

Subclass Restriction and Polyclonality of the Systemic Lupus Erythematosus Marker Antibody Anti-Sm

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Abstract

Anti-Sm antibodies are highly specific markers for the diagnosis of systemic lupus erythematosus (SLE). This specificity suggests that the immunoregulation of these autoantibodies would reflect fundamental immune abnormalities in this disorder. As a clue to this immunoregulation, we have investigated the isotype distribution of anti-Sm antibodies by enzyme-linked immunosorbent assays. We have found that the anti-Sm response is markedly restricted to the IgG1 heavy chain isotype. On the other hand, the light chain distribution reflects that in normal serum, while isoelectric focusing analysis fails to show an oligoclonal pattern. The related specificity, anti-ribonucleoprotein, is also restricted to IgG1, while the SLE-specific antibody anti-double-stranded DNA is mostly IgG1 with a lesser contribution by IgG3. These results suggest that antinuclear antibodies that are strongly associated with SLE are produced by a T cell-dependent response, probably driven by antigen. The immunoregulation of the response to several autoantigens may be quite similar.

Introduction

Systemic lupus erythematosus (SLE)¹ is characterized by the production of a spectrum of autoantibodies that includes a large, but finite, number of different specificities (1). The extensive variety of these abnormal antibodies implies the action of general immunoregulatory deficits. Many immunological abnormalities have been described in SLE, but it is not clear how any of these relate to autoantibody production (2). In general, it has been difficult to study the autoantibody responses themselves (3).

Certain autoantibodies are highly specific for the diagnosis of SLE. These include anti-Sm, directed at a small nuclear ribonuclear protein particle (4, 5), and anti-double-stranded DNA (6). Anti-Sm antibodies probably do not play a critical role in the pathogenesis of the clinical manifestations of SLE, while anti-DNA antibodies are almost certainly important in the development of nephritis in many patients (7). Nevertheless,

the diagnostic specificity of both of these antibodies strongly implies that their production is intimately linked with the basic immunoregulatory abnormalities that cause SLE. In addition, the fact that both these antibodies are relatively specific for murine SLE further underlines their fundamental importance (8, 9).

In the current work, we present a detailed study of the isotype distribution and heterogeneity of anti-Sm antibodies in human SLE sera. The rationale for this work is based on the assumption that the character of a serological response will reflect in vivo immunoregulation of that response. Work with a variety of exogenous antigens in animals has already indicated that certain isotypes are "T-dependent" and others are "T-independent" (10-14). In human studies, protein antigens which would be T-dependent in animals have tended to elicit IgG1 and IgG3 responses, while T-independent antigens elicit IgG2 responses (15-17). IgG4 responses have mainly been seen in sera from patients hypersensitized to allergens (18-21) and in hemophiliacs with anti-factor VIII (22) or anti-factor IX (23). Furthermore, in animals, IgG responses tend to be more T cell-dependent than IgM responses. In mice we have already found that the anti-Sm response is skewed toward the T cell-dependent IgG2a subclass (24). Separate work by ourselves and others have demonstrated a T cell dependence of the MRL disease in general (25), as well as a T cell requirement for the anti-Sm response in particular (26, 27).

In the present report, we also examine the clonality of the anti-Sm response as a further clue to its immunoregulation. The potential mechanisms one could evoke to explain the escape of a single clone of autoantibody-producing cells are different from what would be required to explain a polyclonal response. We have found evidence for polyclonality of the anti-Sm response, in spite of its striking subclass restriction. Finally, we have compared the isotype distribution of other antibody responses in the anti-Sm sera examined, and we have examined the subclass distribution of the related specificity, anti-nuclear ribonucleoprotein (RNP) and another SLE-specific antibody, anti-double-stranded DNA (ds DNA).

Methods

Sera. 42 anti-Sm-positive sera were identified by double immunodiffusion using standard references (28). The sera were among those submitted to the clinical laboratory for routine antinuclear antibody testing. All patients (except one with diffuse interstitial pulmonary fibrosis) with anti-Sm antibody had the clinical diagnosis of SLE (including overlaps), although this was not a criterion for their inclusion. Sera had been stored at -20°C prior to use. During the course of the current studies, they were held at 4°C with 0.1% sodium azide.

Antigens for enzyme-linked immunosorbent assays (ELISA). Sm antigen was affinity purified from RNase- and DNase-digested rabbit thymus extract as previously described (29). It was free of RNP, SS-A, and SS-B, as determined by double immunodiffusion. It showed polypeptide bands of 15,000 and 17,000 mol wt. RNP antigen was

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1. *Abbreviations used in this paper:* ds DNA, double-stranded DNA; ELISA, enzyme-linked immunosorbent assay; SLE, systemic lupus erythematosus; TNP-BSA, trinitrophenylated bovine serum albumin.

