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Serologic markers of lupus nephritis in patients: use of a tissue-based ELISA and evidence for immunopathogenic heterogeneity

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SUMMARY

In order to assess the ability of various serologic assays to correlate with lupus nephritis, we analysed sera obtained from 60 patients with systemic lupus erythematosus (SLE). Patients were categorized as having active nephritis (group 1), active lupus without nephritis (group 2), inactive lupus with prior nephritis (group 3), or inactive lupus without prior nephritis (group 4). Three parameters were assessed including anti-dsDNA antibodies (Farr assay), immune complexes (C1q binding), and anti-C1q antibodies (salt-stable C1q binding). Additionally, glomerular binding activity (GBA) was measured using a new solid-phase immunoassay that detects immune elements by their ability to bind glomerular tissue. We found that patients with nephritis (group 1) exhibited higher mean values for each assay than patients in each of the other three groups ($P = 0.001$, 0·009, 0·14, and 0·23 in the GBA, C1q, anti-dsDNA, and anti-C1q assays, respectively). The only assay which distinguished patients with nephritis (group 1) from patients having active disease without nephritis (group 2) was the GBA (mean 0.48 ± 0.09 *versus* 0.15 ± 0.04, $P < 0.05$). In terms of utility, all tests were specific for diagnosing nephritis among patients with lupus; however, only the GBA was reasonably sensitive. The information provided by the anti-dsDNA and C1q assays were not correlated with one another, nor additive to the GBA. Patients with false negative GBA tended to have received more intensive immunosuppression. The qualitative characteristics of GBA varied among patients with nephritis. These data suggest the pathogenesis of lupus nephritis is complex, and may be mediated by an array of immune elements. Moreover, the data indicate the potential utility for a broad tissue-based approach to detection of pathogenic immune elements over other, specific immunologic markers.

Keywords lupus glomerulonephritis anti-DNA

INTRODUCTION

The renal disease associated with systemic lupus erythematosus (SLE), as well as its treatment, is a significant source of morbidity and mortality in patients with SLE [1,2]. Nonetheless, despite several decades of research, the immunopathogenesis of lupus nephritis remains controversial and uncertain [3–6]. Although there is general agreement that lupus nephritis resembles experimental immune complex-mediated glomerulonephritis, and that anti-dsDNA antibodies are important for disease pathogenesis [4–6], it has been difficult to detect reliably the presence of DNA/anti-DNA immune complexes or to identify the composition of pathogenic immune complexes [3]. Moreover, the presence of anti-dsDNA antibodies appears to be neither necessary nor sufficient for the expression of lupus nephritis in either human lupus or murine models of the disease [7–11].

Several alternative pathogenic mechanisms for lupus nephritis have been proposed. To explain the absence of detectable circulating DNA/anti-DNA immune complexes, it has been suggested that anti-dsDNA antibodies may directly mediate renal disease either by binding to DNA which adheres to the glomerulus (planted antigen hypothesis) [12,13], or by cross-reacting with anionic constituents of the glomerular basement membrane (cross-reactive autoantibody hypothesis) [14–17]. Additionally, because renal disease can occur in the absence of anti-dsDNA antibodies, other autoantibodies directed against non-nuclear antigens (laminin, C1q, histones, RNA polymerase) have been postulated as pathogenic in nephritis [18–22]. Although there is experimental evidence supporting each of the above hypotheses, their relative contributions to lupus nephritis remain uncertain. A practical corollary to this

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uncertainty is that there is no single immunologic assay which can be relied upon as a sensitive and consistent marker for the presence of nephritis in SLE patients [1,2].

