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Presentation of autoantibody to proliferating cell nuclear antigen in patients with chronic hepatitis B and C virus infection

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Abstract

Objectives—To study the association of antibodies to proliferating cell nuclear antigen (PCNA) in patients with chronic hepatitis B (HBV) and C (HCV) virus infection.

Methods—Sera from 243 patients with chronic HBV infection; 379 patients with chronic HCV infection; 80 patients with systemic lupus erythematosus (SLE); 28 patients with rheumatoid arthritis; 15 patients with Sjogren's syndrome; eight with polymyositis; eight with primary biliary cirrhosis; and 33 healthy control subjects were tested for the presentation of anti-PCNA antibodies by enzyme linked immunosorbent assay (ELISA) and immunoblotting using recombinant PCNA as antigen. The distribution of immunoglobulin isotypes of anti-PCNA antibody was measured by ELISA assay.

Results—By ELISA, anti-PCNA antibodies were detected in 30 (12.3%) patients with chronic HBV infection, 71 (18.7%) patients with chronic HCV infection, and five (6.3%) patients with SLE. The inhibition of binding with these sera by purified PCNA was shown to exceed 71%. By immunoblotting, the frequency of anti-PCNA in patients with chronic HBV and HCV infection was 17 of 243 (7%) and 41 of 379 (11%), respectively. Absorption studies on indirect immunofluorescence showed the typical nuclear speckled staining pattern by anti-PCNA sera was abolished by preincubation of sera with PCNA. Anti-PCNA antibody was not detected in sera from patients with autoimmune diseases except SLE. Anti-PCNA antibodies in patients with chronic HBV and HCV infection were predominantly IgG.

Conclusion—These data suggest that anti-PCNA antibody are also present in patients with chronic HBV and HCV infection. Anti-PCNA antibody may not be specific for SLE.

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Autoantibodies to proliferating cell nuclear antigen (PCNA) are detected in the sera of 3-5% of patients with systemic lupus erythematosus (SLE).^{1,2} Anti-PCNA antibody has not been detected in other autoimmune diseases and was thought to be specific for SLE.^{3,4} Despite their low frequency, anti-PCNA antibodies are useful as a serological

marker for SLE. It can be detected by the characteristic speckled immunofluorescence pattern of variable immunolocalisation during mitotic stages because the bulk of its expression occurs during late G1 and early S phase of the cell cycle just before DNA synthesis.⁵

PCNA is a cell cycle regulated protein essential for DNA replication.^{6,7} It is synthesised in proliferative cell⁸ and was identified as DNA polymerase-delta auxiliary protein.⁸⁻¹⁰ Several reports indicated that SLE patients with anti-PCNA showed a high frequency of renal and central nerve system (CNS) involvements, nephropathy, thrombocytopenia, and seizure.¹¹⁻¹³

Chronic hepatitis B (HBV) and C (HCV) virus infections are important infection worldwide. It can cause acute or chronic hepatitis, cirrhosis of the liver and hepatocellular carcinoma. Several studies have suggested that chronic HBV and HCV may act as a trigger mechanism for the development of autoimmune rheumatic diseases.^{14,15} Viral infections have also been found to be associated with the development of autoantibodies.¹⁶⁻¹⁸ Taiwan is a hyperendemic area for chronic HBV and HCV infection. The HBsAg carrier rate in its general population is about 10% to 20%.¹⁹ It was the aim of this study to analyse whether anti-PCNA antibody was associated with chronic HBV and HCV infection.

Methods

SERA

Serum samples were obtained from 243 patients with chronic HBV infection and 379 patients with HCV infection. Diagnosis of chronic HBV or HCV infection is based on the presence of hepatitis B surface antigen (HBsAg) or anti-HCV antibodies (anti-HCV), respectively. The presence of anti-HCV antibodies was tested by enzyme linked immunosorbent assay (ELISA). All patients were regularly followed up as outpatients at Divisions of Gastroenterology, Chung Shan Medical and Dental College Hospital. In addition, serum samples were collected from 80 patients with SLE, 28 rheumatoid arthritis (RA), 15 Sjogren's syndrome (SS), eight polymyositis (PM), and eight primary biliary cirrhosis (PBC). The diagnoses of SLE and RA were made in patients who met the American College of Rheumatology criteria for SLE²⁰ and RA.²¹ Criteria required for a diagnosis of SS were as previously described.²² The criteria for diagnosis of PBC were made by accepted clinical and histological criteria.²³ These patients

Department of
Medicine, Chung Shan
Medical and Dental
College, Taichung,
Taiwan
T-Y Chen
T-C Hsu
G J Tsay

Department of Life
Science, National
Tsing-Hua University,
Hsin-Chu, Taiwan
B-S Tzang
Y-C Liu

Correspondence to:
Dr G J Tsay, Department of
Medicine, Chung Shan
Medical and Dental College,
110 Section 1, Chien Kuo N
Road, Taichung 402, Taiwan.

