Multiple Autoantibodies Form the Glomerular Immune Deposits in Patients with Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To determine the specific antibodies present in glomerular immune deposits in patients with systemic lupus erythematosus (SLE).

Methods. Kidney tissue was obtained at autopsy and stored frozen until used. Glomeruli were isolated from the renal cortex, sonicated, and the glomerular basement membrane fragments were extracted with a pH 2.5 buffer or 6 M guanidine hydrochloride. The latter method provided a higher yield and was used in most of the 23 specimens studied. The extracted IgG was quantified by a capture assay using an ELISAwith chemiluminescence. IgG antibodies to 14 different antigens were quantified by the same type of assay. The enrichment of antibodies in the extracts was determined in comparison to the initial supernatant of glomeruli that served as a serum surrogate.

Results. Antibodies to dsDNA, the collagen-like region of C1q, Sm, SSA, SSB, and chromatin were enriched in glomerular extracts, mainly from patients with proliferative lupus glomerulonephritis. The IgG binding to histones resulted from the presence of immune or non-immune aggregates of IgG. In some specimens all 6 of the above-listed antibodies were enriched. In one specimen antibodies to myeloperoxidase were enriched. Antibodies to cathepsin G, lactoferrin, and ß$_2$-glycoprotein I were not detected. Antibodies to Epstein-Barr viral capsid antigen and nuclear antigen 1 and antibodies to tetanus toxoid were detected in the serum surrogate, but were not enriched in extracts of glomerular basement membrane fragments.

Conclusion. Autoantibodies with multiple different specificities form the immune deposits in glomeruli of patients with SLE, including antibodies to dsDNA, Sm, SSA, SSB, the collagen-like region of C1q, and chromatin. (J Rheumatol 2003;30:1495–504)

Key Indexing Terms:
HUMAN SYSTEMIC LUPUS ERYTHEMATOSUS ANTIBODIES KIDNEYS

Renal disease is a major cause of morbidity and significant mortality in patients with systemic lupus erythematosus (SLE). About 50% of patients with this disease have clinical evidence of kidney involvement, manifested by proteinuria and abnormalities of urinary sediment[1,2]. Renal biopsies, however, have shown the presence of immune deposits in renal glomeruli of patients with SLE without proteinuria and without abnormalities of the urinary sediment. The formation of glomerular immune deposits is a major event that initiates glomerular injury and the subsequent loss of renal function. To understand the antigen-antibody systems involved in the formation of glomerular immune deposits in patients with SLE, several reports have described the recovery of specific antibodies from the glomeruli of patients with this disease. The seminal studies of Koffler, Schur, and Kunkel and of Krishnan and Kaplan identified antibodies to dsDNA, ssDNA, and nucleoproteins in extracts of glomeruli from postmortem kidney tissue of patients with SLE[3-5]. Since then, antibodies to the cytoplasmic antigen Ro/SSA were shown to be enriched in kidney tissue of 2 patients with SLE, and subsequently in one of these 2 specimens also antibodies to ribosomal P protein were enriched[6,7]. In another report antibodies to SSA, SSB, and ssDNAwere enriched in low pH extracts of 2 samples of kidney tissue; the 3 mg specimens were obtained by biopsy from patients with proliferative lupus glomerulonephritis[8]. Further, rheumatoid factors were recovered from extracts of glomeruli from some patients with SLE[9]. We reported the enrichment of antibodies to the collagen-like region of C1q in extracts of glomerular basement membrane (GBM) fragments in 4 of 12 patients with SLE[10]. In addition, antibodies to human myeloperoxidase (MPO) were enriched in the extracts of GBM fragments in one of 19 kidneys from patients with SLE[11].

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Since the early studies of antibodies recovered from kidney tissue from patients with SLE, several specific antibodies to nuclear antigens have been identified. Further, the methods for detection and quantitation of recovered antibodies have improved. Therefore, we examined the presence of 14 specific antibodies in the IgG extracted from GBM fragments prepared from postmortem kidney specimens from patients with SLE. The goal of these investigations was to determine if autoantibodies with multiple different specificities form the immune deposits in renal glomeruli of patients with SLE.

MATERIALS AND METHODS

Preparation and extraction of GBM fragments. Renal tissue was obtained at autopsy from 25 patients with the diagnosis of SLE, regardless of the cause of death. The acquisition of kidney specimens at autopsy was reviewed and approved by the Institutional Review Board. The kidney specimens were collected from May 1991 through March 2000 from the University of Washington Medical Center and other institutions, and shipped to us frozen on dry ice. The specimens were labeled K1 through K25 and stored at –20°C in aliquots, submerged in 0.1 M phosphate, 0.15 M NaCl, pH 7.2 (phosphate buffered saline, PBS), containing a mixture of protease inhibitors (PBS-I)[10]. K11 was not studied because the renal cortex was destroyed by multiple cysts, and extraction of K17 was unreliable as the samples had been placed in formaldehyde. In addition, kidney tissue was obtained from 3 patients who died from other causes. These 3 specimens were processed exactly the same as the specimens from patients with SLE.

When kidney tissue was processed for extraction, it was thawed at room temperature and processed as described[10]. Briefly, the kidney cortex was dissected, weighed, diced, forced through a sieve, and glomeruli were isolated by differential settling. The glomeruli were sonicated, sieved to remove the Bowman's capsules, and the GBM fragments were washed 6 times with about 100-fold excess volume of PBS-I and pelleted by centrifugation to remove entrapped plasma proteins. After the final wash the pellet of the GBM fragments was extracted with a low pH buffer (0.1 M glycine HCl, 0.15 M NaCl, pH 2.5) or with 6 M guanidine hydrochloride in the presence of 10^{-4} M iodoacetamide. The supernatants were dialyzed 3 times in 100-fold excess of PBS-I and 10^{-4} M iodoacetamide to allow gradual refolding of the recovered molecules.

