Original Article

Anti-DNA antibodies in the urine of lupus nephritis patients

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Abstract

Background. It has previously been reported that patients with systemic lupus erythematosus (SLE) and glomerulonephritis do not have anti- (deoxyribonucleic acid) DNA antibodies in their urine. This finding was attributed to specific entrapment of anti-DNA antibodies by the immune complexes in the glomerular capillary walls.

Methods. This phenomenon has been re-investigated as part of a study of the use of desoxyribonuclease 1 (DNase 1) to treat lupus nephritis (LN). For this purpose an ELISA was developed for the detection of anti-DNA antibodies in urine. It was found that such an assay was very susceptible to the presence of DNase in urine which destroys the antigen coating the plates and gives rise to false negative results. For this reason, it is essential that all tests for anti-DNA antibodies in the urine are carried out in the presence of EDTA to inhibit the endogenous DNase 1 activity.

Results. Using this assay to test the urine from 24 patients with LN and non-selective proteinuria, it was found that they all contained anti-DNA antibodies. The amount of anti-DNA antibodies detected in the urine was compared with that expected by calculations from the anti-DNA antibody titre in the serum and total immunoglobulin levels in serum and in urine. It showed that in 20 patients there was neither specific entrapment nor specific excretion of anti-DNA in urine, only the expected amount of leakage. In only three patients was any appreciable entrapment demonstrated and in only one, any excess excretion.

Conclusions. It is suggested that the failure to detect anti-DNA antibodies in the urine in the previous work was due to failure to inhibit the endogenous urinary DNase. It remains to be determined whether the retention of anti-DNA antibodies or excessive secretion is correlated with clinical phases of LN.

Key words: anti-DNA antibodies; glomerulonephritis; NZB/NZW F1 hybrids; proteinuria; systemic lupus erythematosus; urine

Introduction

Serum anti-DNA antibodies are a hallmark of systemic lupus erythematosus SLE. Their presence is a diagnostic parameter and their concentration is related to disease activity. High concentrations of anti-DNA antibodies have also been demonstrated in acid buf fer eluate from nephritic kidneys [1–4]. However, despite numerous reports and techniques on the detection of anti-nucleoprotein antibodies in the sera and kidneys of SLE patients, there are few reports on these antibodies in urine. Some groups have claimed that there are no anti-DNA antibodies in the urine [5–7]. Yamada et al. [5] postulated that the fall in serum concentration of anti-DNA antibodies in some patients with SLE was due to specific absorption of DNA-containing immune complexes in glomeruli.

In our previous study [8], we demonstrated the excretion of anti-DNA antibodies in the urine of lupus mice (NZB/W F1 hybrids) starting at the age of 5 months and increasing up to the age of 7 months. The purpose of this study was to measure the excretion of anti-DNA antibodies in the urine of SLE patients with LN and to determine whether there was evidence of specific entrapment of these antibodies in the glomerular immune complexes.

Subjects and methods

Calf thymus deoxyribonucleic acid (DNA), bovine serum albumin (BSA), ethylenediaminetetraacetic acid (EDTA) and 2,2'-azinobis-3-ethylbenzthiazolinesulfonic acid (ABTS) were all obtained from Sigma-Aldrich Co, Ltd, Poole, Dorset, UK.

Patients

Forty-three patients from the Department of Rheumatology, Hammersmith Hospital, London and the Renal Medicine Department, Addenbrooke’s Hospital, Cambridge were selected for analysis. Diagnosis of SLE was based on the standard American Rheumatism Association criteria [9]. Urine and blood samples were obtained on the same day. Total urinary protein and blood in the urine were tested by a multistix test (Bayer Diagnostics, Ames).
**Globulin concentrations**

The concentrations of globulin in the serum and in urine were determined by rocket immunoelectrophoresis [10]. To 10 ml of hot 1% agarose (in barbitone buffer/10 mM EDTA) 75 μl of rabbit polyclonal anti-human IgG serum was added and poured on to an 8×8-cm plate. Wells, 2 mm diameter were cut and 4 μl of sample/well was added. The samples were electrophoresed for 12 h at 20 mA/plate. Subsequently the plates were dried and stained. Protein standard serum LC-V (human) Behring (Behring Werke AG, D-35001, Marburg) was used as a standard for human IgG. Samples for IgG determination were mixed with equal volumes of freshly prepared 2 M KCN (at least 50 μl of each) and reacted at 50°C for 20 min. Final dilutions of sera were 1:200 and urine, 1:7.

