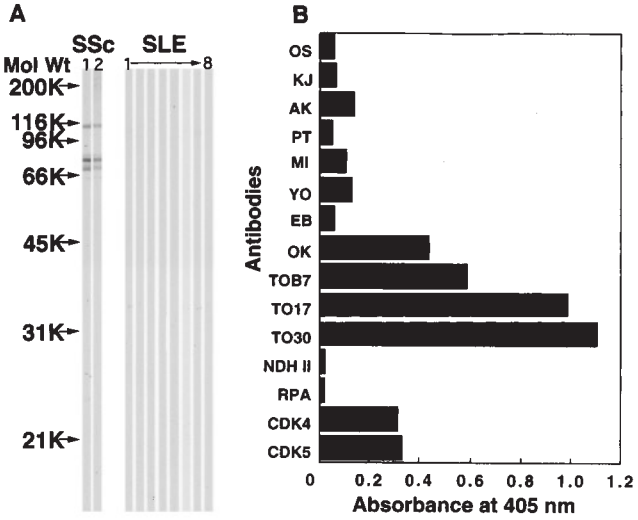

Figure 3. Reactivity to topo I of SSc and lupus sera reactive with 70, 85, and 100 kDa proteins in the PCNA complex tested by immunoblotting and ELISA. In immunoblotting using purified topo I as antigen source (A), 2 SSc sera (serum OS and serum KJ) reactive with 70, 85, and 100 kDa proteins in the PCNA complex reacted with 100 kDa topo I and its degraded products (lanes 1 and 2), whereas 8 lupus sera that were also reactive with the 70 kDa protein in the complex could not react with the topo I polypeptides (lanes 1–8). These findings indicated that the 100, 85, and 70 kDa protein reacting with SSc sera was topo I, and that SLE sera react with a different protein in the PCNA complex. When reactivity of these anti-topo I sera to the PCNA complex (TOB7-PCNA) were analyzed by ELISA (Figure 3B), they failed to react with the complex in ELISA, as was the case for most of the anti-PCNA sera (samples denoted AK, PT, MI, YO, and EB) except serum OK, although they could react with topo I in the PCNA complex in immunoblots. These results suggested that the epitopes reacting with anti-topo I antibodies were hidden when topo I was integrated into the PCNA complex, as was the case for the autoreactive epitopes on PCNA as previously reported[17].

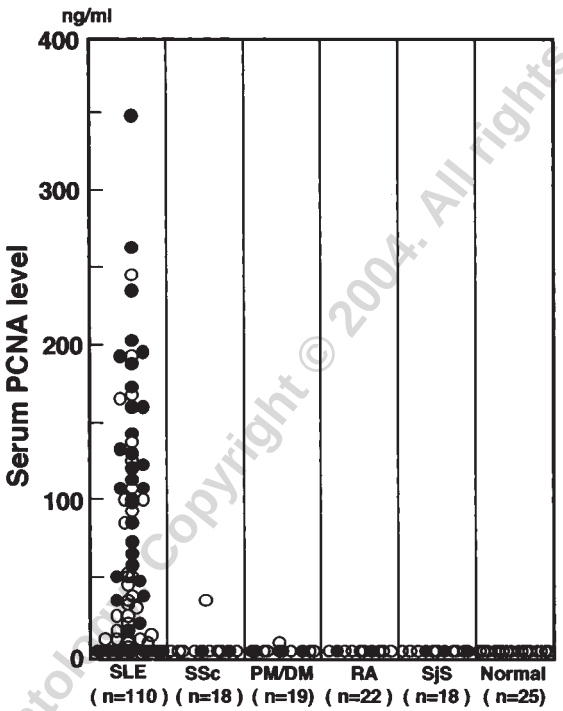

Figure 4. Serum level of PCNA detected by sandwich ELISA using TOB7 and TO17. Serum PCNA was detected by the ELISA at concentrations higher than 6 ng/ml, and it was detected in 68 out of 110 lupus patients (62%, concentration 7–348 ng/ml), but was not detected in sera from patients with other CTD with 2 exceptions (one in SSc serum, 36 ng/ml, one in PM/DM serum, 11 ng/ml). These data revealed that the increased level of serum PCNA is specific for SLE. In addition, 28 of 68 patients (41%) who had elevated serum levels of PCNA (shown as ●) had antibodies to the elements of PCNA complex, and its frequency was more than 2 times higher than that in patients without the elevated level of PCNA in serum (7 out of 42, 17%).

