CONCISE REPORT

A prospective study of anti-chromatin and anti-C1q autoantibodies in patients with proliferative lupus nephritis treated with cyclophosphamide pulses or azathioprine/methylprednisolone

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Objective: To study the prevalence and course of anti-chromatin (anti-nucleosome, anti-double-stranded (ds) DNA and anti-histone) and anti-C1q autoantibodies in patients with proliferative lupus nephritis (LN), treated in a randomised controlled trial with either cyclophosphamide or azathioprine plus methylprednisolone.

Methods: Autoantibody levels were measured and analysed in 52 patients with proliferative LN, during their first year of treatment. Levels in both treatment arms were compared and associations with clinical, serological and outcome parameters were studied.

Results: At study entry, prevalences for anti-nucleosome, anti-dsDNA, anti-histone and anti-C1q autoantibodies were 81%, 96%, 23% and 65%, respectively. Anti-chromatin autoantibodies correlated with each other, but not with anti-C1q levels. If patients were divided for their autoantibody titre at the start of treatment above or below the median, the only significant differences were higher SLE disease activity index with higher anti-nucleosome, and higher creatinine with higher anti-C1q autoantibodies. During the first year, a comparable rapid decline in the levels of anti-nucleosome, anti-dsDNA and anti-C1q autoantibodies was seen in both treatment arms. Anti-histone autoantibody levels were low and did not change. Renal flares were not preceded by rises in autoantibody titres.

Conclusions: These results indicate that measurement of anti-chromatin and anti-C1q autoantibodies is useful for diagnosing LN, but not for monitoring disease course.

The nucleosome, the basic unit of chromatin, has been proposed as a major autoantigen in systemic lupus erythematosus (SLE).¹ Anti-chromatin autoantibodies can be divided into antibodies that recognise a component of the nucleosome—that is, DNA or histones—and autoantibodies that recognise the intact nucleosome (nucleosome-specific antibodies). Anti-nucleosome autoantibodies are found in most patients with SLE, especially in those with lupus nephritis (LN).² Anti-nucleosome and anti-double-stranded (ds)DNA reactivity have been associated with disease activity and flares of LN.^{3,4} In patients deficient for C1q, lupus-like disorders are commonly found, and about 30% develop glomerulonephritis.⁵ Paradoxically, anti-C1q autoantibodies are frequently found,⁶ especially in those with LN. They prognosticate renal flares.⁷ We conducted a multicentre randomised controlled trial comparing cyclophosphamide pulses with azathioprine/methylprednisolone in patients with proliferative LN. Recently, the first results were published.⁸

In the current study, the levels of anti-nucleosome, anti-dsDNA, anti-histone and anti-C1q autoantibodies were assessed in the participants, during their first year of treatment. Autoantibody titres were analysed for association with serological, clinical and outcome parameters.

METHODS

Patients

In all, 87 patients with proliferative LN were randomised to either cyclophosphamide pulses (CY) or azathioprine and methylprednisolone (AZA).⁸ Levels of complement C3 and C4, and anti-dsDNA titres were measured locally. Disease activity was measured with SLE disease activity index (SLEDAI).⁹ Plasma samples at study entry and after 4, 12, 26 and 52 weeks were available from 52 patients. Their baseline and outcome characteristics were representative for the whole group (data not shown).

Definitions

Renal relapse is doubling of the lowest serum creatinine observed so far and/or developing either nephrotic syndrome while the lowest proteinuria had been <2.0 g/day repeatedly, or proteinuria >1.5 g/day without other causes in a patient who does not have proteinuria.

Complete remission (CR) is serum creatinine <130% of lowest serum creatinine since entry, proteinuria <0.5 g/day and <10 erythrocytes/high-power field.

