ANTIBODIES RECOGNIZING GLOBULAR DOMAIN OF C1q – CURRENT VIEW ON THE ASSOCIATION BETWEEN LUPUS NEPHRITIS ACTIVITY AND ANTI-gC1q AUTOANTIBODIES

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ABSTRACT

INTRODUCTION: Lupus nephritis (LN) is a serious complication of the systemic lupus erythematosus (SLE). Anti-C1q antibodies correlate with the occurrence and high clinical activity of LN, especially proliferative LN. The first reported anti-C1q antibodies recognized autoepitopes within a collagen-like region (CLR) of C1q. Recently we have found autoantibodies against globular C1q domain (gC1q antibodies) in LN patients. The aim of the present study was to evaluate the potential pathological consequences of the presence of anti-gC1q antibodies in LN.

MATERIAL AND METHODS: The recombinant globular head region of the three chains of C1q -A, -B and -C were expressed in *E. coli* BL21 and purified. Anti-C1q, anti-gC1q autoantibodies, complement proteins - C1q, C4, C3 and IgG-, IgM-CICs levels were screened by ELISA in 53 sera from LN patients. Sera from 196 normal controls served as controls.

RESULTS: We found that patients positive for anti-B-gC1q antibodies presented with significantly lower serum C4 levels than patients positive for anti-A and anti-C-gC1q antibodies ($p=0.014$) and with significantly lower levels of C3 than patients positive for anti-A and anti-C-gC1q antibodies and patients without anti-C1q antibodies ($p=0.005$; $p=0.018$). Significant correlations to IgG CICs were detected for anti-C1q ($r=0.371$, $p=0.001$) and anti-B-gC1q antibodies ($r=0.431$, $p=0.003$).

CONCLUSIONS: These findings suggest that the binding of anti-B-gC1q autoantibodies with C1q may possibly trigger mechanical stress and induce a structural change within the CLR domain of C1q, compatible with C1r-C1s complement activation in the fluid phase.

Keywords: anti-C1q autoantibodies, anti-gC1q autoantibodies, lupus nephritis

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INTRODUCTION

Lupus nephritis (LN) is a major and severe complication of the systemic lupus erythematosus (SLE) with kidney inflammation and flare. SLE is characterized by the production of various autoantibodies (more than 100 different types of autoantibodies) and immune complexes deposition, which activate the complement. Autoantibodies to C1q, the first subcomponent of C1 in the classical complement activation, are a hallmark of renal involvement and a predictive marker for a flare of LN (1-11). Many research groups put a lot of effort to understand the initial events that trigger autoimmunity against C1q, the role of anti-C1q autoantibodies in the pathogenesis of lupus nephritis and the consequence of the interactions between these serum molecules. However, by now only several hypotheses are presented and many questions remain unclear and the findings are not fully characterized yet.

C1q is the first component of the classical pathway of the complement activation and its main functions are to clear apoptotic cells and immune complexes (ICs). C1q, a major linking molecule between innate and adaptive immunity, also regulates inflammatory mechanisms at cellular and molecular levels (12-16). C1q is a hexameric molecule. Its quaternary structure contains an *N*-terminal triple-helical collagen-like region (CLR) and C-terminal heterotrimeric globular head domain (gC1q). The gC1q domain is composed of C-terminal halves of the A (ghA), B (ghB) and C (ghC) chains. The C1q molecule (460 kDa) is composed of 18 polypeptide chains (6A, 6B, and 6C) (17).

Anti-C1q autoantibodies are mainly against neoepitopes within the CLR of human C1q (4, 7, 18, 19). Recently we have shown that epitopes within the gC1q domain of the C1q molecule may be targeted by anti-C1q autoantibodies (20-22), possibly with functional consequences (22). We found that anti-ghB, but not anti-ghA and anti-ghC autoantibodies, markedly inhibited C1q interaction with IgG as well as with CRP, which suggested that the anti-ghB autoantibodies might partially induce acquired functional C1q deficiency (22). More importantly, the role of these autoantibodies recognizing different epitopes in the large C1q molecule is to be understood, when and how they appear in the serum and

which of them are more specific to initiate autoimmune response and pathology. Therefore, the aim of this study was to evaluate the potential pathological consequences of the presence of anti-gC1q antibodies in lupus nephritis.

MATERIAL AND METHODS

Lupus Nephritis Patients and Healthy Volunteers

The LN serum samples were collected from patients with biopsy-proven lupus nephritis from the Clinic of Nephrology, University Hospital "Tzaritza Ioanna – ISUL" - Sofia. The analysed cohort included 46 (86.79%) women and 7 (13.21%) men with a mean age of 40 (± 13.77) years. All patients fulfilled at least 4 of 11 of the American College of Rheumatology classification criteria for SLE (23), and the diagnosis of lupus nephritis was based on clinical and laboratory parameters including proteinuria, urinary sediment, creatinine level, and erythrocyte sedimentation rate. The disease activity was evaluated by SLE-DAI (SLE Disease Activity Index). Inclusion criteria were defined as proteinuria 500 mg/24 hr or higher in the last 10 days, erythrocyturia as 8 RBC per microliter or higher, renal dysfunction as any increase in creatinine value at any time in the history, and renal involvement as any of the two above variables.

