Spectrotype Distributions of Circulating IgG from Patients with Systemic Lupus Erythematosus

Giorgio Barbi,1 Lynn B. Kel1,2 Andrea D. Gaito,2 and Vincent A. DeBar1,3

Pathogenic autoantibodies from patients with systemic lupus erythematosus (SLE) may represent a relatively cationic fraction of IgG. We compared the spectrotype distributions of affinity-purified IgG from the sera of 10 SLE patients and 10 age- and sex-matched control subjects. Purified IgG was subjected to isoelectric focusing between pl 3 and 9. No significant difference was observed for pl 6.0–6.5 and 7.5–8.0. However, control patients had a higher percent of total IgG at 6.5–7.0 (15.4 ± 4.0 vs 11.1 ± 2.0, P = 0.008) and at 7.0–7.5 (22.4 ± 4.8 vs 18.2 ± 3.8, P = 0.04) whereas SLE patients had a higher percent of total IgG at 8.0–8.5 (24.3 ± 3.0 vs 20.5 ± 4.0, P = 0.03) and at 8.5–9.0 (21.9 ± 5.9 vs 15.1 ± 3.7, P = 0.006). Spectrotype distributions of circulating IgG from SLE patients are skewed toward higher pl, providing further evidence of proliferation of B-cell clones that express more cationic IgG in patients with SLE. Longitudinal studies of serum IgG from several patients for >1 year reveal distinct changes in both cationic and anionic clonotypes, suggesting clonal expansion of idiotypes to autoantibodies.

Additional Keyphrases: autoantibodies · autoimmune disease · immunoglobulins · isoelectric focusing

Systemic lupus erythematosus (SLE) is an autoimmune disease that may provoke an inflammatory response in many organ systems.4 There is evidence that much of the pathophysiology of this disease is due to hypersensitivity reactions (including immune-complex-mediated and antibody-dependent cytotoxicity) caused by autoantibodies (1).

An important group of autoantibodies found in SLE patients is directed against well-established anionic antigens such as DNA (2), a variety of the so-called extractable nuclear antigens (3), cardiolipin (4), and some more-recently recognized antigens such as the cytoskeletal intermediate filament vimentin (5, 6) and the glycosaminoglycans (7–9). Ionic interactions are believed to play a role in antigen–antibody binding, and the presumption has been that cationic antibodies would bind these antigens. This binding has not been consistently demonstrated in studies of eluates from the kidneys of murine SLE models, although there has been evidence of some immunoglobulin restriction in these models (10, 11).

This immunoglobulin restriction raises the question of whether these cationic populations of autoantibodies are sequestered in tissue-bound immune complexes and, by extension, whether they can be detected in the circulation of human subjects with SLE. Here we describe the use of affinity-purified IgG from the serum of patients with SLE in conjunction with isoelectric focusing (IEF) to study the spectrotypes of circulating IgG in this group of subjects.

Materials and Methods

Serum Specimens

Patients whose serum was used in this study fulfilled the revised American College of Rheumatology criteria for SLE (12) and all had anti-DNA antibody titers of ≥1:10. Control sera were drawn from a group of apparently healthy, age- (±5 year) and sex-matched subjects selected from laboratory personnel and other hospital employees, their relatives, and friends. Serum specimens were obtained by routine venipuncture. The protocol was approved by the institutional review board responsible for human subject experimentation at St. Joseph’s Hospital and Medical Center. After separation, sera were stored at −70°C until studied.

For comparative purposes, sera from four HIV-positive patients were studied. These were included to determine whether another group of hypergammaglobulinemic subjects in whom autoantibodies are frequently found (13) would give comparable spectrotype distributions. These specimens were also from the bank maintained at −70°C.