Taking a different approach to address these issues, we recently developed a solid-phase ELISA which uses whole glomeruli as the binding substrate [23]. This assay was designed to detect immune elements based upon a pathologic property (i.e. binding to glomeruli) as opposed to nuclear antigen binding or physical properties. We have previously demonstrated that the immune elements detected by this assay (termed glomerular binding activity (GBA)) are present in all models of murine lupus tested to date, and correlate strongly with the presence of nephritis [23,24]. Of clinical importance, the correlation between GBA and renal disease in cyclophosphamide-treated MRL/*lpr* mice was significantly stronger than that obtained with the anti-dsDNA assay [24]. Qualitatively, the GBA in MRL/*lpr* mice may be a mixture of immune elements; however, binding is substantially inhibited by DNase type I, indicating that DNA participates in the binding interaction [23].

In the current study, we applied this assay to human lupus, comparing it with several other parameters of the autoimmune response which have previously been reported to correlate with nephritis. We measured anti-dsDNA antibodies using the Farr technique, given that the high-avidity antibodies detected by this assay are felt to be the most nephritogenic [25]. Additionally, we assayed for the presence of immune complexes using the solid-phase C1q assay [20]. We chose this particular method because, in addition to measuring immune complexes, the salt-stable component of the C1q assay measures anti-C1q autoantibodies, which as noted above have been associated with nephritis [20,26–28]. The aim of this study was to determine the utility of several of the commonly used immunologic correlates of nephritis in predicting the presence of nephritis in patients with SLE, and to compare these assays with the alternative approach of using a tissue-based detection method.

PATIENTS AND METHODS

Patients

Blood was collected from 60 patients previously diagnosed with SLE by American College of Rheumatology (ACR) criteria [29] who were being followed in the Washington University Rheumatology clinic. The specimens were then stored at −70°C. Before performing the immunologic assays listed below, medical records were evaluated and patients were categorized into four groups according to clinical status at the time of specimen collection. Patients in group 1 had active nephritis confirmed either by renal biopsy or by criteria of the ACR (proteinuria $\geqslant$ 500 mg/day or $>3+$ on dipstick, or cellular casts) [29]. Group 2 patients had active non-renal SLE with no evidence of nephritis for at least 1 year prior to, and 1 year following the specimen collection date. Group 3 patients had inactive SLE, but a history of nephritis at least 1 year before specimen collection. Group 4 patients had inactive SLE and no previous history of nephritis. Three patients who had sera collected initially during inactive disease were restudied when their disease flared; both sets of values were used in the statistical analyses. Two additional patients had serial samples drawn during and following treatment of nephritis; only the initial values were included in the analysis.

GBA ELISA

The GBA ELISA, previously described in detail, is a solid-phase immunoassay which uses isolated permeablized whole rat glomeruli on a glassfibre membrane as substrate [23]. Goat anti-human IgG horseradish peroxidase (HRP) and goat anti-mouse IgG HRP (Biorad, Hercules, CA, and Sigma, St Louis, MO, respectively) served as secondary antibodies. In this study, all sera were assayed at a dilution of 1:100 in PBS with 10% goat serum. The resulting membranes were photographed, and the photographs scanned by laser densitometry (Pharmacia LKB Biotechnology, Piscataway, NJ). All samples were run in duplicate and averaged. Serum from healthy laboratory volunteers was used as a normal control and zero point for optical densitometry. A pool of MRL/*lpr* sera (from animals 24–26 weeks old) was used as the positive control (relative OD of 1·0). Values are expressed relative to positive and negative controls. Patients with nephritis and positive GBA assays were also assessed for sensitivity of binding to DNase treatment. Dilutions of sera were treated with DNase type I (Sigma) 50 μg/ml at 37°C for 60 min. The DNase-treated sera were run side-by-side with untreated sera on the GBA assay. The degree of DNase inhibition was then expressed as a percentage of the OD of the untreated sera.

To address the issue of tissue specificity, sera from patients with nephritis and positive GBA assays were preadsorbed to washed rat erythrocytes (5×10^7 cells/ml) for 3 h at 4°C. Adsorbed sera were then compared with non-adsorbed sera using the GBA assay.

Other immunoassays

The anti-DNA assay used in this study was a radioimmunoassay (RIA) purchased from Kodak Clinical Diagnostics (Amersham, UK), and performed according to the manufacturer's specifications. Values are expressed as units relative to the control reference sera included. The normal range is 0–7 U.