The concentration of the recovered IgG was determined in the initial supernatant of glomeruli, the sonication supernatant, and in the extracts of GBM fragments by using a capture assay with antibodies to κ and λ chains and a chemiluminescence detection system as described[11]. Unknowns were analyzed by the same method, using a blank well for each specimen without coating the wells with antibodies to κ and λ chains[11]. Human serum albumin (HSA) was quantified in the extracts with a similar assay as described[10].

Quantitation of specific antibodies in glomerular extracts. Fourteen antigens were employed to detect and quantitate the amount of specific IgG antibodies present in the extracts. In all these experiments black Fluoro Nunc MaxiSorp wells (Nalge Nunc International Corp., Naperville, IL, USA) were used. Helix Diagnostics (West Sacramento, CA, USA) coated wells for us with purified lambda phage dsDNAusing 0.5% bovine serum albumin (BSA) in PBS for blocking. The collagen-like region (CLR) of human C1q was prepared in our laboratories and wells were coated with 250 ng/well. Sm, SSA, and SSB antigens, purified by affinity chromatography from bovine tissues, were used at 1 unit per well, the units being defined by the provider (ImmunoVision, Springdale, AR, USA). The SSA antigen contained small amounts of human IgG and was purified by incubation of the reagent with agarose beads coupled with $F(ab')_2$ fragments of goat antibodies to human Fc fragments. Calf thymus histones (Worthington Biochemical Corp., Lakewood, NJ, USA) and calf thymus chromatin stripped of H1 histone (kindly provided by Dr. R.W. Burlingame, Inova Diagnostics Inc., San Diego, CA, USA) were used at 500 ng/well for coating. Purified Epstein-Barr viral capsid antigen (VCA) and recombinant nuclear antigen type-1 (EBNA-1) (Advanced Biotechnologies Inc., Columbia, MD, USA) were used at 0.5 and 1 unit/well, respectively, to coat the wells, the units being defined by the provider. Human MPO (Calbiochem-Novabiochem, La Jolla, CA, USA), at 400 ng/well, and human neutrophil cathepsin G (ICN Biochemicals, Aurora, OH, USA), at 500 ng/well, were used as described[11]. Human $ß_2$-glycoprotein I ($ß_2$-GPI) and human lactoferrin (ICN Biochemicals) were used at 500 ng/well and tetanus toxoid (Calbiochem-Novabiochem) was used at 400 ng/well. After overnight incubation the wells in all these assays were washed, blocked, incubated overnight with test samples, washed, and developed exactly the same as for the standard curve for IgG, using $F(ab')_2$ fragments of purified goat antibodies to human Fc fragments of IgG, conjugated with horseradish peroxidase, diluted 1:10,000 (Jackson Immuno Research Laboratories, West Grove, PA, USA). The blocking solution was 2% nonfat milk in PBS, 0.05% Tween 20, except for histones and chromatin the blocking solution was 0.5% BSA (Sigma, St. Louis, MO, USA) in PBS, 0.05% Tween 20. Several dilutions of each extract were tested so that titration curves could be constructed. Blank wells without the test antigen were used for each specimen at each dilution. The relative light units (RLU) for each blank were subtracted from the corresponding test RLU. With each assay a standard curve for IgG was repeated and used to quantitate the amount of IgG bound at each dilution to a specific antigen. From this information the µg/ml of a specific IgG antibody was calculated. With each specific antibody test a positive serum with high titer and a negative serum were titrated to assure reproducibility of the test in each experiment. The coefficient of variation for the antibody tests ranged from 2.0 to 4.9%.

With each antibody system the first supernatant of glomeruli, considered a serum surrogate, and the supernatant after sonication were screened for antibody activity. The use of the first supernatant of glomeruli as a serum surrogate was justified by the finding that the titration curves of the surrogate and antemortem serum, when available, were superimposable for antibody activity (Reference 10 and unpublished observations, M. Mannik and M.H. Wener). In these and previous studies by us the sonication supernatant always contained more IgG than the preceding supernatant of settled glomeruli and the IgG:HSA molar ratio increased, indicating that during sonication IgG was released from the immune deposits in glomeruli. If these experiments indicated more antibody activity in the sonication supernatant than in the serum surrogate, then a titration was carried out with the serum surrogate, the sonication supernatant, and the low pH or guanidine extract of the GBM fragments. If the screening experiments did not indicate increased antibody activity in the sonication supernatant as compared to the serum surrogate, the detailed titration studies were performed on 4 to 8 kidney specimens to be sure that the antibody activity was not enriched in the extracts of GBM fragments. In the titration experiments the amount of specific antibody in µg/ml was calculated for the serum surrogate and the low pH or guanidine extract using the standard curve for IgG. Knowing the IgG concentration of these samples, the percentage of IgG for a given antibody was calculated. Dividing the percentage of a given antibody in the extract from GBM fragments by the percentage of the same antibody in the serum surrogate indicated how many fold the specific antibody was enriched in the extract. Enrichment of antibodies in the glomerular extracts greater than 2.0-fold was chosen as a reportable enrichment, in part based on the finding that only one specimen had a value between 1.0- and 2.0-fold enrichment.

Other Methods

Sucrose density gradient ultracentrifugation was used to determine if the specific antibodies sedimented as monomeric IgG or higher polymers. Precautions were taken as described to prevent loss of IgG and immune complexes[12]. The centrifuge tubes were precoated with 0.5% BSAin PBS and the 0.5 ml fractions were collected into 0.1 ml of 2.5% BSA in PBS,

0.25% Tween 20. Some extracts were treated with DNase I from bovine pancreas (Sigma) to determine if antibody titers were altered. In these experiments 20 units of DNase were added per 0.1 ml of extract, rendered 5 mM with $MgSO_4$, incubated at 37°C overnight, and then rendered 6 mM with EDTA.