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with autoimmune diseases were followed up at the Division of Rheumatology, Chung Shan Medical and Dental College Hospital. Thirty three normal, age and sex matched subjects were used as a control group. Patients with chronic HBV or HCV infection had no evidence for diagnosis of SLE. An anti-PCNA mouse monoclonal antibody (PCNA (Ab-1)/PC-10, Oncogene Research products, Cambridge, MA) was also used as control.

PREPARATION OF RECOMBINANT RAT PCNA

Wild type PCNA expression vector was constructed with a rat PCNA cDNA containing plasmid, PCNA/pGEM-1²⁴ as the DNA template in the polymerase chain reaction (PCR). The forward and reverse primers in the reaction were 5'GCCGGATCCATGTTTGAGGCA3', 5'CCCGTCCGACCAACGCCTAAGA3' respectively, in which the initial and stop codons are in bold face and restriction enzyme sites (Bam HI and Sal I sites) for facilitating the cloning are underlined. The PCR product was purified and inserted into a prokaryotic expression plasmid, pET-30a(+)(Novagene, Cambridge, MA). The BL21 (pLys) strain of *Escherichia coli* that contained a full length cDNA of PCNA was used as the bacterial host for expressing the recombinant proteins.²⁵ When the OD 600 reached 0.7–0.9, protein expression was induced by addition of IPTG to a concentration of 2 mM and incubated for another three hours. The cells were harvested by centrifugation at 4000 *g* for 20 minutes and resuspended in 20 ml sonication buffer (50 mM NaPO₄ pH 8/0.25 mM EDTA). Lysozyme was added to a final concentration of 1 mg/ml and kept on ice for 30 minutes. The cells were sonicated (W385, Heat systems-ultrasonic, INC) for a total of 30 minutes at five minute intervals, centrifuged 10 000 *g* for 30 minutes and the supernatant was collected. Sodium chloride was added to a final concentration of 100 mM. The supernatant was loaded onto a 2 ml Ni²⁺-NTA column (Qiagen, Chatsworth, CA, USA) and washed with PES buffer (50 mM NaPO₄ pH 8/0.25mM EDTA/100 mM NaCl). Protein then was eluted with 20 mM–100 mM imidazole and analysed by SDS-PAGE for further use.

ELISA

ELISA was performed according to the method of Rubin *et al.*²⁶ All sera were assayed at a dilution of 1/200. Briefly, microwell plates were coated overnight at 4°C with 100 µl/well of 2.5 µg/well antigens in 50 mmol/l Na₂CO₃/NaHCO₃ buffer, pH 7.2. Wells were blocked with gelatin, washed with phosphate buffered saline (PBS)-Tween and sequentially incubated with human sera (1:200 dilution) and peroxidase conjugated goat antihuman immunoglobulin. The peroxidase conjugated goat antihuman Ig was used 1:1000 dilution. Substrate solution containing 2,2'-azino-di-(3-ethylbenzthiazolin-6-sulphonic acid) 1 mg/ml and 0.005% hydrogen peroxide in 0.1 mol/l McIlvaine's buffer was used for the peroxidase reaction. Absorption experiments were performed by incubating sera with different concentra-

tions of purified PCNA for one hour at 37°C, whereafter ELISA was performed.

IMMUNOBLOTTING

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), using 12.5% acrylamide slab gel with 5% acrylamide stacking gel, was performed according to the method of Laemmli.²⁷ Samples were reduced for five minutes in boiling water with 0.0625M Tris-HCl buffer, pH 6.8, containing 2.3% SDS, 5% 2-mercaptoethanol, and 10% glycerol. Samples applied to the gel were run off 100–150V for 1.5 hours. They were then electrophoretically transferred to nitrocellulose, according to the method of Towbin *et al.*²⁸ The nitrocellulose transferred proteins were cut into strips and soaked in 5% non-fat dry milk in PBS, for 30 minutes at room temperature, to saturate irrelevant protein binding sites. Antiserum diluted with 5% non-fat dry milk in PBS were reacted with the nitrocellulose strips and incubated for 1.5 hours at room temperature. The strips were washed twice with PBS-Tween for one hour and adding secondary antibody consisting of alkaline phosphatase conjugated goat antihuman or mouse IgG antibodies. The substrate NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3 indolyl phosphate) was used to detect antigen-antibody complexes.

