Are the Clonotypes of Serum IgG Anti-DNA Antibodies Associated with Lupus Nephritis in Humans?

AKIRA HATAKEYAMA, TAKESHI SASAKI, TAI MURYOI, CHIHIRO MURAI and KAORU YOSHINAGA

The Second Department of Internal Medicine, Tohoku University School of Medicine, Sendai 980

HATAKEYAMA, A., SASAKI, T., MURYOI, T., MURAI, C. and YOSHINAGA, K.  Are the Clonotypes of Serum IgG Anti-DNA Antibodies Associated with Lupus Nephritis in Humans?  Tohoku J. Exp. Med., 1990, 160 (3), 213-221 —— We analyzed isoelectrofocusing (IEF) patterns of anti-DNA antibodies originated from sera and the renal eluates of patients with systemic lupus erythematosus (SLE). The spectrotypic patterns of serum anti-DNA-antibodies were heterogenous and bands with single-stranded (ss) and double-stranded (ds) DNA were detected in the PI 5.5-6.5 and PI 8-9.5 regions when SLE sera were tested, whereas healthy subjects failed to form bands even at different saline concentrations. The renal eluates from normal subjects never bound to DNA whereas those from SLE glomeruli showed relatively restricted IEF patterns which were detected mainly in PI 6.0 and PI 8.5, showing that some anti-DNA antibodies may be nephritogenic. However, the spectrotypic patterns of serum anti-DNA antibodies in patients with active lupus nephritis were similar with those in patients lacking renal lesions. The reasons why IEF analysis failed to indentify specific clonotypes of nephritogenic anti-DNA antibodies are discussed in association with pathogenesis of lupus nephritis. This study also suggests that the use of a high concentration of 6M urea in an IEF analysis may be able to expose antigen-binding sites of the circulating immune complex (IC)-derived antibodies. —— systemic lupus erythematosus; anti-DNA antibodies; clonotypes

Anti-DNA antibodies occur in patients with systemic lupus erythematosus (SLE). Such characteristics of the anti-DNA antibodies as Ig class, antigen specificity, avidity and complement-fixing ability have been shown to be linked with the development of lupus nephritis (Koffler et al. 1967; Tojo and Friou 1968; Winfield et al. 1977). Cationic charge of anti-DNA antibodies might be an important factor for pathogenesis of renal lesions in murine lupus since this type of anti-DNA antibodies might bind directly to glomerular basement membrane antigens, forming in situ immune complexes in renal glomeruli (Ebling and Hahn 1980; Dang and Harbeck 1981). IgG anti-DNA antibodies with cationic charge are also present in humans (Fishbach et al. 1981; Yoshida et al. 1987). It is, however, still unknown whether anti-DNA antibodies with cationic charge are
associated with the occurrence of lupus nephritis in humans.

The clonotypes of anti-DNA antibodies reflect the repertoire of anti-DNA-producing B cell clones. An isoelectrofocusing (IEF) analysis enables to study the clonotypes of anti-DNA antibodies (Fishbach et al. 1981; Yoshida et al. 1987). It will be important to identify specific clonotypes associated with nephritogenesis because the studies may lead to the understanding of pathogenesis of lupus nephritis, and in addition, bring into consideration of therapeutic strategies for specific manipulation of anti-DNA-producing clones responsible for tissue injuries in SLE. In this report, we present an analytic study of clonotypes of anti-DNA antibodies in association with lupus nephritis.