Double-stranded DNA was quantified in some specimens using PicoGreen (Molecular Probes Inc., Eugene, OR, USA) according to procedures recommended by the provider. Calf thymus DNA(Sigma) was used as a standard, and fluorescence was detected with a fluorometer (Packard Instruments Co., Meriden, CT, USA). dsDNA cellulose (Sigma) and histones coupled to agarose beads, prepared in our laboratories, were used to adsorb some specimens. The calf thymus histones (Worthington Biochemical) were coupled to CNBr-activated CL4B agarose beads according to the recommendations of the provider (Pharmacia Fine Chemicals, Piscataway, NJ, USA). The final preparation had 4.5 mg of histones coupled per ml of agarose beads.

With selected specimens experiments were performed to determine if any IgG remained attached to the GBM fragments after extraction with 6 M guanidine. For this purpose, the pellet of GBM fragments after extraction with guanidine was washed 3 times with PBS to remove any entrained IgG. The pellet was divided in 2 equal aliquots, and each half was then suspended in 1.0 ml of 2% sodium dodecyl sulfate (SDS) and made 0.03 M with dithiothreitol or 0.001 M with iodoacetamide to block free sulfhydryl groups. After 3 h incubation at room temperature the GBM fragments were pelleted by centrifugation at 10,000 × g and the supernatant was analyzed for release of IgG or its polypeptide chains with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and Western blotting. IgG in immunoblots was detected with the same antibodies used for detection of IgG in the standard curves, but at 1:1000 dilution, or using a mixture of peroxidase-conjugated goat antibodies to human κ and λ chains, both at a 1:250 dilution, followed by color development with 4-chloro-naphthol and hydrogen peroxide.

RESULTS

Extraction of GBM fragments. In the initial phase of these studies, involving kidney specimens K1 to K13, the GBM fragments were extracted with a pH 2.5 solution and antibodies to collagen-like region of C1q were enriched in 4 of 12 extracts of GBM fragments[10]. During experiments in mice with radiolabeled immune deposits in glomeruli, 6 M guanidine extracted more antibodies than low pH[13]. Therefore, in the next 5 specimens (K14 to K19), extractions with low pH and 6 M guanidine were compared. Since the yield of IgG was higher with guanidine extraction than with low pH, the former method of extraction was adopted for the subsequent specimens, K20 to K25. Enough tissue was available for K1, K2, K3, K5, and K12 to repeat the extraction with guanidine. In 10 specimens the yield of IgG in µg/g of cortex had a median of 0.035 (range 0.002–0.273) with low pH and 0.257 (range 0.059–0.275) with guanidine ($p < 0.01$). The yield of IgG with guanidine extraction of the GBM fragments from the 3 non-SLE specimens was 0.0024, 0.037, and 0.078 µg/g of cortex, respectively. The extracts of these 3 specimens contained no antibodies to dsDNA, Sm, SSA, or MPO; however, antibodies to tetanus toxoid were present, but not enriched in the guanidine extracts of these GBM fragments. In a previous report we showed that the exposure of kidney extracts of K15 to 6 M guanidine did not alter antibody activity to CLR of C1q, SSA, and Sm antigens[11]. Therefore, in subsequent tabulations the reported data are given for extractions with guanidine, unless otherwise stated.

Antibodies to dsDNA. In 6 of 23 kidney specimens the extracts of GBM fragments were enriched 5.7- to 128-fold with antibodies to dsDNA. Two of these 6 extracts (K6 and K7) were obtained by low pH and 4 of the 6 extracts were obtained by guanidine (Table 1). The treatment of the extracts with DNase increased the amount of detected antibodies to dsDNA in 3 specimens, in K19 from 5.7- to 19.8-fold, in K23 from 128- to 323-fold, and in K24 from 58- to 150-fold enrichment. The increase in detection of antibodies to dsDNAby DNase is illustrated by the titration curves for K23 (Figure 1). By sucrose density gradient ultracentrifugation this antibody activity cosedimented with monomeric IgG (data not shown). In guanidine extracts of the GBM fragments in K1, K2, K3, K5, K12, and K15, the screening tests were negative for antibodies to dsDNA, and DNase treatment did not result in detectable antibodies. DNA was quantified in the guanidine extracts of 8 specimens with adequate material available (K3, K5, K12, K16, K19, K21, K23, and K24) using PicoGreen in a fluorometric assay. All tested specimens, regardless of the presence of antibodies to dsDNA or change with DNase I treatment, had small amounts of DNA, with a median of 242 (range 41 to 707) ng/100 µl volume in the wells.

Antibodies to the collagen-like region of C1q. In 8 of 23 specimens the extracts of GBM fragments were enriched 2.5- to 134-fold with antibodies to CLR (Table 1). In a previous report antibodies to CLR were enriched in the sonication supernatants of K2, K3, K6, and K7 and in the low pH extracts of K6 and K7, but the IgG concentration was too low in the pH 2.5 extracts of K2 and K3 to determine antibody activity[10]. Guanidine extraction of the GBM fragments from K2 and K3 showed that there was modest enrichment of antibodies to CLR (Table 1). In a previous report we demonstrated that the antibodies to CLR in the guanidine extract of K15 sedimented with monomeric IgG on sucrose density gradient ultracentrifugation[11]. In patients with proliferative lupus renal lesions, i.e., WHO class III or class IV lesions, antibodies to CLR of C1q were enriched in the guanidine extracts of GBM fragments in 7 of 14 specimens, compared with enrichment of antibodies to CLR in the extracts from only one of 7 specimens with WHO class I or II lesions ($p = 0.040$ by chi-square analysis). Among patients with class III or class IV lesions antibodies to CLR of C1q were present in the guanidine extracts of GBM fragments in 6 of 8 specimens with enrichment of 3 or more antibodies and in only one of 6 specimens with enrichment of less than 3 antibodies. Further, the level of enrichment of antibodies to CLR of C1q in the guanidine extracts of GBM fragments correlated with the level of total IgG extracted from the GBM fragments, expressed in µg of IgG/g of renal cortex ($r = 0.675$, $p = 0.0041$ by analysis of variance). These findings support the hypothesis that antibodies to CLR of

Table 1. Enrichment of specific antibodies extracted from GBM fragments with 6 M guanidine or with a pH 2.5 buffer (*) and WHO class of renal lesions.

