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Serum glomerular binding activity is highly correlated with renal disease in MRL/*lpr* mice

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SUMMARY

The pathogenesis of lupus nephritis is felt to be mediated by anti-DNA antibodies. However, the anti-DNA response and renal disease do not entirely correspond. We recently developed a new assay which detects immune elements based on their ability to bind glomeruli as an alternative approach to understanding the pathogenesis of this disorder. The glomerular binding activity (GBA) defined by this assay consists of immune elements containing IgG which interact specifically with renal tissue, the binding of which is DNase-inhibitable, but which do not bind to DNA directly. In the current study we assessed the relationship between GBA and renal disease in MRL/*lpr* mice (both untreated and cyclophosphamide-treated) and compared it with the anti-DNA assay. Both assays were highly correlated with renal disease in untreated mice in terms of proteinuria. In cyclophosphamide-treated mice, however, only a weak correlation between the anti-DNA assay and proteinuria was apparent. GBA, in contrast, was more strongly correlated with proteinuria in treated mice. This correlation improved substantially when the DNase-sensitive component of the GBA was used. GBA appeared related to, but not covariant with, the anti-DNA response. These results demonstrate that GBA is a better correlate of murine lupus nephritis than the anti-DNA assay, and suggest that the immune elements detected by this assay, the DNase-sensitive component in particular, may be pathogenically important.

Keywords nephritis anti-DNA antibody immune complex lupus

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune illness which is manifest as multi-organ dysfunction resulting from autoantibody production [1–3]. The renal component is particularly serious and represents the major determinant of mortality in murine lupus [3], and a major source of morbidity and mortality in humans [1]. The pathogenesis of the glomerular inflammation is not completely understood; however, anti-DNA antibodies are thought to play a critical role. Evidence for this hypothesis includes a correlation between serum levels of anti-DNA antibodies and renal disease in mice and humans, the recovery of concentrated anti-DNA antibody from nephritic kidneys, instigation of renal inflammation with injection of anti-DNA MoAbs into normal mice, acceleration of renal disease in murine lupus through administration of exogenous DNA, induction of nephritis by immunization of normal mice with bacterial DNA, and the development of nephritis in mice transgenic for anti-DNA IgG [2–11].

However, it is clear that the presence of detectable anti-DNA antibodies is not always correlated with nephritis. Anti-DNA antibodies are found in several murine strains, as well as in humans, without renal disease ensuing [5,12,13]. In addition, not all nephritis can be attributed to the presence of anti-DNA antibodies. Examples exist in human disease and murine lupus where nephritis occurs without detectable levels of anti-DNA antibodies [5,13]. Furthermore, models of induced lupus nephritis (i.e. bacterial DNA immunization [11], injection of anti-DNA MoAbs [7], and mice transgenic for anti-DNA antibodies [10]) typically develop attenuated forms of the disease. It thus appears that the relationship between the anti-DNA response and nephritis is a complex one.

This lack of an exact correlation between the anti-DNA response and lupus nephritis prompted us to develop an ELISA which uses freshly isolated rat glomeruli as the substrate [14]. The rationale was to detect immune elements based on their ability to bind to a target tissue of interest, rather than to a nuclear antigen. As we have recently reported, the presence of glomerular binding activity (GBA) as defined by this assay is common to several models of murine lupus. Characterization of the binding activity suggests that it consists of a mixture of immune elements, with IgG as the exclusive immunoglobulin component, which binds specifically to renal tissue. GBA does not adsorb to agarose-DNA (although anti-DNA antibodies do), thus indicating that this assay is distinct from the anti-DNA

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assay. GBA, however, is inhibited by DNase, indicating the possible presence of DNA–anti-DNA immune complexes.

Based on these studies, we conjectured that serum GBA should be highly correlated with the renal lesion in lupus nephritis. In the present study we thus sought to probe the relationship between GBA and renal disease (in terms of proteinuria) in MRL/*lpr* mice. Secondarily, we also sought to compare GBA to the anti-DNA response. In order to test more stringently correlations between the assays and nephritis, we treated animals with cyclophosphamide, an immunosuppressive agent which has been shown to decrease autoantibody formation, prevent immune deposits in the glomerulus and thereby slow the progression of renal disease and decrease mortality [15–17].