**MATERIALS AND METHODS**

*Sera*

Sera were obtained from 40 patients with SLE including 30 with active lupus nephritis, 3 with mixed connective tissue disease, 3 with rheumatoid arthritis, 4 with progressive systemic sclerosis and 12 from healthy controls. All SLE patients studied here fulfilled the 1982 criteria of American Rheumatism Association (ARA) for the diagnosis of SLE. The average age of SLE patients was 28 (15-43) years and that of the others 29 (19-48). The disease activity was defined by the clinical signs and symptoms (nephritis, arthritis, serositis, cerebral signs) according to the criteria of Budman et al. (1977). All of the patients with active lupus nephritis presented here showed abnormal urinalysis, a reduced total hemolytic complement level (CH50) and active nephropathy as determined by renal biopsy (Pollak et al. 1977; Churg and Sobin 1982). The immunoglobulins (Ig) were precipitated repeatedly with 33% saturated ammonium sulphate and isolated by DEAE-Sepharose chromatography. Then 20 μg of Ig from SLE or from other categories were used at IEF analysis.

*Elution of immunoglobulins from renal tissues*

The kidneys from autopsy cases with lupus nephritis were minced into small pieces, suspended in 0.15 M phosphate buffered saline (PBS), pH 7.2 and then homogenized in a chilled Waring Blender. The homogenates were washed repeatedly with PBS by centrifugation at 2,000 × g until the optical density of the supernatant read less than 0.05 at 280 nm, suspended in citrate buffer, pH 3.2 and then incubated at 37°C for 1 hr with continuous shaking. After centrifugated at 2,000 × g at 4°C for 15 min, the eluates were pooled and dialyzed against water for 2 hr, then against 0.02M PBS, pH 7.2 for 24 hr. The eluates were concentrated approximately 25-fold with an Amicon filter and stored at -70°C until use.

*DNA*

The antigenic DNA fragments were obtained as follows; the plasmid, pNDPC 1 was digested with restriction enzyme Bam H 1 to obtain two DNA fragments of 1.1 kbp and 1.2 kbp, respectively. These DNA fragments were labeled with 125I-dCTP by using DNA polymerase. Then the labeled DNA was precipitated with 70% ethanol at −80°C and washed with cold 70% ethanol to obtain pure 125I-DNA fragments. The resulting 125I-DNA was used as double-stranded (ds) DNA, because of insensitivity to nuclease S1 (Sasaki 1981) and failure to react with single-strand (ss) DNA -specific human monoclonal anti-DNA antibodies, 0-81 (Sasaki et al. 1985). The molecular size of 125I-dsDNA was 1000 bp. 125I-dsDNA was thermally denatured and used as 125I-ssDNA.
Absorption of antibody activity in sera by a DNA column

A DNA-coupled Sepharose 4B affinity column was prepared using the sonicated salmon sperm DNA (Kitagawa and Sasaki 1987). The samples were passed through DNA-coupled Sepharose column and then tested for the spectrotypes of anti-DNA antibodies.

Isoelectric focusing and immunoblotting

Flat bed isoelectric focusing (IEF) was performed in a Pharmacia apparatus at 4°C. Five percent polyacrylamide gels (10 × 20 × 0.1 cm) containing pharmalyte were made with/without 6 M urea. Twenty µl samples of sera (10–15 µg of IgG) were applied to the gel surface using Whatman No. 1 filter paper wicks, and focused at constant power (30 W) to a maximum of 3,000 V at 4°C for 2 hr. The pH gradient was measured using a flat membrane pH electrode or IEF calibration kit. The focused gel was carefully removed from the plate, placed in a gel transfer holder, and blotted electrophoretically onto a nitrocellulose membrane at (1.45 µm) in transfer buffer (0.7% acetic acid) for 30 min at 30 V and 30 min at 150 V. After blotting, free binding sites on the nitrocellulose membrane were blocked by incubating for 1 hr at 37°C on a rocker platform in 100 ml of 2% BSA in phosphate buffered saline containing 0.1% Tween 20. The membranes were rinsed three times with PBS containing 2% BSA and 0.1% Tween 20 (washing buffer) and then overlayed with 125I-DNA for 90 min at room temperature with rocking. Finally the membranes were rinsed overnight with several changes of washing buffer of the rocker at room temperature. The membranes were dried and exposed to Kodak X-Omat x-ray film at −70°C.