CELLS AND CULTURE

The Chinese hamster ovary K1 (CHO-K1) cells were originally obtained from American type culture collection (ATCC) CCL61 and were maintained in 1 × McCoy's 5A medium containing 10% fetal bovine serum at 37°C in 5% CO₂. The cells were collected by centrifugation and the pellets were added with twice the packed cell volume of Buffer A (150 mM NaCl, 10 mM Tris-HCl, pH 7.2, 0.5% Nonidet P-40) for 10 minutes to allow cell lysis. The supernatant obtained by centrifugation at 10 000 *g* for 10 minutes was stored at –70°C for immunoblotting use. Blood obtained from a healthy donor was mixed with heparin sulphate (20 units/ml) as control. Lymphocytes were separated by Ficoll-Hypaque density gradient centrifugation as described by manufacturer. Cells were suspended in RPMI1640, supplemented with 2 mM glutamine, vitamins, non-essential amino acids, sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 15% fetal bovine serum.²⁹ For PHA stimulation, PHA (0.2 ml/10 ml medium) was added to the culture and incubated for 72 hours. Cyto-centrifugation spreads of these cells were prepared from a 0.1 ml suspension 1 × 10⁶ cells/ml. The cells were fixed in acetone for 10 minutes at room temperature and 0.2% Triton-X 100 for 10 minutes at room temperature.

INDIRECT IMMUNOFLUORESCENCE (IIF)

IIF technique was used with commercially prepared HEP-2 slides (Quantafluor TM, Sanofi, Pasteur, France) and human peripheral blood lymphocyte preparations. The conjugate used FITC labelled goat antihuman IgG as directed

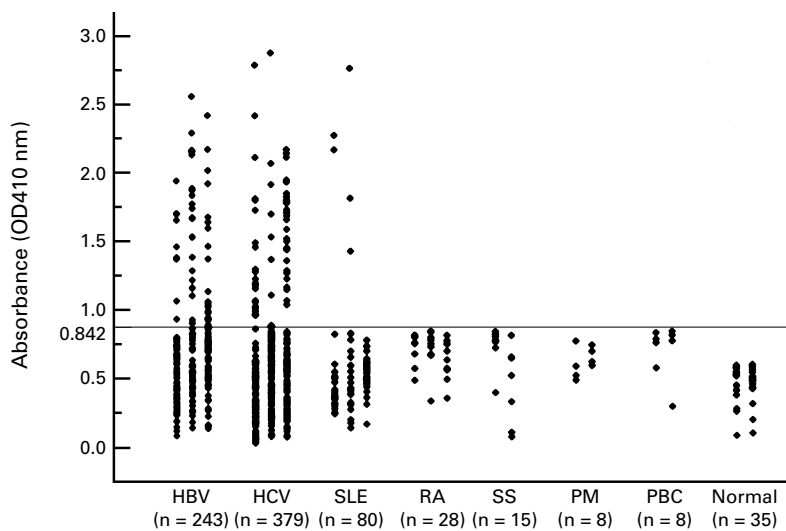


Figure 1 Anti-PCNA antibody concentrations in patients with hepatitis B and C virus infection, different autoimmune diseases, and normal controls. Purified recombinant PCNA was used as antigen and determined by ELISA. Values above 0.842 were regarded as increased anti-PCNA. Anti-PCNA antibody was only present in the patients with hepatitis B and C infection and SLE.

Table 1 Absorption of anti-PCNA with PCNA

Patients	Anti-PCNA after absorption with PCNA (OD)						Per cent binding inhibition
	0 μ g	0.1 μ g	0.5 μ g	1 μ g	3 μ g	6 μ g	
HBV							
8	1.473	0.953	0.547	0.629	0.423	0.499	71.3
50	2.290	1.546	0.498	1.043	0.470	0.658	79.5
51	3.142	1.393	0.699	1.462	1.011	0.514	77.8
103	3.151	0.873	0.604	0.880	0.759	0	80.8
113	4.100	1.651	1.437	1.936	1.229	0.395	90.4
HCV							
46	2.082	1.349	1.427	1.112	0.751	0.322	84.5
55	3.233	2.896	1.292	2.375	1.955	0.607	81.7
78	1.798	0.985	0.158	0.355	0.458	0.193	89.3
SLE							
	3.729	2.914	1.668	1.672	1.096	0.858	77.0

by the manufacturer. The slides for indirect immunofluorescence were counterstained with Evans blue.