MATERIALS AND METHODS

General experimental design

Two groups of 20 MRL/*lpr* female mice were obtained from Jackson Laboratories (Bar Harbor, ME) at approximately 4 weeks old. One group served as the untreated control. The second received cyclophosphamide (Mead Johnson, Evansville, IN), 25 mg/kg intraperitoneally twice each week starting at 6 weeks of age. Urine and serum samples were acquired at 4 week intervals from each animal. Urine protein concentration was assessed using urine dipsticks (Uristix; Miles Inc, Elkhart, IN). Serum samples were used in the GBA and anti-DNA assays detailed below.

GBA ELISA

The GBA ELISA is a solid-phase immunoassay using a peroxidase-linked secondary antibody that is able to detect IgG binding to glomeruli [14]. In this study we screened for positivity in individual animals at a dilution of 1:1000. Because of limited amounts of sera available from each animal at a given point in time, more quantitative information was obtained using pooled samples of serum. The GBA assay was run with pooled serum samples at dilutions of 1:100, 1:1000, and 1:10000. The resulting membranes were photographed, and the photographs scanned by laser densitometry (Pharmacia LKB Biotechnology, Piscataway, NJ). Relative OD ranged from zero (determined by negative control, see below) to a maximum of 1 (determined by positive control, see below). The relative ODs for the 1:100, 1:1000 and 1:10000 dilutions were multiplied by 1, 10 or 100, respectively. All samples were run in duplicate and averaged. Serum from BALB/c mice (Jackson Laboratories) was used as the negative control and the arbitrary zero point for optical densitometry. Known positive MRL/*lpr* sera from proteinuric animals (at a 1:100 dilution) served as a consistent positive control (OD set at 1·0).

As a further manipulation, dilutions of serum were treated with DNase I (Sigma, St Louis, MO), 50 U/ml at 37°C for 60 min. The DNase-treated sera were run side-by-side with untreated sera on the GBA assay. Differences in relative OD between the DNase-treated and the untreated sera were then calculated using the data obtained by laser densitometry (i.e. OD in untreated serum − OD in DNase-treated serum, referred to as DNase-sensitive component).

Anti-DNA ELISA

The anti-DNA assay was performed using a published ELISA protocol [18]. Lambda phage double-stranded DNA (Gibco/BRL, Gaithersburg, MD) 0·33 mg/well coated on poly-L-lysine (10 μg/ml in H_2O; Sigma) was used as the substrate. BALB/c sera was used as negative control. An anti-DNA MoAb (MAB030, Chemicon International Inc., Temecula, CA) served as positive control (OD = 1·0 at a 1:100 dilution).

Statistical analysis

For proteinuria and the anti-DNA assays, data are presented as means ± s.e.m. For the GBA assay, the data are presented as either per cent of the colony positive at a dilution of 1:1000 at each time point, or as an OD measurement using pooled serum from each time point. STATA (Computing Resource Centre, Los Angeles, CA) was used to perform statistical analysis. Comparisons between means were performed using Student's *t*-test; and comparisons between frequencies using χ^2-test. Correlations were performed using both standard linear regression (least squares method) and non-parametric regression (Spearman's rank correlation and Kendall's τ-test). As the non-parametric tests gave results virtually identical to linear regression, only the latter are presented for clarity.

RESULTS

*Clinical parameters of disease in MRL/*lpr *mice*

Proteinuria and mortality were followed in both untreated and cyclophosphamide-treated MRL/*lpr* mice as clinical parameters of disease activity and progression (Fig. 1). The untreated animals exhibited a steady rise in proteinuria between 12 and 20 weeks of age. Thereafter, proteinuria stabilized at concentration 10 mg/ml. Mortality was apparent by 24 weeks, and reached 30% by 28 weeks (Fig. 1). In cyclophosphamide-treated animals, the onset of proteinuria was somewhat accelerated and peaked at approximately 5 mg/ml at 12 weeks (Fig. 1).