The presence of antinuclear antibodies (ANAs) and anti-dsDNA autoantibodies were detected by double immunodiffusion assays and indirect immunofluorescence in University Hospital "Tzaritza Ioanna – ISUL" - Sofia.

The group of healthy volunteers included 118 (60.20%) women and 78 (39.80%) men with a mean age of 46 (± 14.36) years.

The study had the approval of the Ethics Review Board of Medical University of Varna and each patient and healthy volunteer signed a consent form of enrolment.

ELISA for Determination of Serum Complement Proteins Levels

The concentrations of C1q, C4 and C3 antigens in serum were measured by means of a double-ligand ELISA as described previously (22). Normal values, established with pooled plasma from 196 healthy blood donors, ranged between 59 – 178 $\mu\text{g/ml}$, 84 – 396 $\mu\text{g/ml}$ and 612 – 1 444 $\mu\text{g/ml}$ (mean $\pm 2\text{SD}$) for C1q, C4 and C3, respectively.

CH50

CH50 test (samples plasma) was used as a functional measurement of the activity of the whole complement pathway from classical pathway activation. CH50 was determined according to standard procedure (24). Results were expressed as a percentage of the CH50 of a reference plasma pool obtained from 196 healthy blood donors. Normal values for CH50 were 53 – 146% ($\pm 2SD$).

Expression and Purification of the Recombinant Globular Head Regions of the Three Chains of C1q

The recombinant globular head regions of the A chain (ghA, residues 88-223), the B chain (ghB, 90-226), and the C chain (ghC, 87-217) were expressed as fusion proteins linked to the maltose-binding protein (MBP) in *Escherichia coli* BL21 and purified, as described previously (17).

ELISA for Detection of Anti-C1q Autoantibodies Levels

Anti-C1q autoantibody levels were measured in human serum samples under 0.75 M NaCl conditions as described previously (21, 25). Values were expressed in terms of fold increase compared to the pooled normal human serum (NHS) from 196 healthy volunteers (norm=1).

ELISA for Detection of IgG- and IgM-containing Circulating Immune Complexes

Serum levels of IgG- or IgM-containing circulating immune complexes (CICs) levels were determined using the same principle, as ELISA for anti-C1q antibodies, except that the plates were incubated with patients' sera, diluted 1:100 in PBS/0.15 M NaCl (25). Values were expressed in terms of fold increase compared to the pooled normal human serum (NHS) from 196 healthy volunteers.

Statistical Analysis

Statistical analysis was carried out using software GraphPad Prism 5.01. Quantitative data were expressed as mean $\pm$ SD. For comparison between groups of patients and of healthy volunteers, the Mann-Whitney U test for continuous variables for 2-group comparisons was used. The Spearman Correlation was used to analyse the correlations. P values less than 0.05 were considered significant. All tests were 2-tailed.

RESULTS

Main Complement Features and Levels of Anti-C1q, Anti-gC1q Autoantibodies and Circulating Immune Complexes (CICs) in Sera of Lupus Nephritis Patients

Measurement of complement hemolytical activity, levels of serum C1q, C4 and C3 concentration, the presence of anti-C1q, ANA, anti-dsDNA antibodies, and circulating immune complexes (CICs) were used to monitor the disease. Three patients had low levels of C1q, CH50 and C4. C1q binding IgG-CICs were present in these sera. Only one serum of them was seropositive for anti-C1q autoantibodies. We found low levels of C1q and CH50 in five different sera with detected presence of C1q-IgG-CICs. Three of them were seropositive for anti-C1q antibodies and for anti-gC1q autoantibodies against A- and/or C-chains of C1q. 17 out of 53 patients (32.08%) were positive for anti-C1q autoantibodies. Nevertheless, 33.96% (18/53) were positive for anti-gC1q autoantibodies. 10 out of 53 LN patients (18.87%) were seropositive only for anti-A and/or anti-C-gC1q. Eight patients from all 53 (15.09%) were positive predominantly for anti-B-gC1q autoantibodies and only one was positive also for anti-C1q antibodies. In 43.40% of lupus ne-

Fig. 1. Correlation between serum levels of C4 and C1q in lupus nephritis patients and normal controls (A.). Correlation between serum levels of C3 and C1q in lupus nephritis patients and normal controls (B.).

phritis patients (23/53) we did not find anti-C1q and anti-gC1q antibodies. We detected that 60.00% of the patients were positive for ANA and 44.00% of the

patients were positive for anti-dsDNA autoantibodies. The levels of C1q significantly correlated with C4

Fig. 2. Comparison of serum C1q (A.), C4 (B.) and C3 (C.) levels in normal controls and in patients: seropositive for anti-C1q antibodies; seropositive for anti-B-gC1q antibodies; seropositive for anti-A and anti-C-gC1q antibodies and negative for anti-C1q antibodies. The horizontal line indicates the cut-off value.