Preparative Chromatography

IgG was purified on a 65 x 6.7 mm semi-preparative column containing 2.2 mL of recombinant Protein G-silica by using a Genex System 100 (Genex Corp., Gaithersburg, MD) chromatograph as previously described (14). This instrument is programmed to operate as follows: the column is washed, serum is automatically loaded from an injection loop onto the column, unbound protein is washed away, the bound IgG is eluted, and the column is regenerated.

Elution buffer was exchanged with phosphate-buffered saline (PBS; per liter, 0.15 mol of sodium chloride
and 15 nmol of sodium phosphate, pH 7.30 ± 0.05) with simultaneous concentration of IgG by centrifugal ultrafiltration (Centriprep-30; Amicon Div., W. R. Grace & Co., Danvers, MA) according to the manufacturer’s instructions. Eluates were then reconstituted to their original serum volume with PBS and rechromatographed and concentrated, and the buffer was exchanged a second time.

Isoelectric Focusing

IEF and staining of the gels were accomplished with a Pharmacia (Piscataway, NJ) PhastSystem® apparatus and 43 × 50 mm gels. Ampholytes (pI 3–9) are incorporated into these gels by the manufacturer. The focusing and staining processes are microprocessor controlled. By means of a comb, 4 μL of sample (eluates were diluted so that 4 μL contained 0.8–1.8 μg of IgG) was applied to a prefocused gel and focused at 3.5 W for 410 V·h. Pharmacia PhastGel® silver-stain kits were used according to the manufacturer’s instructions except that we decreased the second developing step by 30 s and increased the background reducer step by 30 s. Scanning of the gels was done with a Helena Laboratories (Beaumont, TX) AutoScan Flur-vis densitometer. The gels were scanned in the visible mode with narrow bandwidth (0.2 mm) setting; the scan speed was 18 cm/min.

Immunoblotting

Transfer of the separated IgG from the IEF gels to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) was a modification of the direct immunoblot procedure of Fasullo et al. (15). Blotting reagents were obtained from Pan-Data Systems, Inc. (Rockville, MD). After hydration, nitrocellulose was overlaid onto the gel, bubbles were gently rolled out, and the sandwich was incubated for 1 h in a humid chamber. The blocking procedure was modified as follows: after a PBS rinse, gels were blocked for 1 h with milk (50 g of nonfat dry milk powder per liter of 1X blotting buffer, a proprietary buffer containing normal goat serum, fetal bovine serum, and 0.02 g of thimerosal). After washing (three 5-min washes in washing buffer: PBS plus 0.01 g of thimerosal and 0.025 mL of Tween 20 per liter), gels were incubated with affinity-purified alkaline phosphatase conjugated goat anti-human IgG (1:1500 dilution in a proprietary conjugate buffer containing normal goat serum, PBS, Tween 20, and thimerosal) for 2 h with gentle agitation. After another wash (three 5-min washes in washing buffer), bands were developed for 15 min with substrate, prepared as recommended by the manufacturer. Development was stopped by rapidly rinsing with distilled water, followed by two further washes.

Autoantibody Assays

Anti-DNA antibody titers for patients who were studied longitudinally were determined by using the immunohistochemical Crithidia luciliae assay (16). Reagents were obtained from Immuno Concepts (Sacramento, CA).

IgG-specific anti-DNA antibody (Ga-DNA) values were obtained by using RheumELISA® Microwell Assay Kits (LipoGen, Inc., Knoxville, TN). Specimens were assayed according to the manufacturer’s directions with reagents supplied with the kit except that peroxidase-conjugated goat anti-human IgG (Tago, Inc., Burlingame, CA) diluted 1:5000 with assay diluent from the kit was used in place of the RheumELISA® conjugate reagent. To quantify results, we arbitrarily set the value of the calibrator from the kit to 100 kU/L and calculated relative test specimen values by multiplying their absorbances by a conversion factor (100 divided by the absorbance of the calibrator).

IgG anti-cardiolipin antibodies (Ga-CL) were measured with Reaads® enzyme-linked immunosorbent assay kits (Reaads Medical Products, Inc., Westminster, CO). Assays were performed according to manufacturer’s directions.