RESULTS

Influence of salt concentrations on spectrotype profiles

The avidity studies for anti-DNA antibodies were tested because bands with DNA formed after IEF might be salt concentration-dependent. Most SLE sera formed bands showing similar IEF patterns in the range of PI 5.5–9.5 whereas, the samples from other diseases or from normal subjects never bind to dsDNA and ssDNA at any salt concentrations (data not shown). The band formation occurred at least at 0.15 to 1.0 M NaCl. Based on these results, the reaction on the blotted membranes with DNA was performed at 0.15 M Borate-buffered saline pH 8.0 in the following experiments.

The spectrotypes of anti-DNA antibodies

Sera from patients with SLE were first electrofocused in the absence of 6 M urea, immunobotted to nitrocellulose membrane and finally tested for the binding ability to 125I-labeled DNA. The resulting IEF patterns of IgG antibodies were composed of heterologous bands in the range between PI 5.5 and PI 9.5. There were no significant differences between the spectrotypic patterns of lupus nephritis and those of SLE without renal lesions (Fig. 1A). The analysis using the purified IgG from SLE sera also showed the patterns analogous to the serum samples (data not shown).

Then, spectrotype analysis was tested in the presence of 6 M urea at the focusing in order to detect spectrotypes of possible anti-DNA antibodies in immune complexes. The results are shown in Fig. 1B, which also revealed the
Fig. 1. The spectropatterns of anti-ssDNA antibodies in sera. The IEF analysis was performed in the absence (A) or the presence (B) of 6 M urea as described in Materials and Methods. Lane 1–6: active SLE cases with lupus nephritis. Lane 7–12: active SLE cases lacking renal lesions.

TABLE 1. The incidence of anti-DNA antibodies detected in an IEF analysis

<table>
<thead>
<tr>
<th>Cases</th>
<th>Treatment with 6 M urea</th>
<th>Frequency of the band formation with ssDNA</th>
<th>dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Active lupus nephritis</td>
<td>-</td>
<td>13/17(76)</td>
<td>13/17(76)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>17/17(100)</td>
<td>16/17(94)</td>
</tr>
<tr>
<td>Active SLE without lupus nephritis</td>
<td>-</td>
<td>13/13(100)</td>
<td>10/13(77)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>13/13(100)</td>
<td>10/13(77)</td>
</tr>
<tr>
<td>Inactive SLE</td>
<td>-</td>
<td>0/10(0)</td>
<td>0/10(0)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0/10(0)</td>
<td>0/10(0)</td>
</tr>
<tr>
<td>Other autoimmune states&lt;sup&gt;c&lt;/sup&gt;</td>
<td>+</td>
<td>0/10(0)</td>
<td>0/10(0)</td>
</tr>
<tr>
<td>Healthy</td>
<td>-</td>
<td>0/11(0)</td>
<td>0/11(0)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0/11(0)</td>
<td>0/11(0)</td>
</tr>
</tbody>
</table>

Each sample obtained from the above mentioned cases was tested for the ability to form bands with the indicated probes by IEF analysis.

<sup>a</sup>IEF analysis was performed in the presence (+) or absence (−) of 6 M urea, as described in Materials and Methods.

<sup>b</sup>positive/total cases (% positive).

<sup>c</sup>3 with mixed connective tissue disease, 3 with rheumatoid arthritis and 4 with progressive systemic sclerosis.
IEF patterns similar to those in the absence of 6M urea. It was, however, noted that new bands with ssDNA were detected in the presence of 6M urea-containing gels in 4 cases with active lupus nephritis (Table 1). We did not detect IgM activity by this procedure (data not shown). Absorption of Ig with a DNA-Sepharose column resulted in a failure to form bands with ssDNA and to dsDNA confirming the specific antigen-antibody reaction with DNA (Fig. 2).