**C3 and C4**

The concentration of serum C3 and C4 was measured by nephelometry and the CH₅₀ by functional assay [11].

**Anti-DNA**

Anti-DNA was measured by ELISA. Several methods of coating the ELISA plates with DNA were investigated. In preliminary studies, before incubation with DNA, the plates were treated with poly-l-lysine at 10 mg/ml [12–15], 0.1% protamine sulphate [16], 0.05% protamine chloride [6,17] and 0.01% methylated BSA [18–23]. Subsequently, the plates were washed and incubated with the solution of DNA. Calf thymus DNA was dissolved in distilled water to a concentration of 5 mg/ml, sonicated 2×2 min at maximum intensity, digested with proteinase K, phenol/chloroform extracted ×3 and ethanol precipitated, vacuum dried and finally dissolved in distilled water. The wells of ELISA plates were incubated with DNA in PBS/1 mM EDTA at concentrations of 2.5–100 μg/ml (100 μl/well). Preliminary tests were done with non-digested DNA or undigested but phenol-chloroform-extracted DNA. After overnight incubation and washing, the plates were blocked with 3% BSA for 30 min at room temperature. The plates were then washed ×3 with 1 mM EDTA/PBS 0.05% Tween-20. The standardization of the assay was performed testing the sera of NZB/W F1 hybrid lupus mice [8] at the time of maximum antibody level (6–7 months of age) and after the decline of serum antibody titre (9 months of age). Sera were diluted 1:200 in washing buffer (urines for urinary anti-DNA were diluted 1:2) and incubated for 60 min at room temperature. After washing, the plates were incubated with 1:1000 dilution of peroxidase-labelled goat anti-mouse IgG (anti-human IgG for human sera), washed again and then revealed with ABTS in ABTS buffer + H₂O₂. The plates were read at dual wave length measurements, 405 and 490 nm in a plate reader (Bio-Rad). The standard curve was obtained by plotting the ODs of serial dilutions of 1/100 diluted strongly positive SLE serum. The results were expressed as follows:

Units equal to 1000× sample absorbance/absorbance of highly positive human SLE serum.

For mice sera (used as standards), the results were expressed as follows:

Units equal to 100× sample absorbance/absorbance obtained with a pool of 9 month old NZB/W F1 hybrid sera.

**Specificity of the assay**

The specificity of the assay was demonstrated by testing serum with a high concentration of anti-DNA antibodies after incubation with DNA. The concentrations of DNA used in the inhibition studies were 0.01–25 μg/tube 60 μl of highly +ve serum diluted 1/100 + 60 μl of dilutions of DNA were incubated for 30 min +37°C. 100 μl of this solution (final serum dilution 1/200) was added to the wells of DNA coated ELISA plates and the standard assay performed.

All anti-DNA positive SLE patients sera were tested (in duplicate) in DNA uncoated plates (blocked with 3% BSA).

**The effect of inactivation of endogenous urine DNase by EDTA on anti-DNA antibody binding ELISA**

Plates were coated with calf thymus DNA as described previously. The plates were finally washed in 10 mM MOPS/10 mM azide, pH 7.2 (MOPS). Urine from patients with LN were adjusted to 1 mg/ml BSA and dialysed overnight in MOPS. ELISA plates were prepared in duplicates of 100 μl/well as follows: SLE patients’ serum with a high titre of anti-DNA antibodies were diluted in MOPS and added to the urine samples to give a final concentration of 1/100. One set of samples were prepared in MOPS/10 mM EDTA. Solutions were incubated for 60 min and then the anti-DNA ELISA was completed as previously described. The results (anti-DNA antibody titre) were expressed as absorbance at 405 and 490 nm.

**Demonstration that the DNA binding activity was of the molecular weight expected for IgG**

The urine of four lupus patients and four LN patients were tested: 15 ml of urine was concentrated down to 3 ml using AMICON B15 concentrators. The pH of the concentrate was adjusted to 5.0 with 3 M acetic acid and 0.5 ml caprylic acid added. The solution was stirred for 30 min at room temperature then spun at 20000 r.p.m. for 15 min. The supernatant was removed and precipitated with saturated ammonium sulphate (SAS) at a ratio of 6 ml SAS: 10 ml solution. The solution was then centrifuged for 15 min at 10000 r.p.m. and the precipitate collected in 1 ml PBS. The precipitate was dialysed against PBS overnight and the protein concentration measured by the method of Lowry et al. [24]. The IgG fractions were then tested by the standard antinuclear antibody test assay (ANF) and by the anti-DNA ELISA.