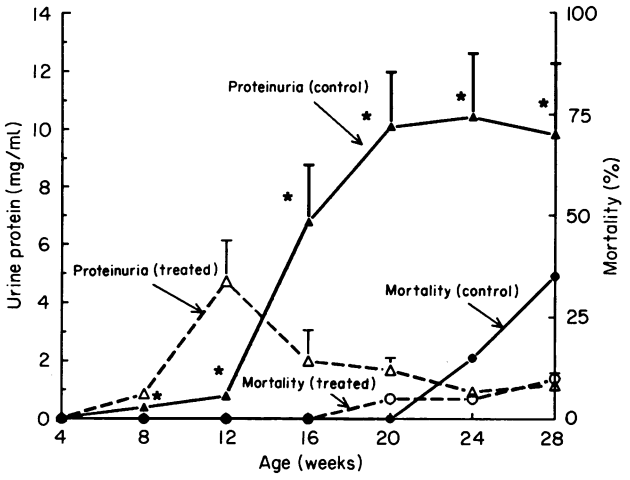

Fig. 1. Clinical parameters of disease in MRL/*lpr* mice as a function of age. Two colonies of 20 MRL/*lpr* mice were followed up to 28 weeks of age. Solid symbols/solid lines and open symbols/dashed lines represent untreated and cyclophosphamide-treated mice, respectively. Triangles represent proteinuria and circles represent mortality. * Significant differences between the two colonies ($P < 0.05$). $P = 0.07$ for the difference in mortality between the two colonies at 28 weeks.