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affinity purified in a parallel manner, except that it was not digested with nucleases. Pneumococcal polysaccharides were in the form of Pneumovax (Merck, Sharp and Dohme, West Point, PA) and were used at 10 $\mu\text{g}/\text{ml}$. Trinitrophenylated bovine serum albumin (TNP-BSA) was made by conjugating trinitrobenzene sulfonate with BSA. The resultant product had a molar ratio (TNP/BSA) of 16. It was utilized in the ELISA assay at 10 $\mu\text{g}/\text{ml}$. Tetanus toxoid was obtained as a purified preparation from the Massachusetts Department of Public Health (Boston, MA) and was used at a concentration of 1 $\mu\text{g}/\text{ml}$. Streptococcal cell wall was prepared as previously described (30) and used at a concentration of 10 $\mu\text{g}/\text{ml}$.

Isotype reagents and isotype-specific proteins. Myeloma proteins, purified by preparative electrophoresis as previously described (15, 31), were used for isolation of polyclonal isotype reagents, testing their specificity, determining standard curves, and as solid-phase reagents for further absorptions as necessary. The proteins were all >99% pure, as determined by double-antibody radioimmune assay (32). Polyclonal anti-isotype antisera were produced in rabbits or in monkeys. For anti-IgA, anti-IgD, anti-IgE, and anti-IgM, rabbits were immunized with myeloma proteins. Resulting antisera were absorbed with solid-phase gels of IgG myelomas of both light chain types and then affinity-purified on gels of the appropriate isotype. Anti-IgG1 and anti-IgG3 antibodies were produced in rabbits that were previously tolerized to IgGs of the other subclasses (33). Anti-IgG2 and anti-IgG4 reagents were produced in monkeys immunized with isolated heavy chains. The specific antibodies were absorbed and affinity-purified in a manner similar to that described for the anticlass reagents. In all cases, the purifications were monitored by double immunodiffusion using purified myeloma proteins. In spite of specificities shown by this technique for each reagent, further absorptions were necessary for the ELISA assays, as described below. Anti-light chain antisera were produced in rabbits. Goat anti-F(ab')₂ was obtained from a goat immunized with polyclonal human IgG and was affinity-purified on the pepsin F(ab')₂ fragments of polyclonal human IgG.

Monoclonal antisubclass reagents were the kind gift of Dr. Charles Reimer (Immunological Products Branch, Center for Disease Control, Atlanta, GA). The following clones were used: 6012 (originally from Dr. R. Jefferis, Birmingham, England [34]), anti-IgG1; 6014, anti-IgG2; 6051, anti-IgG3; and 6022, anti-IgG4. The specificity of these clones was confirmed with standard curve assays utilizing appropriate and inappropriate myeloma proteins, either directly bound to microtiter plates or bound by anti-light chain reagents. Each of the clones showed no significant cross-reactivity, except 6012, which in some situations recognized IgG3 at ~5% the level of IgG1, and IgG2 and IgG4 at <1%. The monoclonal antibodies were all obtained as lyophilized ascitic fluids and were reconstituted in 0.5% BSA with 0.1% azide.

ELISA assays. Specific antibodies and their isotypes were detected in ELISA assays, which were modifications of a previously described technique (24). Sm or other antigens were added to polyvinyl chloride microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) in 0.2 M borate-buffered saline, pH 8.4, with five protease inhibitors (0.5 mM paramethylsulfonyl fluoride, 10 mM EDTA acid, 50 mM ϵ -aminocaproic acid, 1 mM *o*-phenanthroline, and 10 mM benzamide [Sigma Chemical Co., St. Louis, MO]) (BBS-PI). After 5-h incubation at 4°C, the plates were washed and were nonspecifically coated with BBS-PI plus 0.5% BSA and 0.4% Tween 80. In the IgG subclass assays, 0.1% normal goat serum was also added to the coating buffer to decrease background. After 1 h at 4°C, the plates were aspirated and samples were added in coating buffer. Each sample was tested at three dilutions for every isotype. The appropriate dilutions (that fell in the linear portion of the standard curves) were determined empirically in preliminary assays with each serum and ranged from 10⁻³–10⁻⁵. The plates were incubated overnight at 4°C. They were washed again on the following day, and affinity-purified anti-isotype biotinylated reagents were added for a further incubation at 4°C for 3 h. The concentrations of the anti-isotype reagents used were determined empirically in preliminary experiments to give approximate comparable optical densities (ODs). After this incubation, the plates were again washed, and

avidin-alkaline phosphatase was added in coating buffer for a further 3-h incubation. The plates were then again washed, and the substrate paranitrophenylphosphate was added at a concentration of 1 mg/ml in 0.01 M diethanolamine, pH 9.8. The development of OD₄₅₀ was followed at appropriate intervals with a Dynatech microELISA auto-reader (MR580; Dynatech Laboratories).

The assays with monoclonal antibodies were performed in a similar manner, up to the second day of assay. At that time, the monoclonals were added in diluting buffer. The dilutions utilized were determined in preliminary experiments to give comparable ODs when tested against myeloma proteins that had been coated directly onto microtiter plates: 6012, 1:25,000; 6014, 1:7500; 6051, 1:10,000; and 6022, 1:40,000. The plates were incubated for 2 h at 4°C, washed, and then a 1:1000 dilution of affinity-purified biotinylated rabbit anti-mouse IgG was added for a further 2-h incubation at 4°C. After further washing, avidin-alkaline phosphatase at a dilution of 1:500 was added for 2 h at 4°C. The plates were then washed again, and substrate was added for color development as described above.