Serum level of PCNA in sera from patients with SLE or other CTD. To further analyze different autoimmune responses to the PCNA complex in SLE and other CTD, the serum level of PCNA, which may be associated with antigen presentation by the PCNA complex, was measured in those patients by a sandwich ELISA using TOB7 and TO17 (Figure 4) as described[16,24-26]. PCNA was detectable by the ELISA at concentrations higher than 6 ng/ml, and it was detected in 68 of 110 lupus sera (62%) at a level of 7 ng to 348 ng/ml, but was not detected in most sera from the patients with other CTD. The 2 exceptions were one patient with SSc serum (36 ng/ml) and one patient with PM/DM (11 ng/ml). Twenty-eight of 68 patients (41%) who had elevated serum levels of PCNA (Figure 4) also shared antibodies to the elements of PCNA complex, and the frequency of such antibodies was more than 2 times higher than that in the patients without the elevated serum PCNA levels (7 out of 42, 17%).

These findings revealed that an increased serum PCNA level is specific for SLE, and suggested that the circulating level of PCNA is an important determinant of the production of antibodies to the PCNA complex in lupus patients.

Longitudinal analysis of reactivity of serum ES with the PCNA complex. The above observations suggested an important role of the antigen-driven system in inducing the autoimmune responses observed in SLE. Therefore, the association of serum level of PCNA to the autoimmune response to the PCNA complex was longitudinally analyzed in Patient ES using a series of serum samples obtained during the clinical course. The reactivity of serum from Patient ES was analyzed over time by immunoblotting as shown in the lower panel of Figure 5. Initially, serum of patient ES showed a reaction with the 58 kDa protein (lane 1), but over time its reactivity spread to other components of the complex (lanes 2–4). The spreading of the autoimmune response was observed in parallel with the increase of serum PCNA levels detected by ELISA, as shown in the upper panel of Figure 5. The patient developed proteinuria and reactivity of antibodies directed against the PCNA complex became very weak (lane 5) after treatment with prednisolone (black bar indicates administration of 40–60 mg prednisolone/day), and the serum PCNA level became undetectable. This patient subsequently had 3 more disease flares, and each

time the spreading (lanes 6–8, lanes 10–17, and lanes 24–28) and suppression of the immune response to the PCNA complex occurred almost in parallel with the increase of serum PCNA level and administration of corticosteroid therapy, respectively.

Clinical characteristics of SLE patients with antibodies to the PCNA complex. The clinical characteristics of SLE patients with antibodies to at least one element of the PCNA complex were analyzed in comparison with the features of patients without such antibodies (Table 3). Although proteinuria was less common and fever was significantly more common in the patients with anti-PCNA complex antibodies compared to those without the antibodies ($p < 0.001$), the clinical manifestations of both groups were quite similar.