**Statistical analysis**

The differences in the concentrations of IgG and anti-DNA antibodies between normal controls and SLE patients were analysed by Student's t test.
Results

The detection of anti-DNA antibodies

The highest sensitivity and the lowest background were achieved with plates coated only with DNA at a concentration of 5 µg/ml without treatment with methylated BSA, poly-l-lysine, protamine chloride, or protamine sulphate (Figure 1). The most effective DNA preparation in the anti-DNA antibody binding tests was proteinase K digested DNA.

Twelve twofold dilutions of strongly anti-DNA antibody positive human serum (1/100-1/204800) were used to plot a standard curve and subsequently calculate the anti-DNA antibody titre in tested human sera. The OD of a positive control standard serum diluted 1/100 was marked as 1000 units.

Serum antibody activity was inhibited by adding free DNA to the test samples. Proteinase K digested DNA was more effective then other DNA preparations (Figure 2).

All 43 patients included in the study had significant titres (P<0.001) of serum anti-DNA antibodies. In all the patients total urinary protein, haematuria and albuminuria were measured (data not shown). Twenty-four patients presented with proteinuria and globulinuria and these patients only had anti-DNA antibodies in the urine (Table 1). Ten patients in this latter group had a renal biopsy diagnosis showing renal lesions (2 LN type III and 8 LN type IV by World Health Organisation classification criteria [25]. The results of testing human sera for anti-DNA antibodies and IgG concentration and human urine for anti-DNA antibodies and IgG and the results of the determination of C3, C4 and CH50 in human sera are presented in Table 1. Serum anti-DNA antibodies were also measured in the 20 SLE patients without LN (data not shown). However, these patients without proteinuria had no anti-DNA antibodies in their urine. The presence of anti-DNA antibodies in urine was correlated with proteinuria and especially with urinary IgG excretion. One patient with florid SLE was followed thereafter (SM). In this girl, the initial high concentration of anti-DNA antibodies of 93.329 units dropped to 11.690 units in a period of 2 weeks during which time she had heavy proteinuria. Later on, proteinuria subsided, anuria and renal failure developed and anti-DNA antibodies disappeared from the circulation.

Control sera from all 43 patients were tested in plates non-coated with DNA (but blocked with 3% BSA). There was no binding in this assay.

The effect of inactivation of endogenous urine DNase by EDTA on anti-DNA antibody binding ELISA

Urine from 14 SLE patients and six healthy persons was tested. To all the urine samples, serum from SLE patients with a high titre of anti-DNA antibodies (1/1000 final dilution) was added. After incubation in MOPS buffer with or without EDTA, the samples were tested by the ELISA anti-DNA antibody assay (Figure 3). It is evident that both the urine from SLE patients and that from the controls had less anti-DNA antibody when tested in buffer without EDTA. This reduction in antibody binding is due to deoxyribonuclease, i.e. hydrolysis of DNA on the coated plates. Urine from LN patients had more anti-DNA antibody because they contained not only the antibody added from SLE patients’ serum but also that from the patients’ urine. Some of the LN patients (especially those with high anti-DNA antibody concentration) had no significant reduction in anti-DNA binding in the absence of EDTA. This may be caused by low DNase levels in these LN patients.

Specificity of the ELISA anti-DNA antibody assay for measuring urine anti-DNA antibodies

Anti-DNA antibody binding of the urine of SLE patients pre-incubated with DNA at the concentration...
Table 1. Estimation of retention of anti-DNA antibodies in the kidney