Specimen	WHO Class	Anti-dsDNA	Anti-CLR	Anti-Sm	Anti-SSA	Anti-SSB	IgG Binding to Histones[a]	Anti-Chromatin
K1	V	—**	—	—	11.8	—	39.8	—
K2	III	—	6.9	—	8.9	12.9	102	—
K3	IV	—	2.5	—	1121	—	19.4	—
K4*	III	—	—	+	NE	—	+	—
K5	I[c]	—	NE	30	28.1	23.1	70.7	—
K6*	IV	99.8	6.0	+	+	+	+	—
K7*	III	5.7	134	—	+	+	+	+
K8*	I[c]	—	—	—	—	—	—	—
K9*	IV[b]	—	—	—	—	—	21.0	+
K10*	IV[b]	NE	—	5.0	NE	11.3	+	NE
K12	III	—	—	8.7	—	—	17.7	29.0
K13*	I[c]	—	—	—	NE	—	—	—
K14	I	—	4.0	—	—	—	—	—
K15	III	—	48.5	—	5.3	—	17.3	2.73
K16	III	NE	NE	6.0	12.4	9.4	314	127
K18[d]	I	—	—	—	2.5	—	NE	NE
K19	III[c]	5.7	NE	168	49.5	86.2	393	78.4
K20	I	—	—	8.2	2.2	—	221	NE
K21	II[c]	7.5	—	3.2	7.0	5.8	86.6	28.4
K22[d]		—	—	—	100	—	ND	—
K23	IV[b]	128	6.8	74.4	75.8	186	37.7	108
K24	IV	58.0	26.4	107	42.0	22.4	147	4.1
K25	III	—	—	6.0	—	NE	ND	—

** Tests for the given antibodies were negative in the serum surrogate and extracts. +: Antibodies were enriched in the sonication supernatant, but sufficient extract from GBM fragments was not available to determine enrichment of these antibodies. For the same reason, the percentage of these antibodies of total recovered IgG could not be calculated for Table 2. [a] IgG binding to histones was measured after digestion with DNase I, except for K5, K9, K15, and K 20. [b] K9 had endstage renal disease (ESRD) and was dialyzed for 3 years; K10 and K23 had ESRD and were dialyzed for 4 months. [c] These 5 patients died with the antiphospholipid syndrome. [d] K18 had a clinical diagnosis of SLE, died with pulmonary hypertension, organizing pulmonary thrombi, and had normal kidneys, including normal electron microscopy. K22 had a history of SLE and died with pancreatic carcinoma; the autopsy report was not available. NE: Antibodies were present in the serum surrogate but were not enriched in the GBM extracts; ND: Test was not done.

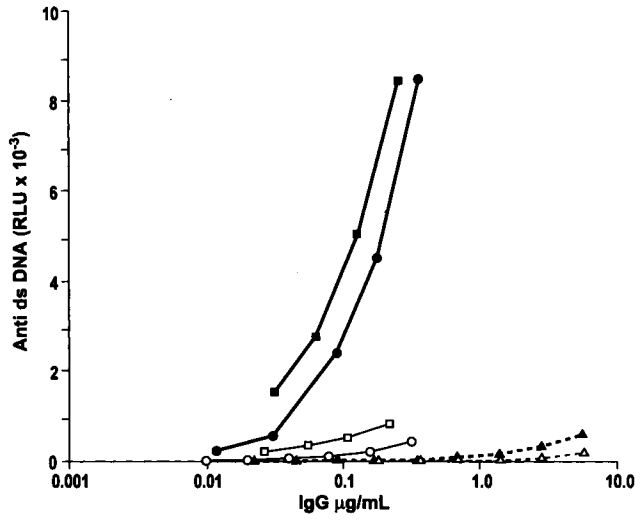

Figure 1. Titration curves for antibodies to dsDNA in K23 before (white symbols) and after DNase treatment (black symbols). The serum surrogate before (△) and after DNase (▲), the sonication supernatant before (○) and after DNase (•), and the guanidine extract of glomerular basement membrane fragments before (□) and after DNase (■) are shown. Quantity (µg/ml) of IgG anti-dsDNAwas determined by the standard curve for IgG, and the enrichment of anti-dsDNAin the guanidine extract compared to the serum surrogate was calculated (Table 1).

C1q contribute to the proliferative lesions of lupus glomerulonephritis.

Antibodies to Sm. In 10 of 23 specimens the extracts of GBM fragments were enriched 3.2- to 168-fold with antibodies to Sm (Table 1). The antibodies to Sm in the guanidine extract of K19 and in the sonication supernatant of K10 and K19 sedimented as monomeric IgG with a small heavier shoulder on sucrose density gradient ultracentrifugation (data not shown). For K4 and K6 not enough low pH extract was available to determine the enrichment of antibodies to Sm; however, these antibodies were enriched in the sonication supernatant as compared to the serum surrogate. This finding is indicated with (+) in Table 1 for antibodies to Sm and other specificities.

Antibodies to SSA. In 13 of 23 specimens, the extracts of GBM fragments were enriched 2.2- to 1121-fold with antibodies to SSA (Table 1). For K6 no extract of GBM fragments was available, but the antibodies to SSA were concentrated in the sonication supernatant as compared to the serum surrogate. The antibodies to SSA in guanidine extract of K15 sedimented as monomeric IgG on sucrose density gradient ultracentrifugation[11].

Antibodies to SSB. In 7 of 23 specimens the extracts of

GBM fragments were enriched 5.8- to 186-fold with antibodies to SSB (Table 1).

Antibodies to histones. The titration curves of antibodies to histones of serum surrogates, sonication supernatants, and guanidine extracts of GBM fragments had anomalous patterns in nearly all kidney specimens. With decreasing IgG concentrations the binding to histones increased, and upon further dilutions decreased. This point is illustrated for one guanidine extract (Figure 2). The possibility was considered that the presence of DNA may interfere with the binding of IgG to histones. Therefore, the extracts were digested with DNase I, and the test for antibodies to histones was repeated. With this procedure the binding curves had an expected pattern (Figure 2). Further, when calf thymus DNA was added to the specimen or when each histone well was preincubated with calf thymus DNA, binding of IgG to the histone-coated wells was blocked (Figure 2).