The C1q binding assay was performed as detailed by Uwatoko & Mannik [20]. C1q (Sigma), diluted to 20 μg/ml in 150 mM Tris buffer (TB) pH 7·6 , was coated on a polystyrene microtitre plate (50 μl/well) and incubated overnight at 4°C. The plate was then washed and blocked using TB + 10 mg/ml of bovine serum albumin (BSA) (TBB). Samples were diluted 1:100 in TBB and then added to the plate in duplicate and incubated overnight at 4°C. The plate was then washed and 50 μl/well of HRP-conjugated anti-human IgG $F(ab')_2$ (Sigma) and HRP-conjugated anti-mouse IgG $F(ab')_2$ (Sigma), each diluted 1:1000 in TBB, were added and allowed to incubate for 1 h at room temperature. The plate was then developed with *o*-phenylenediamine dihydrochloride (Sigma) as per manufacturer's instructions, and read with a microtitre plate reader (Dynotech Labs, Chantilly, VA) at 450 nm. Normal human sera served as negative control, and the MRL/*lpr* serum pool was used as positive control (0·5 OD).

High ionic strength has been shown to inhibit immune complex binding to C1q while having little effect on the binding of anti-C1q autoantibodies [20,26]. Thus, in order to detect anti-C1q antibodies, serum samples were diluted with 1 M NaCl in TBB during the binding step, and then processed as described above.

Statistical analysis

CLINFO data management system was used to perform

statistical analysis. Results of assays performed on stored sera were compared between the patient groups using Wilcoxon rank sum testing. Interrelationships between these assays were examined with regression analysis. For calculation of the sensitivity, specificity and positive predictive value for each assay, a positive value was defined as greater than the mean + 2 s.d. of the value among patients with inactive SLE (groups 3 and 4). A negative test was defined as any value within this range.

RESULTS

As shown in Fig. 1, patients with nephritis (group 1) displayed higher mean values for all four immunologic parameters than patients in the other three groups together. This was statistically significant for the GBA and the C1q binding assays ($P = 0{\cdot}001$ and 0·009, respectively) with trends noted for the Farr and anti-C1q assays ($P = 0{\cdot}14$ and 0·23, respectively). The GBA was the only assay in which the mean value was significantly higher in nephritis patients (group 1) than in those patients with active disease, but no nephritis (group 2) ($P < 0{\cdot}05$).

In addition to comparing the means in the four groups, we also assessed the predictive utility of each assay utilizing the above mentioned criteria for determining a negative (or positive) test. The purpose of this analysis was to determine the sensitivity, specificity and predictive value of each assay given a prior diagnosis of lupus and, as such, is a more stringent test of the predictive value of the assay. It is noteworthy that the negative ranges defined by this criterion (shown as the dashed lines in Fig. 1) were not the same as those defined by a group of normal controls. For example, with the Farr assay the range for normal controls is < 7 U, whereas the range defined by inactive lupus patients in our study was < 23 U (identical to the range for inactive lupus patients in data provided by the manufacturer).