Anti-chromatin and anti-C1q ELISAs

Anti-dsDNA, anti-histone and anti-nucleosome ELISAs were performed as described.¹⁰ For the anti-nucleosome ELISA, H1-stripped chromatin (kindly provided by Dr R Burlingame, INOVA Diagnostics Inc, San Diego, California, USA), diluted in phosphate-buffered saline (5 µg/ml), was used. In the anti-DNA ELISA, calf thymus dsDNA (Roche, Almere, The Netherlands) was coated overnight in phosphate-buffered saline (20 µg/ml). In the anti-histone ELISA, calf thymus histones (Roche) were coated overnight (2.5 µg/ml) in 0.1 M glycine buffer at pH 9. The titre (in arbitrary units (AU)) was defined as dilution of patients' plasmas yielding absorbance of 0.5. Day-to-day variation of the assay was corrected by using a standardised positive plasma. Cut-off was set at the value of negative control plasmas plus three times SD. The anti-C1q

Abbreviations: AU, arbitrary units; AZA, azathioprine plus methylprednisolone; CR, complete remission; CY, cyclophosphamide pulses; ds DNA, double-stranded DNA; LN, lupus nephritis; IQR, interquartile range; SLE, systemic lupus erythematosus; SLEDAI, SLE disease activity index

Figure 1 Autoantibody reactivity in the two treatment arms during the first year of treatment: (A) anti-nucleosome, (B) anti-dsDNA, (C) anti-histone and (D) anti-C1q reactivity. Results are expressed as medians (AU, arbitrary units). The cut-off value for the different assays is indicated by a dashed line. There were no differences between the two treatment groups. CY, treatment with cyclophosphamide pulses; AZA, treatment with azathioprine/methylprednisolone.

ELISA was performed as described previously.¹¹ A sample positive for anti-C1q was used as calibration standard in each assay. Values >75 AU were regarded positive.

Statistical analysis

Statistical analysis was performed using SPSS V.12.0.1. Correlations between autoantibody levels were studied with non-parametric Spearman's rank correlation. Mann-Whitney U or χ^2 tests were used to analyse differences between groups. Differences between treatment arms and course of laboratory parameters, and SLEDAI were studied with repeated measurements using mixed model tests. A p value of <0.05 was regarded as significant, except for correlation coefficients, where a p value of <0.01 was used, to correct for the effect of multiple comparisons (according to Bonferroni).

RESULTS

Autoantibody titres at study entry

Anti-nucleosome reactivity (cut-off: 18.5 AU) was present in 81% of the patients, whereas anti-dsDNA and anti-histone reactivity

(cut-off: 26.5 AU and 17.5 AU, respectively) were found in 96% and 23%, respectively. Anti-C1q reactivity (above 75 AU) was present in 65% of the patients. The median anti-nucleosome titre was 200 AU (interquartile range (IQR) 41–600). For anti-dsDNA, anti-histone and anti-C1q, median titres were 145 (IQR 72–400), 9 (IQR 8–16) and 123 AU (IQR 60–188), respectively. There were no differences between CY and AZA.

Autoantibody titres during treatment

Although anti-nucleosome and anti-dsDNA reactivity declined (fig 1A,B), reactivity at 52 weeks stayed above cut-off in most patients. Median anti-histone reactivity was initially low and did not change during treatment (fig 1C). Anti-C1q reactivity decreased significantly and became negative within 12 weeks (fig 1D). There were no significant differences between the treatment groups.

Correlation between autoantibody titres

At study entry, a significant correlation between anti-nucleosome and anti-dsDNA reactivity existed, and both also correlated with anti-histone reactivity ($p < 0.001$, table 1).

Table 1 Correlation coefficients at study entry in 52 patients with proliferative lupus nephritis

	Anti-nucleosome	Anti-dsDNA (ELISA)	Anti-histone	Anti-C1q
Anti-nucleosome		0.629*	0.583*	0.110
Anti-dsDNA (ELISA)			0.495*	0.270
Anti-histone				0.115
Anti-dsDNA (Farr)	0.491*	0.531*	0.361	0.203
C3	-0.241	-0.418**	-0.231	-0.180
C4	-0.057	-0.227	-0.088	0.023
SLEDAI	0.288	0.420**	0.358	0.159

C3, complement C3; C4, complement C4; SLEDAI, systemic lupus erythematosus disease activity index.

* $p < 0.001$, ** $p = 0.002$.

In contrast, anti-C1q reactivity was not correlated with any of the anti-chromatin autoantibodies.

Correlation of autoantibody titres with serological markers and disease activity

As the Farr assay is often used as the standard assay for measuring anti-dsDNA autoantibodies, autoantibody titres measured by ELISA were compared with results of the Farr assays. Although the Farr assay showed a strong correlation with autoantibody levels obtained with anti-dsDNA ELISA ($p < 0.001$), and with anti-nucleosome autoantibodies ($p < 0.001$), no correlation with anti-histone and anti-C1q autoantibodies was observed (table 1). C4 was not correlated with any of the autoantibodies, while C3 showed a significant negative correlation with anti-dsDNA autoantibody levels measured with ELISA. SLEDAI correlated with anti-dsDNA reactivity only.