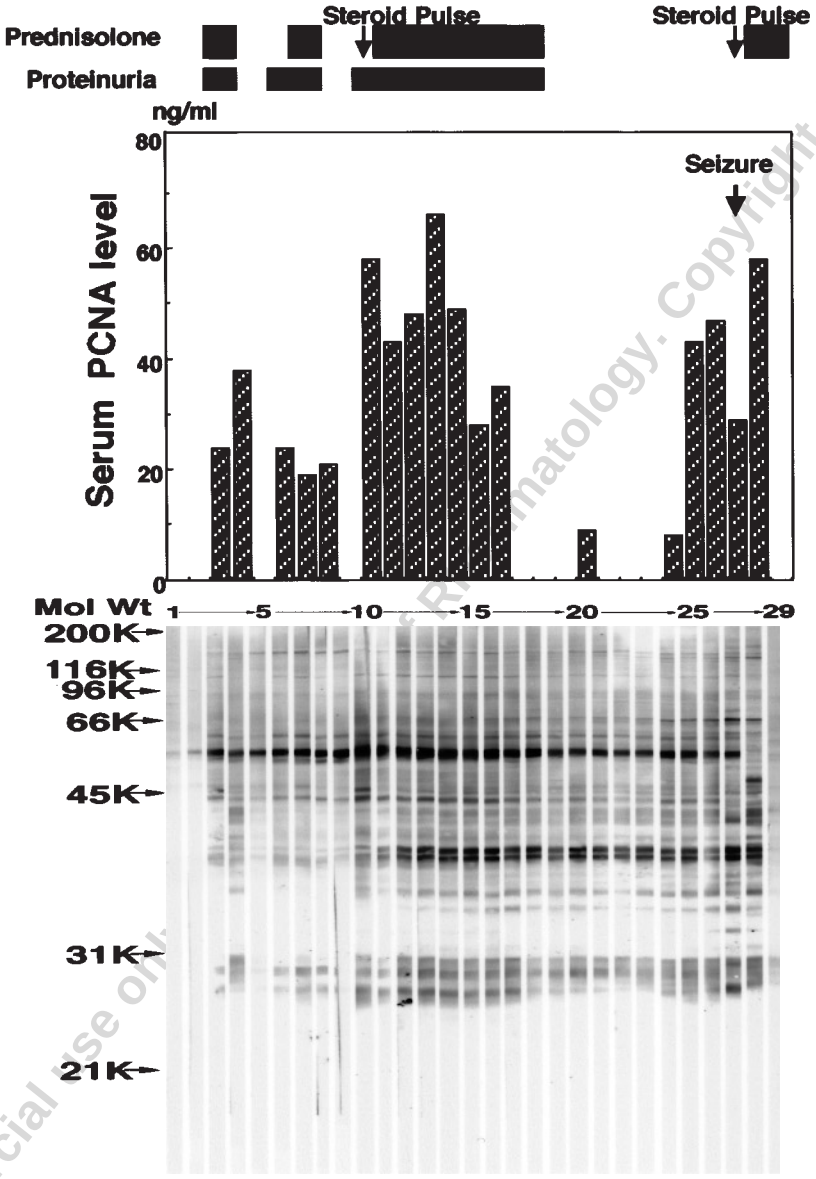

Figure 5. Reactivity of sera obtained from lupus patient ES with the PCNA complex during the clinical course analyzed by immunoblot longitudinally. Initially, serum ES showed a reaction with the 58 kDa protein (lane 1), but over time its reactivity spread to other components of the complex (lanes 2–4). The spreading of the autoimmune response was observed in parallel with the increase of serum PCNA levels detected by sandwich ELISA as shown in the upper panel. After treatment with prednisolone (black bar indicates administration of prednisolone > 40–60 mg/day), reactivity of antibodies directed against the PCNA complex became very weak (lane 5), and the serum PCNA level became undetectable. This patient subsequently had 2 more flares, and each time the spreading (lanes 6–8, lanes 10–17, lanes 24–28) and suppression of the immune response to the PCNA complex occurred almost in parallel with the increase of serum PCNA levels and corticosteroid therapy, respectively.

Table 3. Clinical characteristics of lupus patients with antibodies to the PCNA. Data are percentages.

Clinical Features	Patients with Anti-PCNA Complex, n = 61	Patients without Anti-PCNA Complex, n = 135
Fever	85*	77
Lymphadenopathy	36	32
Malar rash	79	72
Photosensitivity	43	37
Oral ulcer	34	44
Arthritis	93	90
Serositis	5	9
Raynaud's phenomenon	48	57
Alopecia	70	65
Renal disorder		
Proteinuria	87*	96
Cellular casts	83	82
CNS lupus	33	30
Interstitial pneumonitis	3	4
Hematologic disorder		
Hemolytic anemia	13	11
Leukopenia	67	67
Lymphopenia	92	92
Thrombocytopenia	36	36
ANA	97	98
Anti-dsDNA	66	65
Anti-Sm	21	23
Anti-U1-RNP	38	40
Anti-SSA	39	47
Anti-SSB	5	7
Anti-PCNA	6	0
RF	50	47
LE cell positive	36*	22
CH_{50} (< 20)	30	42
Hypergammaglobulinemia	14	21
CRP positive	76	77
ESR > 40 mm/h	76	79

* $p < 0.001$. CNS: central nervous system; ANA: antinuclear antibody; RF: rheumatoid factor; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

However, there was an interesting difference of laboratory findings, since the frequency of lupus erythematosus (LE) cells was significantly higher in patients with anti-PCNA complex antibodies than in those without such antibodies ($p < 0.001$).