The IgG binding to histones was examined in fractions from sucrose density gradient ultracentrifugation. In the serum surrogates, sonication supernatants, and guanidine extracts from K3, K12, K15, K19, and K23, all IgG binding to histones was represented by high molecular weight IgG without the presence of monomeric IgG that bound to histones. The treatment of the guanidine extract with DNase I did not free monomeric IgG that bound to histones, even though the distribution of size of IgG that bound to histones was altered (Figure 3). Further, the bulk of IgG sedimented as monomer and not as aggregates or large immune complexes.

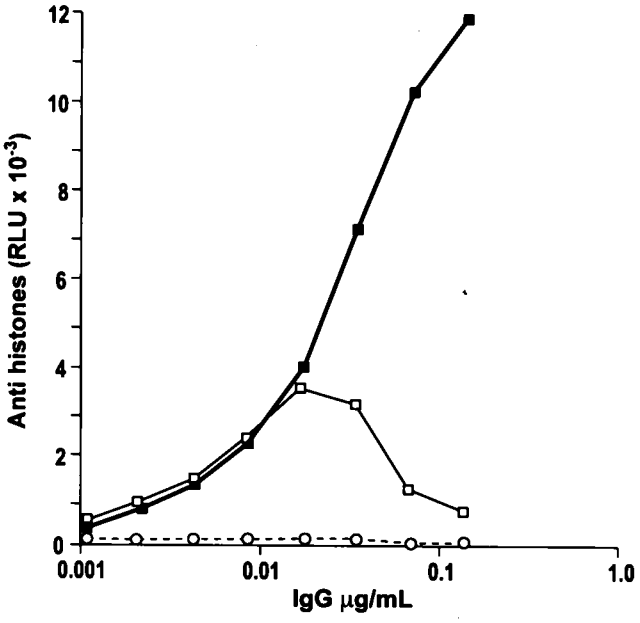

Figure 2. Titration curve for IgG binding to histones in the guanidine extract of glomerular basement membrane fragments of K3 before and after DNase treatment. The relative light units (RLU) for IgG binding to histones for the guanidine extract before DNase treatment increased, then decreased with increasing specimen dilution (□). In contrast, after DNase treatment of the same extract (■) the titration curve had the expected pattern. When 2 µg of calf thymus DNA were added to the DNase treated aliquot of the same extract before the serial dilution, IgG binding to histones was blocked (○).

To determine if aggregates of IgG bound to histones, several experiments were performed. Normal human serum was heated to 56°C for 30 min. This resulted in marked increase of IgG binding to histones. Upon density gradient ultracentrifugation of the heated serum the increased IgG binding to histones was present in fractions denser than monomeric IgG. Purified human IgG was aggregated by heating at 63°C for 10 min. This preparation was then separated by size on sucrose density gradient ultracentrifugation, and the peak fractions of the aggregates were pooled. The marked binding of the aggregates to histone-coated wells was blocked greater than 90% by the addition of 2 µg of calf thymus DNA to the samples before the 2-fold dilutions of the samples were performed, or by preincubating the histone-coated wells with calf thymus DNAfor 3 h (data not shown). These results suggest that the IgG that has aggregated or that exists as immune complexes in the guanidine extracts accounts for a large proportion of IgG binding to histones. These experiments do not allow a statement of what proportion if any of the IgG binding to histones is represented by antibodies to histones.

Antibodies to chromatin. All serum surrogates and sonication supernatants were screened for antibodies to chromatin. The positive specimens and 2 negative specimens (K1 and K2) were submitted to full titration, which included testing of the guanidine extracts. The low pH extracts were not available for K7 and K9, but the antibodies to chromatin were concentrated in the sonication supernatant as compared to the serum surrogate. In the guanidine extracts, antibodies to chromatin were present in 7 specimens with a range of 4.1- to 127-fold enrichment (Table 1). The guanidine extracts of K21 and K24 were absorbed simultaneously with dsDNA cellulose and histones coupled to agarose beads; 5.8% and 3.7% of the IgG binding to chromatin remained, respectively. Not enough aliquots were available in other specimens to carry out the same absorptions. Thus, these experiments do not establish if the detected antibodies were specific for chromatin.

Percentage of recovered IgG antibodies. For each of the specific IgG antibodies in the guanidine or low pH extract, the concentration was calculated from the serial dilutions and the IgG standard curve. Then the percentage of the specific IgG antibody from total recovered IgG was calculated. These percentages are recorded in Table 2. Of note is that most of the antibodies enriched in the guanidine extract of GBM fragments account for less than 1% of the total recovered IgG. For K6 and K7 the percentage could not be calculated, since these specimens were examined with a less sensitive spectrophotometric technique. Antibodies to CLR of C1q exceeded 1.0% of total IgG in only one specimen. The same was true of antibodies to Sm. Antibodies to SSA