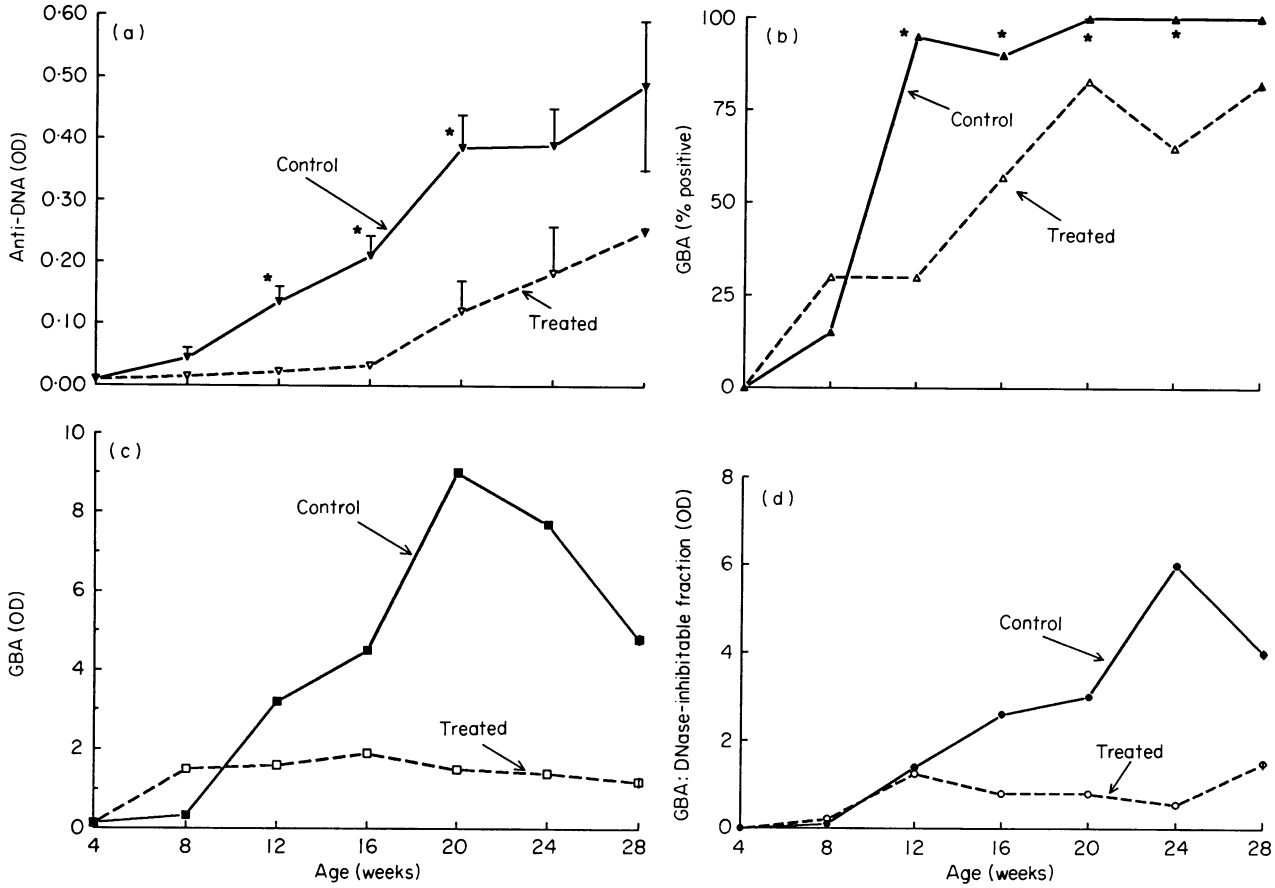

Fig. 2. Immunologic parameters of disease in MRL/*lpr* mice as a function of age. Anti-DNA activity and glomerular binding activity (GBA) were assessed in MRL/*lpr* mice at the ages indicated in both untreated (solid triangles/solid line) and cyclophosphamide-treated groups (open triangles/dashed line). * Significant differences between the two colonies ($P < 0{\cdot}05$). (a) Anti-DNA activity. $P = 0{\cdot}07$ and 0·08 for comparisons at 24 and 28 weeks, respectively. (b) GBA as a percentage of animals positive at a serum dilution of 1:1000. $P = 0{\cdot}11$ for the comparison at 28 weeks. (c) GBA as quantified by optical densitometry using pooled sera. (d) DNase-inhibitable component of GBA as quantified by optical densitometry using pooled sera.

Proteinuria then declined to approximately 1 mg/ml. Overall, mortality was diminished in cyclophosphamide-treated mice, reaching only 10% by 28 weeks.

Immunologic parameters of disease in MRL/lpr *mice*

Anti-DNA activity was undetectable in 4-week-old MRL/*lpr* mice ($\leqslant 0{\cdot}01$ OD units, identical to BALB/c mice). Activity then rose in a time-dependent fashion in the untreated animals through 28 weeks (Fig. 2a). In the cyclophosphamide-treated animals, the anti-DNA level remained nearly completely suppressed through 16 weeks, and then rose to levels approximately 50% of those in the untreated group.

GBA was assessed initially by screening individual animals at a titre of 1:1000, since we have previously found that all MRL/*lpr* animals are positive at a titre of 1:100 from 4 weeks of age and on [14]. None of the 4-week-old animals had detectable binding at a 1:1000 dilution; however, the number of animals with titres $\geqslant$ 1:1000 rose rapidly between 8 and 12 weeks of age, and became 100% by 20 weeks (Fig. 2b). Analysis of the OD of pooled sera total binding gave similar results (Fig. 2c). Discernible GBA was present in 4-week-old animals, but not in BALB/c controls (0·14 *versus* 0·00 OD units, respectively). GBA then rose rapidly between 8 and 20 weeks, declining thereafter. Although pooled sera were used in these assays, variability among individual animals at each time point was assessed separately on cohorts at 8 and 20 weeks ($n = 10$). The s.e.m. for these groups was $< 15\%$ of the mean OD. When the DNase-sensitive component of the GBA was examined (Fig. 2d), no or minimal activity was detectable before 8 weeks. After 8 weeks, however, the DNase-sensitive component rose steadily, peaking at 24 weeks.

With cyclophosphamide treatment, the incidence of animals with a positive GBA and the quantitative GBA were both suppressed. The number of animals with GBA titres $\geqslant$ 1:1000 exceeded those in the control group at 8 weeks, but then rose at a more gradual rate before stabilizing between 65% and 80% after 20 weeks (Fig. 2b). In the quantitative assessment, a rise in the GBA of the treated animals over the untreated group was detectable at 8 weeks (Fig. 2c). After 8 weeks though, activity remained flat at approximately the same level. The DNase-sensitive portion of GBA in the treated animals was comparable to the untreated group up to 12 weeks (Fig. 2d). After 12 weeks, however, the DNase-sensitive component in the treated animals fell, reaching a nadir at 24 weeks.