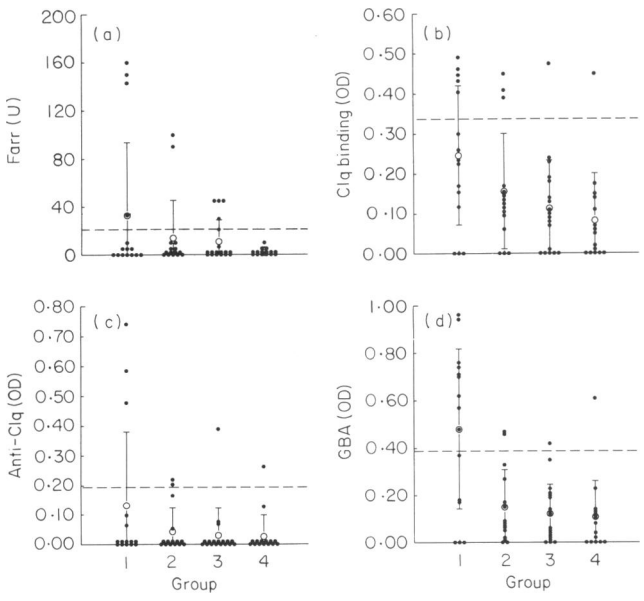

Fig. 1. Immunologic parameters in patients with systemic lupus erythematosus (SLE). Anti-DNA (Farr) (a), C1q (b), anti-C1q (c), and glomerular binding activity (GBA) (d) assays were performed on a cohort of SLE patients. ●, Values for individual patients divided into four clinical groups. Group 1 includes patients with active nephritis. Group 2 are patients with active lupus without nephritis. Group 3 are patients with inactive lupus but a history of nephritis. Group 4 are patients with inactive lupus and no history of nephritis. ○, Mean values for the group. Error bars represent 1 s.d. The dashed line is the mean for patients with inactive lupus (groups 3 and 4), plus 2 s.d.

Table 1. Diagnostic utility of immunologic parameters in detecting nephritis among patients with systemic lupus erythematosus (SLE)

	Sensitivity	Specificity	Positive predictive value
GBA	0·60	0·92	0·70
Anti-DNA (Farr)	0·20	0·88	0·38
C1q binding	0·33	0·90	0·50
Anti-C1q	0·20	0·92	0·43

A positive test was defined as any value greater than 2 s.d. above the mean for patients with inactive lupus (groups 3 and 4).

Using this criterion, we found that all of the assay systems were relatively specific for the diagnosis of lupus nephritis (Table 1). However, the anti-dsDNA and both C1q binding assays were relatively insensitive. Only 4/15, 5/15 and 3/15 nephritis patients were positive in the anti-dsDNA, solid-phase C1q binding, and anti-C1q assays, respectively. The GBA was the only assay which was reasonably sensitive (positive in 9/15 nephritis patients) and had a useful positive predictive value.

In order to understand the interrelationships between the various immunologic assays, we examined the correlations between the assays by regression analysis (Table 2). Two facets of this analysis were notable. First, the C1q binding assays and the anti-dsDNA assay appeared to be independent of one another. Second, the GBA assay was moderately well correlated with all of the other assays. The lack of complete correlation of the GBA with any of the other assays suggests that the GBA is not simply a more sensitive version of one of the other immunologic assays.

Individual assay values and clinical data for patients in group 1 are presented in Tables 3 and 4. Only one of the six nephritis patients with a negative GBA was positive in any of the other assays, demonstrating that the immunologic assays other than the GBA did not provide additional information to that already provided by the GBA. The increased sensitivity of the GBA over the other immunologic assays also did not seem to be simply the ability to detect more modest degrees of nephritis. All patients in group 1 had either clinical or histo-

Table 2. Correlations of immunologic parameters using regression analysis

	GBA	Anti-DNA (Farr)	C1q binding
Anti-DNA (Farr)	0·47*		
C1q binding	0·35**	0·08	
Anti-C1q	0·43	−0·08	0·53*

Data are expressed as r values. *$P < 0{\cdot}0003$; **$P < 0{\cdot}005$.

Table 3. Immunologic characteristics for patients in group 1 (active nephritis)

Patient	Anti-DNA Farr (20·8)	C1q binding (0·33)	Anti-C1q (0·19)	GBA (0·39)	DNase sensitivity (% inhibition)
1	0	0·49	0·48	0·76	46
2	0	0·17	0	0·70	41
3	2	0·46	0·74	0·96	12
4	33	0·23	0·10	0·74	2
5	0	0·30	0	0	–
6	143	0·43	0	0·94	0
7	0	0·26	0	0	–
8	0	0·12	0	0·48	14
9	4·8	0	0	0	–
10	0	0·45	0·59	0·62	81
11	153	0	0	0·71	66
12	2	0·40	0·06	0·18	–
13	2	0·15	0	0·37	–
14	0	0	0	0·17	–
15	152	0·23	0	0·57	56

Numbers in parentheses represent the cutoff between a negative and positive test as defined by the mean + 2 s.d. for patients with inactive lupus. DNase sensitivity represents percentage inhibition of GBA-positive sera when treated with DNase type 1.

logic evidence of substantial renal involvement, which required treatment with either prednisone or cytotoxic agents (Table 4).