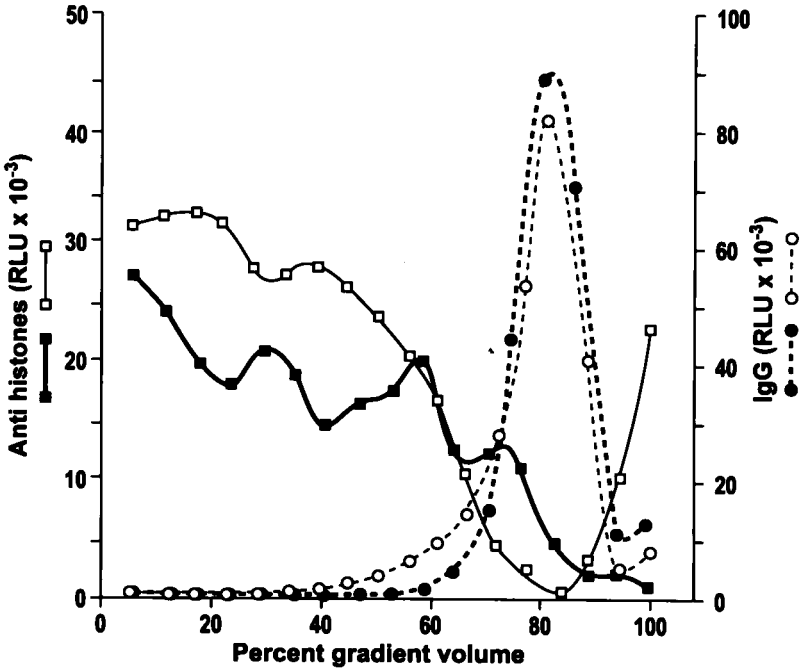

Figure 3. Sucrose density gradient ultracentrifugation patterns of IgG binding to histones of K19 guanidine extract of glomerular basement membrane fragments before (□) and after treatment with DNase (■). For comparison, IgG distribution in the gradient fractions is shown before (○) and after (●) DNase treatment, using a 100 µl aliquot of 1:5 dilution of each fraction. No IgG binding to histones was evident in the monomeric IgG fractions, i.e., sedimenting at 80% of the gradient volume. A shoulder of faster sedimenting IgG is present, but inspection of the relative light units from 0 to 60% of the gradient volume reveals no IgG peaks in the DNase treated and untreated aliquots.

Table 2. Percentage of specific IgG antibodies of total IgG recovered from GBM fragments with 6 M guanidine or with a pH 2.5 buffer (*).

	Anti-dsDNA	Anti-CLR	Anti-Sm	Anti-SSA	Anti-SSB	IgG Binding to Histones[a]	Anti-Chromatin
K1				1.33		7.89	
K2		0.083		4.00	0.072	2.97	
K3		0.049		11.1		4.21	
K4*							
K5[c]			0.135	3.29	0.049	0.205	
K6*	ND[d]	ND	ND	ND	ND	ND	
K7*	ND	ND		ND	ND	ND	ND
K8*[c]							
K9*[b]						0.340	ND
K10*[b]			5.33		0.034	ND	
K12			0.244			3.08	0.232
K13*[c]							
K14		ND					
K15		0.612		0.487		0.443	2.73
K16			0.072	0.116	0.0065	7.07	0.238
K18				0.353			
K19[c]	0.034		0.420	7.92	0.025	32.6	0.345
K20			0.049	4.22		0.618	
K21[c]	0.013		0.194	1.20	0.286	23.9	0.401
K22				0.230			
K23[2]	0.059	0.052	0.065	3.79	0.153	9.6	1.84
K24	0.022	5.56	0.384	0.672	0.016	6.66	0.575
K25			0.0083				

[a] IgG binding to histones was measured after digestion with DNase I, except for K5, K9, K15, and K20. [b] See note b, Table 1. [c] These 5 patients died with the antiphospholipid syndrome. [d] ND: not done, see Table 1.

exceeded 1.0% of total IgG in 8 specimens. The percentage of IgG bound to histones, however, exceeded 1.0% in all specimens that required DNase I digestion to quantify IgG binding to histones. As noted in the preceding section, IgG binding to histones may not represent specific antibodies to histones. Less than 10% of the total IgG is accounted for by identified antibodies, with 2 exceptions: antibodies to SSA in K3 (11.1%) and antibodies to MPO in K15 (11.9%)[11].

Detection of other antibodies. We previously reported that in K15 antibodies to human MPO were concentrated 103-fold in the guanidine extract and that these antibodies were not detected in the remaining 18 specimens studied at that time[11]. In the same report, antibodies to cathepsin G were not detected in the serum surrogates or sonication supernatants in any of 19 specimens. Antibodies to human MPO and to cathepsin G were searched for in K22 to K25, and were not detected. Antibodies to tetanus toxoid were present in serum surrogates of all 23 specimens, but were not concentrated in the extracts of GBM fragments. Since 5 of the 23 kidney specimens studied were obtained from patients who died with the antiphospholipid syndrome, the presence of antibodies to ß$_2$-GPI was examined. The serum surrogates and sonication supernatants were tested for presence of these antibodies in all 23 specimens and all were negative (data not shown). A high prevalence of antibodies to Epstein-Barr VCAand EBNA-1 has been reported in sera of patients with SLE[14]. Therefore, the presence of these antibodies was tested in all specimens. Antibodies to VCA and to EBNA-1 were found in 23 of 23 and 22 of 23 serum surrogates, respectively. The sonication supernatants contained lesser amounts of these antibodies without any evidence for enrichment. In 6 specimens, K1, K3, K12, K15, K24, and K25, the guanidine extract of the GBM fragments was examined for antibodies to VCA and to EBNA-1. Low levels of these antibodies were detected in 4, but were not enriched in the guanidine extracts.

Studies to determine if extraction of IgG with 6 M guanidine was complete. Studies in mice indicated that extraction with 6 M guanidine or with SDS did not release all IgG molecules present in immune deposits in GBM fragments and that some of these molecules were covalently attached to components of the GBM[13]. For this reason, further extractions were performed on the centrifugation pellet after extraction with guanidine, as described in Materials and Methods. The pellet from 6 specimens (K14, K16, K19, K21, K23, and K24) after guanidine extraction was divided into 2 equal aliquots and extracted with 2% SDS, one half also containing 0.03 M dithiothreitol and the other half containing 0.001 M iodoacetamide. The supernatants were analyzed by SDS-PAGE under reducing conditions and Western blotting. All the examined specimens showed release of IgG heavy chains (Figure 4) and light chains (data not shown) in the SDS extract in the presence of dithiothreitol, but not in the SDS extract in the presence of iodoacetamide.