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG concentration</th>
<th>Anti-DNA antibody (units/ml)</th>
<th>Complement</th>
<th>Renal biopsy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum (mg/ml) St Dev</td>
<td>Urine (μg/ml) St Dev</td>
<td>Serum</td>
<td>Urine observed</td>
</tr>
<tr>
<td>1 FM</td>
<td>4.91 0.35</td>
<td>205.00 56.63</td>
<td>4.18%</td>
<td>140.54 37.95</td>
</tr>
<tr>
<td>2 CS</td>
<td>6.94 1.75</td>
<td>118.33 28.10</td>
<td>1.71%</td>
<td>240.30 11.60</td>
</tr>
<tr>
<td>3 SM</td>
<td>5.06 0.48</td>
<td>110.25 53.80</td>
<td>2.18%</td>
<td>83.03 41.78</td>
</tr>
<tr>
<td>4 HR</td>
<td>5.05 1.18</td>
<td>94.67 24.88</td>
<td>1.88%</td>
<td>39.65 11.17</td>
</tr>
<tr>
<td>5 ML</td>
<td>5.30 0.62</td>
<td>114.00 64.11</td>
<td>2.15%</td>
<td>26.66 6.93</td>
</tr>
<tr>
<td>6 CF</td>
<td>4.97 0.72</td>
<td>46.33 14.29</td>
<td>0.93%</td>
<td>25.72 17.42</td>
</tr>
<tr>
<td>7 BI</td>
<td>9.37 0.74</td>
<td>21.75 9.24</td>
<td>0.23%</td>
<td>73.88 9.48</td>
</tr>
<tr>
<td>8 OO</td>
<td>6.83 0.13</td>
<td>50.67 54.35</td>
<td>0.74%</td>
<td>40.56 2.50</td>
</tr>
<tr>
<td>9 MS</td>
<td>3.28 0.50</td>
<td>113.95 3.21</td>
<td>3.47%</td>
<td>9.83 9.97</td>
</tr>
<tr>
<td>10 MW</td>
<td>4.81 3.19</td>
<td>256.33 10.97</td>
<td>5.33%</td>
<td>3.16 11.45</td>
</tr>
<tr>
<td>11 EJ</td>
<td>6.71 0.52</td>
<td>7.33 7.09</td>
<td>0.11%</td>
<td>26.83 11.48</td>
</tr>
<tr>
<td>12 NG</td>
<td>5.58 0.32</td>
<td>40.00 31.56</td>
<td>0.72%</td>
<td>5.26 10.00</td>
</tr>
<tr>
<td>13 FL</td>
<td>5.56 0.32</td>
<td>58.33 3.06</td>
<td>1.05%</td>
<td>4.29 4.30</td>
</tr>
<tr>
<td>14 HS</td>
<td>4.21 0.72</td>
<td>57.75 7.00</td>
<td>1.37%</td>
<td>8.70 3.21</td>
</tr>
<tr>
<td>15 KR</td>
<td>5.49 0.49</td>
<td>3.33 21.31</td>
<td>0.06%</td>
<td>7.83 5.03</td>
</tr>
<tr>
<td>16 SL</td>
<td>11.00 1.80</td>
<td>43.33 6.43</td>
<td>0.39%</td>
<td>18.09 6.00</td>
</tr>
<tr>
<td>17 AL</td>
<td>6.96 0.17</td>
<td>17.67 0.00</td>
<td>0.25%</td>
<td>46.30 7.97</td>
</tr>
<tr>
<td>18 DM</td>
<td>6.30 0.76</td>
<td>0.00 8.08</td>
<td>0.00%</td>
<td>20.02 12.75</td>
</tr>
<tr>
<td>19 SS</td>
<td>3.76 0.44</td>
<td>24.25 8.08</td>
<td>0.64%</td>
<td>18.75 12.16</td>
</tr>
<tr>
<td>20 KK</td>
<td>8.37 3.05</td>
<td>48.33 35.80</td>
<td>0.58%</td>
<td>27.25 3.61</td>
</tr>
<tr>
<td>21 KB</td>
<td>4.89 0.17</td>
<td>39.33 6.85</td>
<td>0.82%</td>
<td>4.29 7.76</td>
</tr>
<tr>
<td>22 PS</td>
<td>4.42 0.23</td>
<td>26.50 37.48</td>
<td>0.60%</td>
<td>5.34 3.55</td>
</tr>
<tr>
<td>23 VG</td>
<td>4.73 0.72</td>
<td>27.33 6.11</td>
<td>0.58%</td>
<td>7.03 6.58</td>
</tr>
<tr>
<td>24 SD</td>
<td>7.35 0.85</td>
<td>33.33 16.04</td>
<td>0.45%</td>
<td>50.01 10.99</td>
</tr>
</tbody>
</table>

Values for IgG and anti-DNA antibodies are means of up to 5 tests (done in triplicate).
The leakage ratio is urine IgG/serum IgG.
The expected anti-DNA level in urine = serum anti-DNA * leakage ratio.
Difference = observed − expected. When this is negative there is retention of anti-DNA; when positive there is excretion of anti-DNA.
Values greater than 1 for excretion and less than −1 for retention are bold. The patients are ranked in the order of the difference.
Complement: Low, C3, C4 or CH50 less than normal mean−2 SD; Normal, within normal range; n.a., not available.
Renal biopsy: 3, focal proliferative glomerulonephritis; 4, diffuse proliferative lupus nephritis; n.a., not available.
of 25 µg/ml was significantly reduced. Analogous experiments were done with the serum of SLE patients and a similar reduction of anti-DNA activity was demonstrated (Figure 4).