Statistics and Curve Fitting

GB-Stat® software (Dynamic Microsystems, Inc., Silver Spring, MD) was used with an IBM PC AT to perform statistical analyses (Student’s t-test for paired samples) and curve fitting.

Results

Preparation of IgG from SLE Serum

As can be seen in Table 1, the SLE patients had mean serum IgG concentrations greater than twice that of the control subjects. After purification (two passes through the ligand affinity column), we observed an average recovery of 42% for the SLE patients and 70% for the control subjects. These data compare favorably with those in our previous work on this method (14) in which ~80% of IgG from specimens averaging 12.4 g/L was recovered after one pass. Assuming that 80% was recovered after each pass, a simple calculation led us to expect 64% recovery after two passes. For the SLE sera, we observed that as loading approached the saturation capacity of the column (30 mg of IgG), recovery fell to ~75%. It is not unexpected, then, that our average yield for the SLE patients, whose serum IgG concentration approached the column capacity, was proportionately lower.

In an earlier study (17) we purified IgG from SLE

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* Mean ± SD; n = 10. Units anti-DNA binding or anti-cardiolipin binding per mg of IgG; i.e., binding activity of IgG is standardized to mass (mg) of IgG and referenced to serum standards for both Ga-DNA and Ga-CL.

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patients whose serum IgG concentrations were 14.7–23.1 g/L. We found a fraction that required strong acidic (>2.0 mol/L acetic acid, pH < 2.5) conditions for elution. This fraction contained a trace of a component (which we suspected to be, but could not positively identify as, C3), which suggests that the fraction may have been circulating immune complexes. However, these eluates contained only 157.2 ± 31.6 mg/L of IgG (detected with a particle-enhanced radial immunodiffusion assay), or ~1% of the serum IgG concentration. These eluates did not appear to be enriched in cationic IgG, but the amount of IgG present was not sufficient to alter the relative distribution of the various pI fractions.

Analytical Aspects of IgG Spectrotyping

Regarding the isofocusing system, in preliminary experiments not reported here, we attempted to use higher IgG loading and stain the gels with Coomassie Blue. We found poor resolution at higher loading. At the low concentrations of IgG that we applied, silver stain gave excellent detection and discrimination of the bands (Figures 1A and 2) and, within the range of IgG concentrations applied to the gels, loading did not affect distribution of the spectrotypes. Using the narrow-beam-width setting on the densitometer, we were able to discriminate the bands adequately for this study, i.e., to calculate the relative percentage of IgG in discrete regions of the spectrotype. We observed that within the range of IgG loading of the gels, distribution of bands within the spectrotype resulted in identical spectrotypes of sera run on different gels and on different days. This is best exemplified by the points in the standard curve in Figure 3, where the variability of migration distance (for 12 runs) was so small that the standard error (0.09 at pI 9.3 to 0.17 at pI 5.85) fell within the circles at the plotting points. Coefficients of variation ranged from 14.5% for the pI 9.3 marker to 6.2% for the 5.85 pI marker.

The isoelectric focusing gel in Figure 1A compares the IgG spectrotype pattern of a representative SLE patient (P) and a control subject (C). Although the range of pI observed is similar, i.e., from ~5.5 to 9.0 (the most cationic ampholyte incorporated in this gel is actually 9.0; the band is the 9.3 marker), the patient's serum clearly demonstrates increased density at pI > 7.35. The densitometric scan of this gel (Figure 1B) provides a more quantitative measure of this skew toward higher pI in the patient's spectrotype.

In experiments published earlier by another group (15), specific subclasses of IgG in myeloma sera were identified by immunoblotting of isofocusing gels and probing with specific antisera. We used a similar approach to demonstrate that the relatively wide range of pI observed in the spectrotypes that we measured was, in fact, IgG (even though we used twice-chromatographed samples). The developed immunoblots are shown in Figure 2 and indicate that the range of pI observed in the gels is comparable with that of the immunoblots. Furthermore, definition is considerably better in the gels, even when blots were generated from gels with higher IgG loading (not shown).