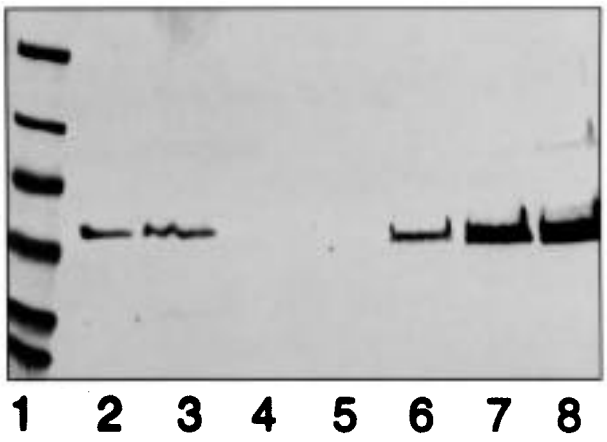

Figure 4. Immunoblot developed with antibodies to the Fc fragments of human IgG. SDS-PAGE was run under reducing conditions using 0.01 M dithiothreitol. Lane 1 contains molecular weight markers, the fourth band from the top having size 47 kDa. Lane 2 is K19 sonication supernatant, containing 9 ng of IgG. Lane 3 is 25 µl of SDS and 0.03 M dithiothreitol extract of half of the K19 pellet of glomerular basement membrane fragments after extraction with guanidine. Lane 4 is 25 µl of SDS and 0.001 M iodoacetamide extract of the other half of the same K19 pellet. Lanes 5, 6, 7, and 8 are human IgG in increasing amounts per lane (5, 20, 50, 100 ng/lane). IgG heavy chains are present in the SDS extract in the presence of a reducing agent and absent in the SDS extract in the presence of iodoacetamide.

DISCUSSION

The notable finding in our study was that autoantibodies with multiple different specificities were enriched in the glomerular immune deposits in patients with SLE who had died. At the inception of the present investigations we sought to include postmortem kidneys from patients with SLE regardless of the cause of death. Therefore, among the 23 studied specimens a spectrum of renal involvement existed at the time of death. Proliferative lupus glomerulonephritis, i.e., WHO class III or IV lesions, was present in 14 specimens. Three of these specimens came from patients with endstage renal disease on dialysis for variable periods of time. In the guanidine extract of GBM fragments of K9 no specific antibodies were enriched, and in K10 extracts 2 specific antibodies were enriched. In 3 specimens with class III lesions antibodies with one or 2 specificities were recovered from the GBM fragments. In 8 of the 14 specimens with proliferative lupus glomerulonephritis 3 or more antibody specificities were enriched in the extracts of GBM fragments. Although the presence of these autoantibodies in the GBM of kidneys from SLE patients does not prove that they are pathogenic, the fact that they are enriched in the kidneys of patients with proliferative lupus glomerulonephritis supports a role for these autoantibodies in the pathogenesis of lupus glomerulonephritis.

Studies in mice have shown that in experimental renal diseases induced simultaneously with 2 different antigens, individual immune deposits in glomeruli contain only one

antigen[15]. It is likely that the same holds true in human diseases, provided that the different antigenic molecules are not cross-reactive. We have previously suggested that the antibodies to CLR of C1q bind to C1q deposited on immune complexes formed with different antigen-antibody systems[10]. Our observations that enrichment of antibodies to CLR of C1q was associated with multiple autoantibodies and with proliferative forms of glomerulonephritis tend to support the hypothesis that antibodies to CLR of C1q could facilitate formation of larger immune deposits, and thus promote activation of complement and other inflammatory mechanisms. Formation of immune complexes between anti-CLR and fluid-phase C1q, followed by deposition in glomeruli, is unlikely because these antibodies do not react with free fluid-phase C1q[16]. Alternatively, C1q potentially could interact with DNA or other macromolecules and then bind anti-CLR. Regardless of the mechanisms leading to the enrichment of antibodies to the CLR of C1q in glomeruli of patients with proliferative lupus renal disease, these antibodies are likely to enhance the inflammatory processes in glomeruli.

Antibodies to dsDNA, probably the most thoroughly studied of any antibodies, were enriched in 6 extracts from the GBM fragments. DNase I digestion of 3 of these 6 extracts increased the amount of detectable antibodies to dsDNA, yielding a higher enrichment of these antibodies. This finding suggested the presence of DNAin the extracts, possibly as a result of the sonication and incomplete removal of DNA. The presence of small amounts of DNA was confirmed by use of PicoGreen in a fluorometric assay.

The enrichment of antibodies to Sm, SSA, and SSB in the extracts exceeded the frequency of antibodies to dsDNA enriched in the extracts. This is the first demonstration of enrichment of antibodies to Sm in glomerular immune deposits of patients with SLE, and a confirmation and extension of previous reports of the enrichment of antibodies to SSAand SSB in these deposits. We found the enrichment of antibodies to SSA in 15 of the studied 23 extracts of glomeruli, and enrichment of antibodies to SSB in 10 of the 23 extracts.

The detection and presence of antibodies to histones in extracts of GBM remain ambiguous for several reasons. First, the titration curves for IgG binding to histones had aberrant patterns, and DNase I digestion converted the patterns to those expected for serial dilutions. Second, the addition of calf thymus DNAblocked the binding of extracts and of aggregated IgG to histones. These findings indicated that binding of DNA to histones interfered with the binding of IgG to histones. Third, the sucrose density gradient ultracentrifugation experiments showed that the IgG that bound to histones was of high molecular weight and not monomeric IgG. Fourth, previous reports had indicated that aggregated IgG binds to histones in a non-immunospecific manner[17,18] and that in sera from patients with SLE the IgG binding to histones represented primarily immune complexes[17]. Our experiments with heated serum or heated purified human IgG concur with these previous reports. Therefore, we have used the term “IgG binding to histones” and not antibodies to histones. The presence of histones in glomeruli of patients with SLE has been shown by immunofluorescence microscopy using antibodies to histone H3[19]. Whether these histone molecules have bound antibodies to histones or attracted circulating immune complexes has not been clarified. The latter, however, raises the possibility that immune complexes in circulation, containing autoantibodies or containing unrelated exogenous antigens and specific antibodies, may become deposited in glomeruli of patients with SLE by binding to histones in glomeruli.