Demonstration that the DNA binding activity was of the molecular weight expected for IgG

IgG, fractionated from the urine of four patients with LN, was tested by the standard anti-nuclear antibody test (ANF) and by the anti-DNA ELISA (Table 2). All the samples had a high anti-DNA antibody titre in the anti-DNA ELISA and two of the samples were also positive in the ANF test.

**Discussion**

The modified and specific ELISA was used to detect anti-DNA antibodies not only in the sera, but also in the urine of a number of LN patients (type III and IV, in biopsied patients). To avoid the interference of DNase, EDTA was used in all the buffers (EDTA chelates Mg$^{2+}$ and Ca$^{2+}$ which are required for DNase activity). Both serum and urine contain free DNase that may degrade the DNA which is used as an antigen in both ELISA and Farr tests. The presence of DNase in the urine [8] is probably the major reason for the previously reported failure to detect anti-DNA antibodies in the urine of SLE patients or lupus mice. EDTA was not apparently used in the tests reported [5–7].

A decrease in serum anti-DNA antibody titre coin-

**Table 2.** Anti-nucleoprotein antibodies in immunoglobulin fractionated from SLE patient’s urines

<table>
<thead>
<tr>
<th>Patient</th>
<th>IgG concentration (mg/ml)</th>
<th>ANF (0–4)</th>
<th>Anti-DNA ELISA Units (0–1000)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BB</td>
<td>13.06</td>
<td>4</td>
<td>119</td>
</tr>
<tr>
<td>MC</td>
<td>0.751</td>
<td>4</td>
<td>947</td>
</tr>
<tr>
<td>FL</td>
<td>0.178</td>
<td>0</td>
<td>220</td>
</tr>
<tr>
<td>MS</td>
<td>0.120</td>
<td>0</td>
<td>243</td>
</tr>
</tbody>
</table>
cident with the development of nephrotic syndrome has been observed in some SLE patients [5,7,26]. However, the reports on the presence of anti-DNA antibodies in the urine of SLE patients or in experimental SLE are very rare. Yamada et al. [5] looked for antibodies to DNA in the serum and urine of nine patients with SLE presenting with heavy proteinuria (more than 3 g/l) and the activity of anti-DNA antibodies was compared between paired serum and urine samples for each patient. Anti-DNA antibodies were detected invariably in the serum of these patients, but in contrast, anti-DNA activity was not detectable in urine samples from any of the nine patients.

Perez-Vazquez et al. [7] detected antinuclear antibodies (by indirect immunofluorescence using Hep-2 cells as the substrate) in the urine of SLE patients with nephrotic syndrome but they were unable to detect anti-DNA antibodies in the same urine by Farr assay.

Yamamoto et al. [6] used solid phase ELISA test to investigate the excretion of anti-DNA antibodies in the urine of 7-month-old, female NZB/W F1 hybrid mice. No anti-DNA antibodies were found in the urine. Only after unilateral nephrectomy and lysozyme administration did the treated mice excrete significantly larger amounts of anti-DNA antibodies than the saline treated animals. The amount of urinary anti-DNA antibodies was markedly high irrespective of urinary IgG concentration.

In our study on the effects of recombinant mouse deoxyribonuclease 1 in the treatment of nephritis in lupus mice [8], anti-DNA antibodies were regularly detected in the urine of the animals with the highest titre at the age of 7 months. An ELISA was used similar to that in the present study.

IgG and anti-DNA concentrations in the sera and urine of 23 SLE patients with non-selective proteinuria were measured. From the values, an expected urinary anti-DNA level (serum anti-DNA × urine IgG/serum IgG) was calculated. The difference between this expected value and the observed value has been used to assess whether it is due to specific retention of anti-DNA in the kidney or specific excretion. Since the IgG and anti-DNA concentrations were measured on the same samples of serum and urine, the difference between the expected and the observed amount of anti-DNA in the urine is not affected by the state of diuresis in the patient or by the clearance rate of the IgG.

It can be seen in Table 1 that significant retention of anti-DNA in the kidney is observed in only three patients, all of whom had high expected values. In 19, the amount excreted is only close to the expected amount and in one patient there is some excess anti-DNA antibody in the urine, suggesting this may come from the immune complexes in the kidney. Significant binding of anti-DNA to the immune complexes in the kidney therefore seems to be uncommon and seen only in patients where a large amount of anti-DNA antibody is being filtered.

In only one patient was there any suggestion of excess release of anti-DNA into the urine. This sug-

References

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