A standard curve of migration position (distance from the start scan point at the cationic end of the gel) vs pI was made for gel markers from 5.85 to 9.3. This plot, shown in Figure 3, was fitted to a fifth-order polynomial.
regression and the incremental positions (at increments of 0.5 pl units) for pl between 6 and 9 were determined. This was done to provide uniform 0.5-pl increments even though our available pl markers had values that were not in regular increments. The empirical regression obtained from the curve-fitting routine gave a correlation coefficient of 0.9998; the goodness-of-fit was easily ascertained (in Figure 3) by the computer-generated overlay of the regression line and point-by-point plot of the values.

Clinical Studies

Having obtained the positions of the appropriate pl intervals, we used the integrator lines within each interval to calculate the areas under the curves, expressed as percent of total IgG. This was done for specimens from 10 SLE patients and 10 age- and sex-matched control subjects (Table 1). The resulting histograms are presented in Figure 4. The skew toward higher pl is apparent from the histogram. When compared by a parametric statistic (Student's t-test for paired data), the control group had relatively more IgG in the 6.5–7.0 pl range (15.4 ± 4.0 (SD) vs 11.1 ± 2.0; P = 0.008) and in the 7.0–7.5 pl range (22.4 ± 4.8 vs 18.2 ± 3.8; P = 0.04). The SLE patients, however, had relatively more cationic IgG; the percent of total IgG for the 8.0–8.5 pl interval was 24.3 ± 3.0 vs 20.5 ± 4.0 (P = 0.03) and for the 8.5–9.0 pl interval was 21.9 ± 5.9 vs 15.1 ± 3.7 (P = 0.006).

This skew does not appear to be related solely to an increase in polyclonal IgG. We chose four serum samples from HIV-infected subjects and purified IgG from these. Although all had polyclonal increases of IgG, only one demonstrated a cationic shift.

Longitudinal studies of IgG spectrotypes were conducted on SLE patients’ sera that had been stored at −70 ºC for almost 3 years (Figure 5). In Figure 5A, a patient who had anti-DNA titers (Crithidia luciliae assay) of 1:320 in August 1988, 1:640 in February 1989, and 1:1280 in May 1989 demonstrated fairly similar spectrotypes, consistent with the similarity (moderately high) of the Crithidia titers. By April 1991, the titer had fallen to 1:40 and proportionately more IgG appeared in the 6–8 pl range. Another patient (Figure 5B), studied between August 1988 and March 1991, showed a distinct clonotype at pl ~5.5 in a sample collected in November 1988, when the patient's Crithidia titer was highest (1:1280). This anionic clonotype and another group appearing in the same specimen as a cluster centered around pl 5.0 seem not to persist, although the emergence of the peak at 5.5 may be detectable as a slight peak in the specimen harvested several months earlier (August 19, 1988). A clonotype or group of clonotypes at or near pl 5.5 is also observed in the specimens from the patients depicted in Figure 5, C and D. Interestingly, even when distinguishable accompanying clonotypes (Figure 5C, specimen dated April 15, 1988) or broadening (Figure 5D, specimen dated January 11, 1991) occurred, other specimens from the same patient showed the most persistent single peak at 5.5.