($r=0.561$, $p=0.0002$) and with C3 ($r=0.561$, $p<0.0001$) in LN patients (Fig. 1, A. and B.)

Association between the Levels of C1q and the Presence of Anti-C1q and Anti-gC1q Antibodies in Lupus Nephritis Sera

All lupus nephritis patients were divided into four groups – positive for anti-C1q antibodies, but negative for anti-gC1q antibodies (12); positive for anti-B-gC1q antibodies (8, out of which 7 positive also for anti-C1q antibodies); positive for anti-A and/or anti-C-gC1q antibodies (10, out of which 3 positive also for anti-C1q antibodies) and negative for anti-C1q antibodies (23). Serum C1q levels in these groups and normal controls were compared (Fig. 2A). All patients sera from the four groups presented with significantly lower levels of serum C1q than the healthy donors (120.00 $\mu\text{g/ml}$ vs. 55.00 $\mu\text{g/ml}$, $p=0.002$; 67.00

$\mu\text{g/ml}$, $p=0.0002$; 67.00 $\mu\text{g/ml}$, $p<0.0001$; 72.00 $\mu\text{g/ml}$, $p<0.0001$ in groups).

Association between the Serum C4 Levels and the Presence of Anti-C1q and Anti-gC1q Antibodies in Lupus Nephritis Sera

Serum C4 levels in the groups: positive for anti-C1q antibodies; positive for anti-B-gC1q antibodies; positive for anti-A and anti-C-gC1q antibodies and negative for anti-C1q antibodies, were analysed and compared with the normal controls (Fig. 2B). We found significantly lower serum C4 levels in all four groups of lupus nephritis patients in comparison with the levels in the healthy donors (231.00 $\mu\text{g/ml}$ vs. 130.00 $\mu\text{g/ml}$, $p=0.004$; 99.00 $\mu\text{g/ml}$, $p<0.0001$; 161.00 $\mu\text{g/ml}$, $p=0.001$; 110.00 $\mu\text{g/ml}$, $p<0.0001$ in groups). Patients sera positive for anti-B-gC1q anti-

Fig. 3. Correlation between serum levels of anti-A-gC1q antibodies (A.), anti-B-gC1q antibodies (B.), anti-C-gC1q antibodies (C.), anti-C1q antibodies (D.) and IgG CIC in lupus nephritis patients.

bodies presented with significantly lower serum C4 levels than patients sera positive for anti-A and anti-C-gC1q antibodies (120.00 µg/ml vs. 161.00 µg/ml, $p=0.014$).

Association between the Serum C3 Levels and the Presence of Anti-C1q and Anti-gC1q Antibodies in Lupus Nephritis Sera

Our analyses detected lower serum C3 levels in patients sera positive for anti-B-gC1q antibodies and negative for anti-C1q antibodies compared to the normal controls (1014 µg/ml vs. 634 µg/ml, $p=0.006$; 875 µg/ml, $p=0.007$). Patients positive for anti-B-gC1q antibodies presented with significantly lower levels of C3 in serum than patients positive for anti-A and anti-C-gC1q antibodies and patients without anti-C1q antibodies (634 µg/ml vs. 1013 µg/ml, $p=0.005$; 875 µg/ml, $p=0.018$). The serum C3 levels of patients seropositive for anti-B-gC1q antibodies were also lower than the C3 levels in positive for anti-C1q antibodies patients (634 µg/ml vs. 851 µg/ml, $p=0.068$) (Fig. 2C).

Association between the Levels of ANA and/or Anti-ds DNA Antibodies and Presence of Anti-C1q and Anti-gC1q Antibodies in Lupus Nephritis Sera

The cohort of lupus nephritis patients were divided into two groups – positive for ANA and negative for ANA. Both groups were analysed for the presence of anti-C1q and/or anti-gC1q antibodies. We did not find any significant association between ANA and anti-A-gC1q, anti-B-gC1q, anti-C-gC1q and anti-C1q antibodies. No significant association between anti-ds DNA antibodies and anti-gC1q and/or anti-C1q antibodies was found in the two groups – positive and negative for anti-dsDNA antibodies.

Correlations between the Presence of Anti-C1q and Anti-gC1q Antibodies and Some Markers of Lupus Nephritis Activity.