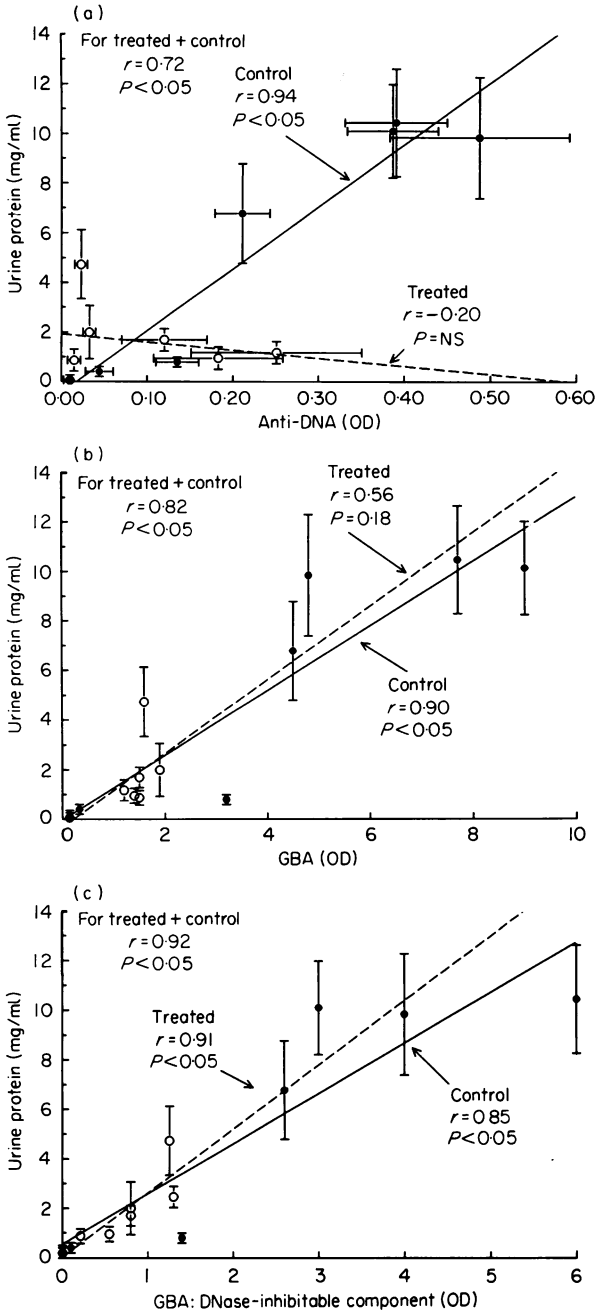

Fig. 3. Correlations between the immunologic assays and proteinuria in MRL/*lpr* mice. Regression analysis between the immunologic assays and proteinuria in MRL mice was performed in the entire cohort and in the untreated (closed circles, solid line) and treated subgroups (open circles, dashed line). Overall correlations are indicated in the insets. (a) Mean serum anti-DNA activity. (b) Mean serum glomerular binding activity (GBA) *versus* proteinuria. (c) Mean serum GBA (DNase-inhibitable component) *versus* proteinuria.

Correlations between the immunological assays and proteinuria

We correlated the immunologic parameters with proteinuria in order to examine the relationship of each assay with renal disease (Fig. 3). Overall, using data from both treated and untreated mice, the correlations between the assays and proteinuria were high, although the correlation with anti-DNA was lowest and that with the DNase-sensitive component of GBA was the best (insets, Figs 3a, b and c). Analysing the data from the untreated animals separately, the correlations of anti-DNA and GBA with proteinuria were also strong and relatively comparable (Fig. 3). In the cyclophosphamide-treated group, however, no correlation between the anti-DNA assay and proteinuria was apparent (Fig. 3a). GBA, in contrast, still positively correlated with proteinuria, although less well than with the untreated group (Fig. 3b). The DNase-sensitive component of the GBA, however, continued to correlate well with proteinuria (Fig. 3c).