STATISTICAL ANALYSES

Statistical analysis of the results was performed by using Student's *t* test.

Results

Recombinant PCNA were purified by nickel column through imidazole gradient for use in ELISA and immunoblotting. Figure 1 shows the results of anti-PCNA antibodies using recombinant PCNA as antigen by ELISA. The normal value of the absorbance was based on the results from 33 normal controls. The normal value of the absorbance was 0.443 (0.133) (mean (3 SD)). Value above 0.842 was regarded as increased anti-PCNA. The frequency of increased anti-PCNA in patients with chronic HBV and HCV infection were 30 of 243 (12.3%) and 71 of 379 (18.7%), respectively. Five of 80 patients with SLE had increased anti-PCNA. The titres of anti-PCNA were higher ($p < 0.05$) in patients with HBV infection than those in patients with SLE. None of the patients with RA, SS, PM, PBC, and normal controls has increased anti-PCNA.

Table 1 gives the results of absorption experiments by ELISA. Sera for five patients with HBV infection, two with HCV and one

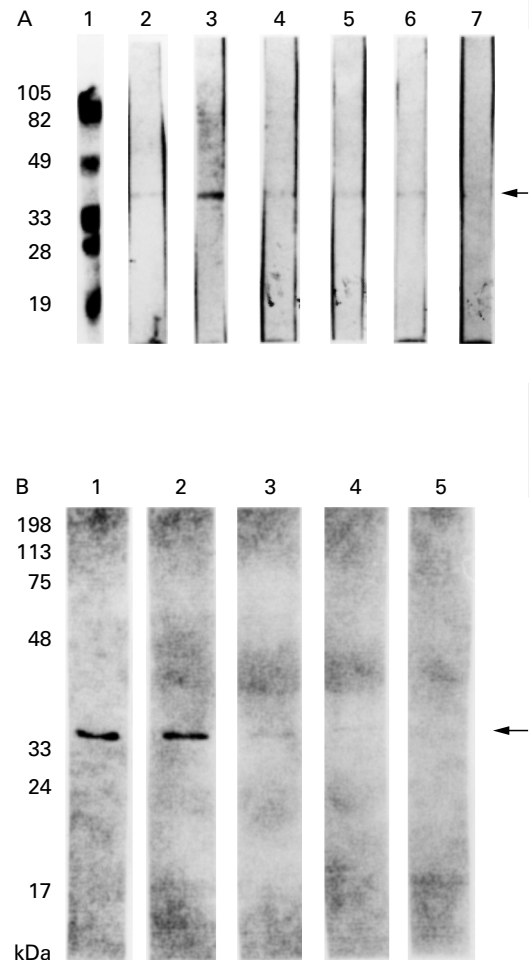


Figure 2 Reactivities of anti-PCNA on immunoblotting using recombinant PCNA proteins (A) and total CHO-K1 cells proteins (B) as antigens. Immunoblots of recombinant PCNA antigens (A) were probed with monoclonal anti-PCNA antibody (lane 2), and sera from patients with HCV infection (lanes 3–7). Immunoblots of total CHO-K1 cells proteins (B) were probed with monoclonal anti-PCNA antibody (lane 1), sera from patients with HBV infection (lanes 2–4), and normal control (lane 5). Sera from patients HBV and HCV infection and monoclonal anti-PCNA antibody reacted with a band of molecular weight of 34 kDa.

with SLE were available for the absorptive experiments. The binding activity in sera from patients with HBV and HCV infection, and SLE was inhibited by purified PCNA at different concentrations. The inhibition of binding by purified PCNA with sera from patients with chronic HBV and HCV was shown to exceed 71%. Inhibition binding by PCNA in sera from patients with SLE was 77%.

Using purified recombinant PCNA and total CHO-K1 cell proteins as the source of antigens for immunoblotting, anti-PCNA binding to 34 kDa antigens was observed (fig 2). Immunoblots of recombinant PCNA antigens (fig 2A) were probed with monoclonal anti-PCNA antibody (lane 2), and sera from patients with HCV infection (lanes 3–7). Monoclonal anti-PCNA antibody and sera (lanes 3–6) from patients with HCV infection recognised the 34 kDa PCNA antigens. Immunoblots of total CHO-K1 cell proteins (fig 2B) were probed with monoclonal anti-PCNA antibody (lane 1), sera from patients with HBV infection

Table 2 Distribution of immunoglobulin isotypes of anti-PCNA in SLE, HBV and HCV

Immunoglobulin isotype(s)	SLE (n=5)	HBV (n=30)	HCV (n=71)
IgG	5	24 (80)	44 (62)
IgM	0	6 (20)	8 (11)
IgA	0	14 (46)	14 (20)

*Numbers in parentheses are percentages.