Association with clinical parameters

As no significant differences were observed in autoantibody titres and time course between the treatment arms, all patients were analysed as one group. No significant differences in serum creatinine or proteinuria existed between patients with high (above the median) or low (below the median) anti-chromatin antibody levels. Patients with initial high anti-C1q had a higher serum creatinine (median 138 vs 100 $\mu\text{mol/l}$, $p = 0.042$). The course of disease in patients with high versus low autoantibody titres at study entry did not differ with regard to the occurrence of renal relapses, or CR, except for patients with initial high anti-dsDNA reactivity who attained CR more often (20 vs 13 patients, $p = 0.046$). For all autoantibody specificities, there were no differences in the time to reach CR. Patients with high anti-chromatin titres had a significantly higher SLEDAI. This was not observed for anti-C1q. Renal relapses were not associated with significant increases in anti-nucleosome or anti-dsDNA titres.

DISCUSSION

We performed a prospective analysis in patients with proliferative LN. As expected, a high prevalence of anti-dsDNA (96%) and anti-nucleosome (81%) autoantibodies was found, as the presence of these autoantibodies correlates with the occurrence of LN.² Remarkably, the prevalence of anti-dsDNA autoantibodies was higher than the presence of anti-nucleosome autoantibodies, whereas in most of the studies this was the other way around. The latter can be expected as all anti-chromatin antibodies (nucleosome-specific, anti-dsDNA, anti-histone) can bind to nucleosomes. The higher prevalence of anti-dsDNA antibodies might be due to the use of anti-dsDNA ELISA, which also detects low-avidity antibodies in contrast to the Farr assay,¹² which indeed was positive in 81% of our patients. On the basis of our results, we cannot support the anticipated superiority of anti-nucleosome testing. The observed correlation between anti-dsDNA and anti-nucleosome reactivity was comparable with values found in unselected patients with SLE.^{3–13} The prevalence (23%) and reactivity of anti-histone autoantibodies was low. This corresponds with the fact that anti-histone autoantibodies are more often found in drug-induced lupus and do not correlate with renal disease.² Anti-C1q reactivity (65%) was comparable with reported prevalences in those with LN.^{6–7} The lack of correlation between anti-chromatin and anti-C1q autoantibody levels indicates a different driving autoantigen—namely, the nucleosome versus C1q.

No differences between CY and AZA were found on the course of autoantibody titres and the rate of decline was equal. This paralleled the earlier published results of the trial—namely, a comparable decline in proteinuria, and rise in C3 and

C4 in both treatment groups. Moreover, the number of patients reaching complete remission and the time to do so were not different between CY and AZA.⁸

Anti-nucleosome and anti-dsDNA autoantibodies have been associated with disease activity.^{2–3} High anti-chromatin reactivity was associated with a higher SLEDAI, comparable with the literature.³ The absence of such a correlation with anti-C1q reactivity was remarkable. Furthermore, the presence of anti-dsDNA autoantibodies seems to be associated with complement consumption, as shown by the negative correlation with C3. Others showed that anti-nucleosome and anti-C1q autoantibodies correlated with renal disease manifestations.^{2–7} However, this was investigated in patients with or without renal involvement. In our patients, a direct correlation between anti-nucleosome titres and renal function parameters was not found. This might be due to the fact that they had biopsy-proven LN with more or less comparable disease manifestations. In contrast, patients with higher anti-C1q reactivity had higher serum creatinine. Although it has been claimed that renal flares are associated with significant increases in anti-dsDNA⁴ or anti-C1q titres,¹⁴ we could not confirm this. This is consistent with only one prospective analysis available so far.¹³ The relatively low sampling frequency in our study may have influenced the results. Comparing our ELISA techniques with other studies is also difficult, although the anti-nucleosome and anti-C1q ELISA were identical to studies which reported predictive increases.^{2–14}

In conclusion, measuring anti-chromatin and anti-C1q autoantibodies serves as an important tool for diagnosis of LN, but not for monitoring disease course or treatment.

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