The lack of correlation between the anti-DNA assay and proteinuria was not primarily due to the earlier onset of proteinuria in the treated group. When data from week 12 were removed from the regression analysis, the correlation between anti-DNA activity and proteinuria remained weak, although it was statistically significant ($r=0{\cdot}18$, $P<0{\cdot}05$). The correlation between the GBA and proteinuria was improved by this alternative analysis ($r=0{\cdot}88$ and 0·96 for the GBA and DNase-sensitive component of GBA, respectively; $P<0{\cdot}05$). It was not the case that the high anti-DNA activity in the treated animals presaged the later development of renal disease. Proteinuria and mortality in this group remained unchanged up to 43 weeks (data not shown).

The data were also analysed to determine if the assays were related to one another. By linear regression, GBA correlated well with the anti-DNA assay: $r=0{\cdot}79$ overall and $r=0{\cdot}85$ for the DNase-inhibitable component ($P<0{\cdot}05$ for both). Similar correlations were seen in analysing the untreated subgroup only: $r=0{\cdot}84$ for GBA and $r=0{\cdot}88$ for the DNase-inhibitable component ($P<0{\cdot}05$ for both). In the treated animals GBA did not correlate with the anti-DNA assay: $r=0{\cdot}06$ overall and $r=0{\cdot}52$ for the DNase-inhibitable component ($P=$ NS for both). We additionally employed multivariate linear regression to determine if the information contained in the two assays was complementary and would achieve a higher correlation with proteinuria when combined. However, utilizing the data from both assays together, the correlation with proteinuria in all the animals (or the two subgroups) did not improve upon those obtained by using GBA alone (data not shown).

DISCUSSION

Lupus nephritis is felt to be mediated by humoral immune elements which bind to or deposit in glomeruli. Anti-DNA antibodies are certainly important to disease pathogenesis; however, data clearly suggest that the presence of circulating anti-DNA antibodies may be neither necessary nor sufficient for the development of lupus nephritis [2–8,12,13]. This lack of correspondence prompted our recent development of the GBA assay, which detects immune elements based on their ability to bind to the end organ of interest, and to compare it with the anti-DNA assay as a correlate of renal disease in the MRL/*lpr* mouse. As detailed above, we found that both assays correlated with renal disease (in terms of proteinuria) in unmanipulated animals. However, when we treated mice with cyclophosphamide to attenuate the autoantibody response, and thereby reduce renal injury, we found a lack of correspondence between the anti-DNA activity and renal disease, whereas the GBA retained its correlation with renal disease.

The lack of correspondence between the anti-DNA assay and renal disease that we observed in the treated animals was

due primarily to substantial increases in levels of anti-DNA activity without the development of renal disease. The weak association between the anti-DNA assay and renal disease that we observed is not unique. Similar results were obtained in studies using NZB/NZW mice, in which cyclophosphamide treatment completely suppressed proteinuria, but only decreased anti-DNA antibodies by 50% [17]. Immunogenetic studies of murine lupus have also shown that the presence of anti-DNA antibodies is not invariably associated with renal disease [12,13]. The explanation behind these observations may be that not all anti-DNA antibodies are equally nephritogenic [7,19].

It is worth noting that suppression of renal disease in murine lupus in the absence of significant effect on anti-DNA antibodies has been observed with other immunosuppressive therapies such as irradiation [20] or cyclosporin A [15]. These observations are probably attributable to the effects of these therapies on other aspects of the inflammatory response subsequent to autoantibody formation. For example, irradiation appears to inhibit macrophage influx into glomeruli in glomerulosclerosis [21]. However, cyclophosphamide's efficacy is clearly associated with a decrease in autoantibody formation and immune element deposition in the kidney [17]. Thus, the dissociation of the anti-DNA response and renal disease that we and others have observed more probably calls into question the relationship between these two disease parameters.