It is noteworthy that many of the patients in group 1 had already received some treatment at the time of sample procurement (Table 4). Five of the six patients with false negative GBA tests were receiving prednisone at a dose $\geqslant$ 20 mg/day at the time of sample acquisition (patients 5, 7, 9, 12 and 14; dose of prednisone 44 $\pm$ 33 mg, mean $\pm$ s.d.); two had received cyclophosphamide (patients 5 and 7), and one had been apheresed twice 1 week before sample procurement (patient 7). The only patient with a false negative GBA who was not receiving treatment (patient 13) had a borderline GBA of 0·37 OD. By comparison, only four of nine patients with true positive GBA were receiving prednisone $\geqslant$ 20 mg/day (mean dose for the nine patients $\pm$ s.d., 22·5 $\pm$ 25 mg). In addition, no true positive patients were receiving cyclophosphamide or had been apheresed.

Three patients had sera available both before and during a

Table 4. Clinical characteristics for patients in group 1 (active nephritis)

Patient	Creatinine (mg/ml)	Creatinine clearance (mg/min)	Cellular casts	Urine prt (mg/day)	Renal biopsy	Treatment
1	1·8	NA	+	4+*	NA	S
2	1·1	55	+	5400	NA	S
3	1·6	65	+	4700	IV	None
4	0·5	NA	+	NA	V	None
5	2·7	65	–	22 500	IV	S,C
6	0·9	NA	+	NA	II	None
7	8·6	NA	NA	NA	IV	S,C,P
8	1·5	NA	–	4+*	NA	S
9	2·4	52	NA	5000	NA	S
10	2·2	72	NA	9100	IV	None
11	5·0	10	NA	7170	NA	None
12	1·5	92	+	NA	IV	S
13	0·7	NA	NA	1720	II	None
14	0·8	131	+	2900	NA	S
15	2·2	43	NA	1400	NA	S

NA, Not available. *Protein concentration by urine dipstick. Renal biopsy data given by WHO classification. S, Treatment with steroids greater than 20 mg of prednisone/day; C, treatment with cytotoxic agents; P, patient underwent apheresis.

lupus flare. One patient went from group 4 to group 1 and, while her anti-DNA activity remained negative (0 U), her GBA became strongly positive (changing from 0 to 0·76 OD). Two patients went from group 4 to group 2. In both instances the anti-DNA activities and GBA remained negative through disease flares. Two patients had sera available both at the time nephritis was first diagnosed and at a point when renal disease was no longer clinically apparent. In one patient who went from group 1 to 2, the GBA fell from 0·74 to 0·18 OD, while the anti-DNA also fell from 33 to 0 U. In another patient who went from group 1 to 3, both the GBA and anti-DNA remained negative.

Because our previous studies in proteinuric MRL/*lpr* mice indicated that GBA binding is inhibited with DNase [23], we examined the effect of DNase I on the sera of GBA-positive SLE patients with nephritis (Table 3). In contrast to the mouse model, DNase inhibition of glomerular binding varied widely among individual patients, ranging between minimal (< 15%) and nearly complete (81%). Also to investigate the tissue specificity of the GBA, we preadsorbed sera from nephritis patients with positive GBA to rat erythrocytes before running the assay. Preadsorption to erythrocytes did not diminish GBA (data not shown).