DISCUSSION

Recent studies have shown that PCNA is involved in various functions associated with cell proliferation, and that it forms multiprotein complexes binding directly to or interacting with various proteins[6-14,16,17,29,30]. The PCNA complexes were purified using a series of centrifugations, polyethylene glycol precipitation, Q-sepharose column chromatography, and sucrose gradient centrifugation by Applegren, *et al*[27] and using PCNA-Sepharose 4B affinity chromatography by Loor, *et al*[28]. Their results showed DNA replication complex to consist of DNA Pol-α, δ and ε, DNA primase, replication factor C, RNase H, DNA ligase I, DNA helicase, RPA, DNA-dependent ATPase, nuclear NDH II and topo I, and a quaternary PCNA/p21/CDK/cyclin complex can be identified as PCNA binding proteins. Our previous studies revealed that there were monomeric and trimeric PCNA that were free from other proteins (free PCNA) and the PCNA complexes in RTE and HEp-2 cell extract[16,17] and mAb to PCNA raised in our laboratory were useful to purify the PCNA complexes reacting with all these PCNA[15-17], whereas autoantibodies to PCNA could react with only the free PCNA.

We analyzed the reaction of lupus sera with the PCNA complex and found that more than 35% of the serum samples reacted with at least one component of the complex, most of which also contained antibodies to several other components of the complex[17]. In contrast, most anti-PCNA-positive sera, recognizing as epitopes the binding sites for other proteins on PCNA[16,17], reacted exclusively with only the 34 kDa PCNA[17]. When reactivity with the PCNA complex was analyzed over time, spreading of the immune response to elements of the PCNA complex was observed in anti-PCNA-negative SLE patients, but 9 out of 10 anti-PCNA-positive patients (except one whose serum reacted with other components of the complex) did not show a reaction with any other proteins in the PCNA complex, although autoimmune response to the 34 kDa PCNA was established in our patients[17].

These findings can be explained by the concept that epitopes recognized by autoantibodies in anti-PCNA-positive sera are hidden or altered when PCNA binds to other proteins associated with cell proliferation[16,17], and that autoreactive B and T cells for the 34 kDa PCNA polypeptide that may be induced by the free PCNA do not recognize PCNA when it forms a complex[16,17,29]. These results strongly suggested the importance of "intermolecular-intrastructural help" in inducing the autoimmune response to elements of the PCNA complex[17,31].

We investigated this issue further by comparing patients with other CTD and SLE patients in this study, and found that lupus sera were more reactive with elements of the PCNA complex than sera from other CTD (Tables 1 and 2, Figure 2). In addition, we found that lupus sera often reacted with various polypeptides in the complex, and that each serum specimen contained autoantibodies to multiple elements of the complex (Figure 2). This phenomenon was rarely observed in sera from patients with other CTD (Table 2, Figure 2). Although the target proteins have not been identified yet, many proteins in the PCNA complex associated with cell proliferation seem to be specifically targeted as autoantigens by antibodies in SLE. Although 2 out of 20 SSc sera reacted with 100, 85, and 70 kDa proteins in the PCNA complex, they were identified as 100 kDa topo I, which is known to be one of the components of this complex[16,17,27,28] and its degraded products (Figure 3A).

Therefore, these sera were positive for anti-topo I antibodies, which are known as a disease marker for SSc[32], and reacted with topo I in the PCNA complex. Eight lupus sera also reacted with 70 kDa proteins in the PCNA complex (Table 2), but these proteins were found to be different from topo I (Figure 3A). These findings suggested the autoimmune response to the PCNA complex is relatively specific for SLE, being more common in SLE patients and showing unique reactivity with multiple proteins from the complex at the same time.