The lack of correspondence between the anti-DNA titre and renal disease that we observed was to a minor degree due to the development of proteinuria in the treated animals at a time when anti-DNA antibodies were not evident. The occurrence of renal disease in the absence of anti-DNA antibodies is also not without precedent. Immunogenetic studies of (NZB × NZW)F_1 backcrosses show that certain lines develop nephritis without manifesting anti-DNA antibodies [13].

In contrast to the results obtained with the anti-DNA assay, the current study shows that the GBA assay correlates closely with renal disease in MRL/*lpr* mice, especially the DNase-sensitive component of the GBA. The apparent advantage of the GBA assay over the anti-DNA assay may be that it detects immune elements based on a presumed pathologic property (i.e. their ability to bind to the glomerulus) rather than ability to simply bind to DNA. Such elements would hypothetically be more likely to be pathogenically important, in that they would be more likely to deposit in glomeruli *in vivo*. Moreover, the GBA assay, in particular, is not restricted to detecting only one type of immune element, but should be capable of detecting all immune complexes or autoantibodies with a propensity to bind to glomerular tissue. The current study suggests that these hypothetical advantages may be, in fact, real ones.

What the GBA assay is detecting has yet to be established. The assay does not appear to detect anti-DNA antibodies directly, in that GBA is not removed from serum by adsorption to DNA-agarose, although anti-DNA antibodies are [14]. The current study supports the conclusion that the assays are measuring different immune elements, in that the GBA and anti-DNA assays were not covariant. A lack of correspondence between the two assays was particularly evident in the treated subgroup of animals. However, the assays are clearly related to one another, as evidenced by their overall correlation in the current study. A relationship between these two immunologic parameters is also supported by more recent data which show GBA in some (though not all) monoclonal anti-DNA lines (Bernstein *et al.*, unpublished observations) and the presence of GBA in the serum and kidneys of DNA-immunized mice [11].

It remains to be determined, however, what this relationship is. The DNase sensitivity of the GBA that we observed previously, and in the current study, suggests that these immune elements may be DNA–anti-DNA complexes [14]. The existence of such complexes has been a subject of debate over the years and remains unresolved [22–24]. Part of the problem may be the intrinsic stability of such complexes to measures which typically dissociate immune complexes [25]. An alternative explanation of the DNase sensitivity of the GBA is that the GBA detects a subset of anti-DNA antibodies which bind to DNase [26]. We feel that this is a less likely explanation, in that the GBA is also diminished by exposure to micrococcal nuclease but not S_1 nuclease [27].

The pathogenic potential of the GBA in lupus nephritis also remains to be conclusively established. Although this study, and our previous one, show a strong correlation between GBA and renal disease, they do not establish a causal relation. Moreover, aspects of GBA binding to glomeruli remain to be clarified. The role of factors such as charge or the participation of locally produced inflammatory mediators, which are felt to potentially mediate immune complex deposition in glomeruli [28], remains to be clarified.

The high degree of correlation between the GBA and renal disease in both untreated and treated mice raises the issue as to whether this assay may be useful to detect and follow renal disease in patients. We have previously reported that GBA is detectable in the serum of patients with SLE, and that this activity appears to be qualitatively similar to that found in the MRL/*lpr* mouse (e.g. both are DNase-inhibitable) [29]. In these preliminary studies, the three patients who were markedly positive for GBA (i.e. relative OD > 1) all had active renal disease. Notably, one of these patients was negative for anti-DNA antibodies. We are currently expanding upon these preliminary observations in order to determine the potential clinical utility of our assay, both in terms of its diagnostic significance as well as its utility as a disease marker during therapy.

In sum, the GBA assay appeared to be strongly correlated to the presence of renal disease in MRL/*lpr* mice, and superior to the anti-DNA assay as a marker of renal disease when mice were treated with cyclophosphamide. The explanation behind this apparent advantage may be that the GBA assay detects immune elements based on their pathogenic potential. The DNase-sensitive component of this assay (which may represent DNA–anti-DNA complexes), in particular, correlated strongly with renal disease in both untreated and treated mice. This close correlation suggests a possible causal relationship. These observations (and our preliminary studies in patients) suggest that this test may be useful in the clinical assessment of SLE, and may help in clarifying the pathogenesis of the human disorder.

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