(lanes 2–4), and normal control (lane 5). Monoclonal anti-PCNA antibody and sera (lanes 2–4) from patients with HBV infection also recognised the 34 kDa PCNA antigens. All sera with anti-PCNA from patients with chronic HBV and HCV infection and monoclonal anti-PCNA antibody reacted only with a band of molecular weight of 34 kDa. The frequency of anti-PCNA was detected in 12 of 243 (5 %) and 41 of 379 (10.8 %) in patients with chronic HBV and HCV infection, respectively. Anti-PCNA was detected in 3 of 80 (3.7 %) patients with SLE.

IIF on lymphocytes with PHA stimulation by sera from patients with chronic HBV and HCV infection was performed. Before mitogen stimulation, these sera with anti-PCNA showed weak stain. After PHA stimulation, nuclear fluorescence was intensified. These sera after absorption with PCNA became negative staining on PHA-stimulated lymphocytes (data not shown). The experiments for IIF counterstained with Evans blue.

Table 2 shows the distribution of immunoglobulin isotypes of anti-PCNA in patients with SLE, HBV and HCV infection. All five SLE had increased IgG anti-PCNA only. Most sera from patients with chronic HBV or HCV infection had predominant IgG anti-PCNA. IgM and IgA anti-PCNA were present to varying extents in sera from patients with chronic HBV and HCV infection.

Discussion

We have provided evidence showing the presence of anti-PCNA antibodies in the sera of patients with chronic HBV and HCV infection. The association of these patients with chronic HBV and HCV infection and SLE patients has been excluded in this study. The prevalence of anti-PCNA in patients with chronic HBV and HCV infection was obviously higher than that of patients with SLE. The prevalence of anti-PCNA in our patients with chronic HBV and HCV infection was 12% and 18.7%, respectively, whereas anti-PCNA was only detected in as many as 5% of SLE patients. Evidence for the presence of anti-PCNA in these patients includes the assays of IIF, ELISA, and immunoblotting. Anti-PCNA is present not only in patients with SLE but also in patients with chronic HBV and HCV infection. This result is in contrast with a general consensus that anti-PCNA antibodies is exclusively detected in sera from patients with SLE.³

Chronic HBV and HCV infections have been associated with the development of autoimmune diseases included vasculitis, SS, and mixed cryoglobulinaemia.^{15–32} Infection with HBV and HCV may produce ANA, RF,

anti-smooth muscle, anti-LKM, anti-microsome, and anti-thyroid antibodies.¹⁶ We further demonstrate that anti-PCNA antibody is also present in patients with chronic HBV and HCV infection.

The significance of anti-PCNA antibody in patients with chronic HBV and HCV infection is not clear. These patients did not exhibit clinical manifestations of SLE. It is known that HBV is not cytopathic. The liver cell damage occurs as a consequence of the host's immune response to virus infected hepatocytes.³³ Therefore, it is not surprising if anti-PCNA antibodies like other autoantibodies in autoimmune disease are detected in patients with HBV or HCV infection.

PCNA is an auxiliary protein for DNA polymerase δ . PCNA is essential for the synthesis of leading strand DNA and it also plays a part in DNA repair.^{6–10} HBV and HCV infection can cause hepatic damage including inflammation, piecemeal necrosis, or nodular regeneration. The production of anti-PCNA antibody may be associated with the abnormal or increased cell proliferation during chronic HBV and HCV infection.

The isotype distribution of anti-PCNA antibody in patients with chronic HBV and HCV infection was predominantly IgG (80% and 62% in patients with HBV and HCV infection, respectively). IgM anti-PCNA antibody still represented a minor isotype (20% and 11% in patients with HBV and HCV infection, respectively). These results are similar to spontaneously arising autoantibodies in SLE, which was predominantly IgG with low level of IgM.^{34–35} The isotype distribution in patients with HBV and HCV infection suggests that anti-PCNA antibody may occur in the autoimmune response.

The association of anti-PCNA with HBV and HCV infection in our study has provided a clue in understanding the linkage between viral infection and autoimmunity. We still do not know when anti-PCNA is produced and how long it will persist in patients with HBV and HCV infection at this moment. HBV and HCV virus induced autoantibody production may provide a clue in understanding the pathogenesis of autoimmune disease.

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