We assessed the correlation of anti-C1q and anti-gC1q antibody with several serologic markers of disease activity such as levels of C1q, C4, C3, IgG- and IgM-CICs. For anti-C1q antibodies there was only a trend for negative correlations with C1q and C3 levels. Similarly, a tendency for negative correlation between anti-B-gC1q antibodies and C3 was observed. For anti-B-gC1q, anti-C-gC1q and anti-C1q antibodies there were significant correlations to IgG CICs ($r=0.431$, $p=0.003$; $r=0.389$, $p=0.016$ and

$r=0.371$, $p=0.001$) and for anti-A-gC1q antibodies to IgG CICs - a correlational trend ($r=0.281$, $p=0.087$) was found (Fig. 3). The levels of IgM CICs decreased in accordance with the increase of anti-C1q antibodies ($r=-0.238$, $p=0.051$).

DISCUSSION

Several studies have shown that anti-C1q autoantibodies correlate with the occurrence and high clinical activity of LN, especially proliferative LN (2,4,7,9,10). The increasing of anti-C1q antibodies was shown to predict renal relapse within 6 months (1,26). On the other side, Katsumata et al., (2011) and Trad et al., (2013) recently revealed a lack of specific association of anti-C1q antibodies with active and also with proliferative nephritis (27,28). Gunnarsson et al., (2002) and Marto et al., (2005) also did not find differences in levels or prevalence of anti-C1q antibodies between patients with proliferative and patients with non-proliferative form of LN (5,6). These few studies contradict the widespread allegation that anti-C1q antibodies are pathogenic.

Flierman and Daha (2007) suggested a hypothesis to explain the role of anti-C1q antibodies in the development of LN (29). According to their model, the presence of anti-C1q antibodies seems to be a necessary, but not sufficient, condition for the development of LN and is associated with C1q deposition and nucleosomes containing CICs accumulation in the glomerular basement membrane. The subsequent local activation of complement leads to renal damage during inflammatory response and hypocomplementemia in serum is indication for this (29). We suggested that probably the pathological role of anti-C1q antibodies was not as much in the activation as in the amplification of prolonged activated complement attack.

Anti-C1q autoantibodies may affect the physiological role of C1q indirectly by elimination of the available C1q in the circulation. Moreover, researchers have demonstrated a negative correlation between anti-C1q antibodies and plasma C1q (30). Chen et al., (2002) found that serum anti-C1q antibody levels were higher in LN patients with renal C1q deposition than without renal C1q deposition (31).

Autoantibodies against C1q were originally detected against the collagen-like tail region of C1q. Recently we have shown that anti-C1q antibodies are

generated also against the A, B and C chains of the globular heads of C1q – anti-gC1q antibodies (20-22). We demonstrated that anti-C1q autoantibodies specifically against the globular domain of the C1qB-chain from LN patient could partially induce acquired functional C1q deficiency and interfere with the biological function of C1q (22).

In this study we explored the associations between the presence of anti-C1q and anti-gC1q antibodies and the levels of complement proteins (C1q, C4, C3) to evaluate the potential pathological effects of anti-gC1q antibodies and especially the effects of anti-B-gC1q antibodies in LN. Interestingly, we found significantly lower serum C4 and C3 levels in patients positive for anti-B-gC1q antibodies in comparison to patients positive for anti-A and anti-C-gC1q antibodies. The levels of C3 in patients seropositive for anti-B-gC1q antibodies were also lower than the C3 levels in patients positive for anti-C1q antibodies (Fig. 2). Julkunen et al., (2012) found that anti-C1q antibodies and complement C3 and C4 were better markers for lupus nephritis activity than anti-dsDNA antibodies (32). Significant correlations were also observed for anti-C1q and anti-B-gC1q antibodies to IgG CICs and to some other renal pathological characteristics in lupus nephritis (unpublished data). Low levels of C1/or C1q, C4 and C3 in the sera positive for anti-B-gC1q autoantibodies patients could be an important correlation indicator for a possible crucial role of anti-B-gC1q antibodies in the initiation of autoimmune response. Stoyanova et al., (2012) suggested that the presence of anti-gC1q in both healthy and diseased humans also implied that these antibodies, unlike anti-CLR, might have a contribution to an onset of autoimmunity (33).

Thus, the population of polyclonal anti-C1q antibodies, which is very heterogeneous may influence on the immune response multidirectionally and to trigger autoimmunity, with severe pathological consequences.

CONCLUSIONS

Our findings and analyses suggest that the anti-gC1q autoantibodies upon interaction and binding to C1q may trigger mechanical stress and structural conformational changes within the CLR domain of C1q, compatible with C1r-C1s activation in fluid phase. This complement activation results to onset of

a strong inflammatory response, tissue deposition of immune complexes, triggering of apoptosis, necrosis and autoimmunity.

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