DISCUSSION

We believe that these data provide evidence to support the contention that the pathogenesis of human lupus nephritis is immunologically complex, in other words that multiple immune elements and mechanisms may contribute to glomerular inflammation. This conclusion is based on several observations from the current study. First, no one single immunologic parameter was consistently elevated in all patients with active nephritis. Second, it appeared that the anti-dsDNA assay and the C1q assay, which were seemingly independent of one another, were nonetheless both correlated with the presence of nephritis. Finally, we found that the qualitative nature of the GBA varied significantly among patients with nephritis. Some exhibited a GBA which was strongly inhibited by DNase (similar to that from MRL/*lpr* mice [23]), whereas others, unlike MRL/*lpr* mice, were relatively DNase-resistant. We believe that these data indicate substantial heterogeneity in the cause of lupus nephritis from patient to patient, and may thus provide an explanation for the difficulty in developing a consensus on the pathophysiologic trigger for lupus nephritis.

While it is clear that the anti-dsDNA response is in some patients associated with the presence of nephritis (an assertion also supported by our observations), it is equally clear that this association is not precise. Immunogenetic and pharmacologic studies in murine lupus show that the anti-dsDNA response alone, although correlated with nephritis, is neither necessary nor sufficient for the development of nephritis [7–9]. Additionally, numerous studies show that not all patients with anti-dsDNA antibodies manifest nephritis, nor do all nephritis patients exhibit anti-dsDNA antibodies [10,11].

Part of this lack of correlation between the anti-DNA responses and nephritis may relate to the fact that only some anti-DNA antibodies may be nephritogenic (e.g. the subset with 16/6 idiotype [30,31]). However, in our study the major shortcoming of the anti-dsDNA assay as a predictor of nephritis was a lack of sensitivity (when inactive SLE patients were used as controls) rather than a lack of specificity. This lack of sensitivity may have several different explanations. It is possible that our findings were due in part to the insensitivity of the Farr assay in detecting anti-dsDNA antibodies [32]. However, when we used anti-dsDNA ELISAs in parallel studies the sensitivity was only marginally better, and we could not distinguish patients with nephritis from those with active non-renal disease as we had done using the GBA assay (i.e. differences between groups 1 and 2 were not significant) [33]. It is also possible that the absence of detectable anti-dsDNA antibodies in nephritis is due to the lability of these antibodies to treatment [34], a decline in antibody titres just before onset of renal disease [35], the formation of immune complexes in circulation [7], or the concentration of low levels of antibody in the kidney [36]. Since our study was retrospective, many specimens were procured on treated patients or at the onset of renal disease (rather than before), and thus the relatively low incidence of anti-dsDNA antibodies in our cohort may reflect these problems. However, even if one assumes that the reason for the low incidence of anti-dsDNA antibodies in nephritis patients is a function of these factors, it still represents a substantial problem with this assay in terms of its clinical utility.

In the light of our current observations, we would propose another explanation for the lack of an association between the anti-dsDNA response and nephritis in certain patients: the multifactorial nature of the disease. In these patients, other pathogenic mechanisms such as anti-DNA/DNA immune complexes [37], anti-ssDNA antibodies [38,39], anti-C1q autoantibodies [20,26–28], anti-RNA polymerase antibodies [21], anti-histone [18,22], or anti-laminin antibodies [18,19] may be operative. Moreover, anti-DNA antibodies may bind to the glomerulus in multiple ways and with differing avidities, further complicating the relationship between quantitative anti-DNA measurements and nephritis [13]. Apropos of these issues, we have recently observed that the GBA of MRL/*lpr* mice may contain both anti-DNA antibodies and anti-nucleosomal antibodies which bind to the glomerulus via DNA/histones adsorbed to components of the glomerular basement membrane (particularly collagen) ([40,41] and Di Valerio *et al.*, unpublished observations).

If one accepts this conclusion, it then becomes clear why assays of individual immunologic parameters may be limited in their sensitivity for the presence of nephritis. Since there may be multiple pathogenic immune elements which may bind in multiple ways, no one immunologic test is ever likely to be an absolutely sensitive predictor of nephritis. Rather, a more integrated approach to detecting nephritogenic immune elements, such as the tissue-based GBA assay or some variant thereof, may be a more useful means to diagnose lupus nephritis than any single immunologic assay.

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