This characteristic reactivity of lupus sera to the PCNA complex that was not seen in patients with other CTD could suggest the importance of the "antigen-driven" system in inducing an autoimmune response to the complex. When we studied the percentage of PCNA-positive peripheral blood mononuclear cells, mainly activated T cells, in patients with various CTD, it was significantly higher in SLE than in the other CTD[33,34]. In addition, the serum PCNA level measured by sandwich ELISA was elevated in SLE patients[26], as reported in patients with lymphoma[25] but not in patients with other CTD. These data suggested that the PCNA complexes may be released into the peripheral blood with other nuclear antigens by activated cells in lupus patients after these cells undergo apoptosis, as reported by Emlen, *et al*[35] and Herrmann, *et al*[36]. However, this phenomenon may rarely occur in other CTD, because an autoimmune response to elements of the PCNA complex was not observed in patients with diseases such as SSc, PM/DM, SS, and RA. To confirm this, we analyzed PCNA levels in the sera used in the present study, and found that an elevated level of PCNA was fairly specific for lupus patients, and was rare in patients with other CTD (Figure 5). In addition, an elevated serum PCNA level was associated with positivity for autoantibodies to elements of the PCNA complex in SLE patients. A significantly higher frequency of LE cells was a characteristic of patients with antibodies to the PCNA complex, which may also support the concept that the PCNA complex is released from apoptotic cells and then induces an autoimmune response to the complex, because recent studies on LE cells have suggested a role in apoptotic events in lupus patients[37]. In addition, the low frequency of autoimmune response to PCNA itself in lupus patients (less than 5%)[1] also suggests the importance of the "antigen-driven" system, because the amount of free PCNA that may be responsible for the induction of autoimmune response to PCNA is very limited compared with that of the complexed PCNA in the cell extracts, according to our analysis by high performance liquid chromatography-gel filtration[16].

Spreading of the autoimmune response to other elements of the PCNA complex along with an increase of serum PCNA levels was observed in this study, which also strongly suggests a vital role of "intermolecular-intrastructural help" in induction of the autoimmune response to the complex in SLE patients. An interesting observation was that none of these sera reacted with topo I, which is part of the PCNA complex[16], although the sera reacted with many other proteins in the complex. Indeed, all 196 lupus sera samples that we tested (including 61 sera reactive with elements of the PCNA complex) were negative for anti-topo I antibodies by DID and immunoblotting. This result was reasonable in light of the reactivity of anti-PCNA sera to the PCNA complex[16,17,27,28]. As described above, anti-PCNA sera can react with the free PCNA, but are unable to react with PCNA once it becomes part of the complex, because some epitopes act as binding sites for other proteins and thus are hidden when PCNA is integrated into the complex[16,17,29]. This may also explain why an autoimmune response to topo I within the PCNA complex was rare in lupus patients. Two out of 20 SSc sera reacted with topo I polypeptides in the PCNA complex on immunoblotting (Figure 3A), but these sera were not reactive with the PCNA complex itself (containing topo I) when tested by ELISA (Figure 3B). It has been reported that autoantibodies to topo I recognize functional sites as epitopes and can inhibit DNA relaxation[38], thus autoreactive epitopes on topo I may be hidden when it is integrated into the PCNA complex, interacting with other proteins making up the complex. The finding that spreading of autoimmune response to topo I is rare in lupus sera reactive with other elements of the PCNA complex also suggests a critical role of "intermolecular and/or interstructural help" in inducing the autoimmune response to the PCNA complex.

When we studied the clinical characteristics of lupus patients with antibodies to the PCNA complex, there were few notable features apart from increased positivity for LE cells, as noted. However, it is possible that we may find clinical features associated with the autoimmune response to particular proteins in the PCNA complex, because some proteins specifically reactive with lupus sera were detected. Further analysis of these proteins may lead to detection of novel autoantibodies and confirm their clinical significance in patients with SLE. Identification of these proteins may also shed light on the mechanisms underlying cell proliferation and the mechanisms of autoantibody production in patients with connective tissue diseases.

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