Antibodies to a number of other antigens have been reported in the sera of patients with SLE. We searched for antibodies to human MPO in all specimens and did not identify additional presence of these antibodies, other than in K15 as reported[11]. We did not detect the presence of antibodies to cathepsin G, lactoferrin, or ß$_2$-GPI. As noted, antibodies to tetanus toxoid, to Epstein-Barr virus VCA, and to EBNA-1 were detected in the serum surrogates, but these antibodies were not enriched in extracts of GBM fragments. These findings indicate that Epstein-Barr viral antigens and specific antibodies to these viral antigens do not participate in the formation of immune complexes in glomeruli. We did not study rheumatoid factors or antibodies to the ribosomal P proteins in the guanidine extracts.

Our studies allowed the calculation of the proportion of total recovered IgG that was represented by the identified antibodies. As seen in Table 2, most of the percentages are small, below 1.0%, and infrequently are between 1.0% and 10%. The percentages for IgG binding to histones are higher, and as discussed above may represent IgG aggregates or immune complexes.

The presence of the bulk of IgG in the extracts remains unexplained, but several possibilities need to be considered. First, the loss of antibody activity by exposure to 6 M guanidine was ruled out by the demonstration that antibody activity was not lost by exposure to this potent molecular perturbant[11]. Second, the recovery of known amounts of a specific antibody was evaluated and found to be complete in an equivalent system[11]. Third, antibodies to yet-unknown antigens may be present. Fourth, small amounts of normal IgG and other serum proteins may be associated with or entrapped in the GBM and not removed by washing with a neutral buffer. This possibility is supported in part by recovering IgG from glomeruli isolated from non-lupus renal tissues and by detecting in some specimens small amounts of antibodies to tetanus toxoid, antibodies to Epstein-Barr virus VCA, and EBNA-1 in the GBM extracts without evidence of being enriched in comparison to the serum surrogate.

The possibility exists that in the postmortem tissues some

antibodies to nuclear antigens had penetrated the cells and bound to nuclei. This is supported by the finding that postmortem frozen and thawed kidney tissue from patients with SLE showed IgG bound to cell nuclei by immunofluorescence microscopy[20]. Therefore, we chose the method of sonication of glomeruli until no intact nuclei were visible, followed by washing of GBM fragments. During the sonication of isolated glomeruli some antibodies were removed from the immune deposits, as shown by increased IgG:HSA molar ratio and by increased concentration of antibodies in the sonication supernatant as compared to the serum surrogate, as described for anti-CLR[10]. Immune deposits remained attached to the GBM fragments in experimental models after sonication, as determined by electron microscopy[21] and by use of radiolabelled antibody molecules[13].

The possibility remains that antigen molecules from immune deposits survived the extraction and re-formed immune complexes, thus limiting antibody detection. In this report and in previous studies[10,11], the specific antibodies and the recovered IgG sedimented largely as monomeric IgG on sucrose density gradient ultracentrifugation with a small heavier shoulder. In these experiments, described methods were employed to prevent losses of large immune complexes[12]. Thus, these findings indicate that large amounts of immune complexes were not present in the neutralized extracts of GBM fragments. Nonetheless, the IgG that bound to histones was all larger than monomeric IgG. Also, DNAwas present in small quantities in the guanidine extracts and the digestion of DNA with DNase increased the detection of antibodies to dsDNA in some specimens. In extracts that contained no detectable antibodies to dsDNA, however, the use of DNase did not reveal previously undetected antibodies to dsDNA. Of note is that the DNase did not alter the binding of antibodies to SSAand to Epstein-Barr VCA antigens[22].

We were also concerned that the sonication of glomeruli may enhance binding of autoantibodies or IgG to components of glomeruli. To test this possibility, an aliquot of a non-SLE kidney was processed as usual, except prior to sonication 1.0 ml of diluted serum was added, containing 20.7 µg of antibodies to SSA. After overnight incubation at 4°C, sonication and subsequent steps were performed by the usual protocol. In the guanidine extract 0.071% and 0.067% of the added antibodies to SSAwere recovered in 2 separate experiments. These findings indicate that autoantibodies entrapped in glomeruli do not significantly contribute to the antibodies recovered from the GBM fragments.

The recovery of IgG from GBM fragments by guanidine extraction was incomplete. Further extraction with SDS in the presence of a reducing agent yielded small amounts of IgG heavy chains and light chains. In contrast, SDS in the presence of iodoacetamide released no detectable IgG polypeptide chains. These findings indicate that some IgG molecules are linked to GBM macromolecules by intermolecular disulfide bonds. The presence of covalent bonds between immune complexes and structural molecules of the GBM has been described in a model of immune complex-induced lesions in mice[13]. The mechanism that leads to covalent attachment of components of immune complexes to molecules of the GBM may be related to the mediators of inflammation[23].

The finding that autoantibodies with multiple specificities form the immune deposits in glomeruli of patients with SLE has implications for developing therapeutic interventions for lupus glomerulonephritis. Suppressing or abrogating the antibody response to one antigen may not suffice to improve the course of lupus glomerulonephritis. Several approaches have been developed and recommended to specifically suppress antibodies to DNA. LJP 394, which consists of 4 double-stranded oligodeoxynucleotides attached to an inert platform, was developed to act as a B cell toleragen[24,25]. Idiotypic vaccination with murine antibodies to dsDNA was recommended to suppress antibodies to dsDNA[26]. Solid-phase DNAimmunoadsorbents for extracorporeal removal of antibodies to DNA was suggested for altering the course of lupus renal disease[27,28]. These interventions or other approaches directed to antibodies with one defined specificity are unlikely to have a profound effect on the course of lupus glomerulonephritis. The presence of discrete immune deposits with different autoantibodies and antigens, possibly cross-linked by C1q and antibodies to the CLR of C1q, may not be altered much by the abrogation or suppression of antibodies to just one specific antigen present in the immune deposits.

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