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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 Sjögren’s syndrome; eight with polymyositis; eight with primary biliary cirrhosis; and 33 healthy control subjects were tested for the presence 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.

(Ann Rheum Dis 1999;58:630–634)

Autoantibodies to proliferating cell nuclear antigen (PCNA) are detected in the sera of 3–5% of patients with systemic lupus erythematosus (SLE). Anti-PCNA antibody has not been detected in other autoimmune diseases and was thought to be specific for SLE. 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."
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-1[α] as the DNA template in the polymerase chain reaction (PCR). The forward and reverse primers in the reaction were 5’GGCGAGATCCCATGTTGAGGCA3’ and 5’CCGGTCGACCAAGGCCATAAGA3’ 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[+] (Novagen, Chatsworth, CA, USA) and washed with PES buffer (0.25mM EDTA/100 mM NaCl). Protein then was eluted with 20 mM–100 mM imidazole (Qiagen, Chatsworth, CA, USA) and washed with 5% Chelex (Bio-Rad, Hercules, CA) followed by centrifugation at 4000 g for 10 minutes. The supernatant was loaded onto a 2 ml Ni2+-NTA column equilibrated with 100 mM. The supernatant obtained by centrifugation was run on 9 March 2005 ard.bmjjournals.comDownloaded from

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.23 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.26 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 reactivated 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.

ELISA
ELISA was performed according to the method of Rubin et al.27 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 9.2. 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 anti-human Ig was used in 1:1000 dilution. Substrate solution containing 2,2’azobis-(3-ethylbenzthiazolin-6-sulphonic acid) 1 mg/ml and 0.005% hydrogen peroxide in 0.1 mol/l McIlvaine’s buffer was used for coloration. Absorption experiments were performed by incubating sera with different concentrations of purified PCNA for one hour at 37°C, whereafter ELISA was performed.

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

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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. 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

<table>
<thead>
<tr>
<th>Patients</th>
<th>Anti-PCNA after absorption with PCNA (OD)</th>
<th>Per cent binding inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 µg</td>
<td>0.1 µg</td>
</tr>
<tr>
<td>HBV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>1.473</td>
<td>0.953</td>
</tr>
<tr>
<td>50</td>
<td>2.290</td>
<td>1.546</td>
</tr>
<tr>
<td>51</td>
<td>3.142</td>
<td>1.393</td>
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<tr>
<td>103</td>
<td>3.151</td>
<td>0.873</td>
</tr>
<tr>
<td>113</td>
<td>4.100</td>
<td>1.651</td>
</tr>
<tr>
<td>HCV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>46</td>
<td>2.082</td>
<td>1.349</td>
</tr>
<tr>
<td>55</td>
<td>3.233</td>
<td>2.096</td>
</tr>
<tr>
<td>78</td>
<td>1.798</td>
<td>0.985</td>
</tr>
<tr>
<td>SLE</td>
<td>3.729</td>
<td>2.914</td>
</tr>
</tbody>
</table>

Using purified recombinant PCNA and total CHO-K1 cells proteins as the source of antigens for immunoblotting, anti-PCNA binding to 34 kDa antigens was observed (fig 2). 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.
Association of autoantibodies to proliferating cell nuclear antigen in HBV and HCV

Frequency of anti-PCNA was detected in 12 of 243 (5%) patients with SLE. Evidence for the presence of anti-PCNA antibodies in the sera of 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. Therefore, it is not surprising if anti-PCNA antibodies like other autoantibodies in autoimmune diseases 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. 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.

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.

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

<table>
<thead>
<tr>
<th>Immunoglobulin</th>
<th>SLE (n=30)</th>
<th>HBV (n=71)</th>
<th>HCV (n=71)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>5</td>
<td>24 (80)</td>
<td>44 (62)</td>
</tr>
<tr>
<td>IgM</td>
<td>0</td>
<td>6 (20)</td>
<td>8 (11)</td>
</tr>
<tr>
<td>IgA</td>
<td>0</td>
<td>14 (46)</td>
<td>14 (20)</td>
</tr>
</tbody>
</table>

*Numbers in parentheses are percentages.

We would like to thank Dr Edward K L Chan, The Scripps Research Institute, La Jolla, CA, USA for his helpful comments and Dr Kiyomitsu Miyachi, MD, Keigyu Clinic, Kanagawa, Japan for providing the anti-PCNA reference serum. This study was supported by a grant NSC 87–2314-B040–009 from the National Science Council, Taiwan.


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 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.

Chronic HBV and HCV infections have been associated with the development of autoimmune diseases like vasculitis, SS, and mixed cryoglobulinemia. Infection with HBV and HCV may produce ANA, RF, anti-smooth muscle, anti-LKM, anticrosome, and anti-thyroid antibodies. 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. Therefore, it is not surprising if anti-PCNA antibodies like other autoantibodies in autoimmune diseases are detected in patients with HBV or HCV infection.

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