The spectrotypic patterns of renal glomerulus-deposited antibodies

In order to determine whether certain spectrotypes of IgG anti-DNA antibodies were deposited in SLE glomeruli, we tested the spectrotypes of the eluates from renal tissues of active lupus nephritis. The IEF patterns in those from cases M and T. were shown in Fig. 3, where the band of the eluted antibodies against DNA were observed in PI 5.5 to PI 8.5.
DISCUSSION

This study demonstrated heterogeneity of IgG anti-DNA autoantibodies in humans. The greater heterogeneity in anti-DNA autoantibodies is not surprising, since there are various types of autoantibodies with different specificity to nucleic acids. As far as we performed an IEF analysis, anti-DNA antibodies were detected only in SLE, but never in healthy subjects at any salt concentrations. One might also ask about the possibility that the plasmid DNA with restriction DNA fragments (1000 bp) may be different from eukaryotic genomic DNA in antigenicity, so that DNA used in this experiment might have failed to detect some types of anti-DNA antibodies in normal subjects. This is, however, unlikely because anti-DNA antibodies could recognize DNA fragments in the range of 100 bp to 500 bp (Sasaki et al. 1985) and, in addition, they have ability to bind to E-coli DNA as well as to mammalian DNA (Seligman and Arana 1968). The main reason why we used the cloned DNA fragments, is that these averaged and relatively small sized DNA enable to prevent nonspecific binding at the tests.
The band formation with ssDNA and dsDNA did not occur after passing of the samples through a DNA-Sepharose column, confirming specific reaction between DNA and the antibodies.

When we compared the bands of serum antibodies against ssDNA and dsDNA, no differences could be found between the samples with renal lesions and those without nephritis. Differed from the heterogeneity of the spectrotypes in serum samples, IgG of the glomerular eluates showed relatively restricted bands with DNA in PI 5.5 to PI 9.5 (Fig. 3). It should be in mind that IEF analysis described here fails to detect IgM type of antibodies because of the use of polyacrylamide gel. Taken together, the clonotypes of anti-DNA antibodies deposited in the kidney were relatively heterogenous and distributed to arange, pH 6.5-9.0. These results provide an interesting information for understanding the pathogenesis of renal injuries in SLE; Recent studies have indicated that cationic charged anti-DNA antibodies in the circulation might form in situ immune complexes at renal glomeruli and then cause the following process to renal injuries in lupus patients (Ebling and Hahn 1980; Faaber et al. 1984; Eilat 1985). Our results are compatible with these observations and add new information that clonotypes of anti-DNA antibodies with cationic charge are deposited in renal glomeruli of human SLE. It should be noted that the renal eluates also included neutral charged anti-DNA antibodies. Our preliminary data indicated that the neutral charged ones in renal deposits might be originated from circulating immune complexes. The precies data will be shown in another paper.

Anti-DNA antibodies with cationic charge, however, were detected even in serum sample lacking active renal lesion (Fig. 1). Thus, DNA binding ability of the antibodies with certain charges is not sole factor responsible for the renal injuries. With this regards, idiotypic analysis of anti-DNA clonotypes may be significant: Isenberg demonstrated preferential deposition of 16/6 anti-DNA idiotypes in SLE glomeruli (Isenberg and Collins 1985). We also found specific idiotypes associated with active lupus nephritis (Takai et al. 1989). In the following paper, we will present the clonotypes of idiotypes probably responsible for the occurrence of renal lesions of SLE.

Another interesting finding is that in the presence of 6M urea, we could detect new bands with DNA by IEF analysis, which were undetectable in the absence of urea. A high concentration of urea might be able to dissociate antigen binding sites in IC-derived antibodies, which had been masked with cognate-binding antigens (Bionda et al. 1984). Thus, newly formed bands at the presence of 6 M urea may be attributed to circulating IC-derived antibodies. Further studies are needed to know whether the use of a high concentration of 6 M urea could be applied for detecting specific antibodies in immune complexes in vivo.

Acknowledgments

We thank Miss M. Suzuki for preparing the manuscript. This work was supported by
References