Discussion

The numerous immune manifestations of SLE include hyperproliferation of B cells accompanied by the appear-
Fig. 5. Longitudinal study of IgG from sera of four patients (A–D), collected over several years.
Isoelectric points are indicated at the ticks on the x-axis; at the right of each scan, the AntiChl titer is given on top and the date on which the sample was harvested is underneath; e.g., for patient A, the top scan is IgG from a specimen collected on August 9, 1988, which had a titer of 1:320.

ance of autoantibodies to a variety of self antigens, i.e., normal components of cells and tissues (18). A group of the earliest recognized and most intensely studied of these autoantibodies is composed of those binding to DNA (2). These are present at a relatively high titer in many cases of SLE, although there is strong evidence that they are present, in lesser amounts, in the serum of healthy individuals (19, 20).
Within the past few years, it has been recognized that there is also a reactivity of serum autoantibodies from SLE patients with other polyanions, such as glycosaminoglycans (7–9). There may be a population of these antibodies that also bind to DNA (7,21,22). This line of evidence and the principle of charge complementarity in antigen binding by antibodies (23) lead to the suggestion that antibodies to the multiplicity of acidic (anionic) antigens observed in SLE are cationic (24). Another reason to expect cationic antibodies in SLE was given by Gauthier et al. (25,26): there is a highly negative charge on the glomerular basement membrane (27) of the kidney, a frequent site of antibody deposition and tissue damage in SLE (28).

These considerations have led to many studies on the role of cationic antibodies in SLE. Several investigations have focused on established murine models of SLE (10,11,29–32), such as the NZB × NZW F1 and the MRL-lpr/lpr, although another approach has been to study the effect of artificially cationized, preformed immune complexes in a control (disease-free) mouse, the C57BL/6 (24,25). These studies have not been conclusive, in that whereas immune complexes bearing artificially cationized antibodies can be deposited in kidneys of control mice (24,25), the renal eluates of the SLE mouse models have either lacked pl restriction or shown a cationic shift in the spectrotypes (10,11,29–32).

Spectrotype analysis has not been so extensively applied to human SLE. In a recent report, Sabbaga et al. (20) isolated anti-DNA antibodies from serum of SLE patients and healthy subjects and found no restriction of pl in either group, both groups having pl 5.4–9.0. They did not report on the quantitative aspects of the spectrotype distributions.

We found that the whole-IgG fractions from serum of our patients and control groups were similarly unrestricted (as would be expected from whole IgG fractions) in pl but that quantitatively there was a statistically significant skew toward the cationic end of the spectrotype. In another study of myeloma immunoglobulins, Fasullo et al. (15) clearly showed that these paraproteins could isofocus at a pl as low as 6.0, demonstrating that a cationic shift is not characteristic of a single paraprotein increase. Moreover, in our study of IgG from HIV-infected patients, it appears that polyclonal increases in IgG in other diseases are not necessarily accompanied by a shift toward cationic pl.

Additionally, we provide data on a small but interesting group of stored SLE serum specimens from four patients. All four patients had predominantly cationic IgG spectrotype changes over the several-year collection period, but additionally several individual sera contained a band or group of bands in the pl 5.0–6.0 range, principally at pl ~5.5. A similar finding with regard to a partial restriction in anionic (pl 5.0–7.0) clonotypes in murine anti-DNA antibodies was reported (32). These clonotypes may represent anti-idiotypic antibodies expected to isofocus in the anionic region. Several common idiotypic determinants are recognized, such as 31 (33) and 16/6 (34), the latter also occurring in other autoimmune diseases (35). The longitudinal studies reported here for a limited number of patients suggest that further clinical and laboratory studies along these lines may provide useful clinical correlations.

In conclusion, we observed that a group of 10 SLE patients had serum IgG spectrotypes that are significantly different from those from a group of age- and sex-matched control subjects. These IgG spectrotype changes do not appear to correlate with anti-DNA antibody titers but do suggest that polyclonal activation of B lymphocytes, a widely recognized phenomenon in this disease (36), leads to the synthesis of IgG clonotypes that tend to react with anionic antigens, another frequently observed serologic phenomenon in SLE. This situation further emphasizes the dichotomy between the specific and nonspecific autoantibody reactivities in systemic autoimmune diseases and the need to better define pathogenic autoantibodies (37) at the molecular level.

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