It should be emphasized that appropriate absorptions were critical to the performance of the ELISA assays described. For example, the reagents used in the first step of the standard curve assays with the polyclonal antisera had to be absorbed against IgG from rabbits and monkeys to prevent direct binding to the polyclonal anti-isotype sera. In addition, each biotinylated polyclonal anti-isotype antibody had to be absorbed with inappropriate isotype myeloma proteins in solid phase. The exact absorptions were determined in extensive preliminary assays using graded amounts of gels of each specificity. The rabbit anti-mouse IgG used to develop the monoclonal antibody assays was absorbed with human IgG. In addition, all polyclonal reagents were absorbed with antigens tested in a given assay.

A critical aspect for the interpretation of the isotype assays was the ability to compare them quantitatively. With the polyclonal anti-isotype reagents, this was accomplished by constructing standard curves for each isotype. For IgA, IgD, IgM, IgE, and IgG, the standard curve ELISA was performed by initially coating the plates with 1 $\mu\text{g}/\text{ml}$ of affinity-purified rabbit anti-human λ -chains. Then, in place of serum samples, graded dilutions of λ -light chain myelomas of the appropriate heavy chain were added. The assays were developed as described above, and standard curves were constructed after subtracting the appropriate controls. The ODs obtained for each serum for each isotype were referred to these standard curves in order to yield an estimate in micrograms per milliliter of anti-Sm antibody of that isotype. For the IgG subclass assays, the initial coating step was a goat anti-human F(ab')₂, which had mainly anti- κ -chain specificity. For the standard curve, dilutions of κ chain myelomas of the appropriate IgG subclasses were utilized. For the light-chain assays, standard curves were constructed by using affinity-purified anti-IgG1 antibodies as the first step, and IgG1 myelomas of κ - and λ -light chains in graded amounts.

With the monoclonal antibody assays, it was not feasible to construct such standard curves. Therefore, the reagents were initially standardized by measuring binding to microtiter plates coated with graded amounts of myeloma proteins. Equivalent binding of the myeloma proteins to the microtiter plates was assured by simultaneous assays with a monoclonal anti-IgG reagent which did not have subclass selectivity. Appropriate dilutions were chosen to insure that each of the four subclass assays gave comparable ODs.

Anti-RNP antibodies. The monoclonal antibodies were utilized in a similar set of assays to determine the IgG subclass distribution of anti-rRNP antibodies. Since the RNP preparation was inevitably contaminated with Sm determinants, all sera in this assay were simultaneously tested against Sm. The increased reactivity of sera typed as anti-RNP with the RNP antigen compared with the Sm antigen indicated that the assay preferentially measured anti-RNP rather than anti-Sm antibodies. Sera typed as anti-Sm-positive, anti-RNP-negative in the same assays showed much higher reactivity with Sm as compared with RNP.

Anti-DNA antibodies. Anti-ds DNA antibodies were measured by

a standard *Crithidia lucilia* assay using commercial slides (Kallestad Laboratories, Inc., Austin, TX). The sera were tested at neat, 1:4, 1:10, and then at serial two-fold dilutions. The assays were developed with fluorescein-conjugated IgG fractions of anti-isotype sera, which were purified by absorptions as described (35). Each IgG was used at a concentration fourfold less than the lowest concentration that gave a visible immunoprecipitin line against an appropriate myeloma protein. C3 fixation test by *Crithidia* was done as previously described (36).

Isoelectric focusing of anti-Sm antibodies. Isoelectric focusing was performed in 1% agarose (IsoGel; Marine Colloids, Rockland, ME) with 2% ampholytes, pH 3.5–9.5 (Marine Colloids). The gels were focused for 10 min at constant power of 1 W, then for 30 min with constant power of 10 W. The adequacy of focusing of each run was monitored with easily visualized standard proteins, including horse myoglobin, sperm whale myoglobin, cytochrome C, and ferritin (Sigma Chemical Co., St. Louis, MO). Complete focusing was further assured by adding a strong anti-Sm serum in both the acidic and alkaline ranges and determining that the final banding patterns were comparable. Sera to be tested were dialyzed against ampholytes and added by a filter paper strip near the expected acidic portion of the gel. When the focusing was complete, the pH gradient was read by microelectrode, and the proteins were precipitated in 18% sodium sulfate for 1 h at room temperature. Precipitated bands were fixed with 0.5% glutaraldehyde in 18% sodium sulfate for 1 h at room temperature, followed by sodium borohydride at 10 $\mu\text{g}/\text{ml}$ for 2 h at room temperature. The fixed gels were then washed overnight. The following day, they were overlaid with a cellulose acetate sheet impregnated with 100 $\mu\text{g}/\text{ml}$ of purified Sm conjugated to biotin. After further washing, the gels were exposed to avidin conjugated with horseradish peroxidase, followed by further washing, and then developed with substrate aminoethylcarbazole plus hydrogen peroxide.

Results

Specificity of the anti-Sm ELISA assay. In preliminary investigations, we determined the antigen specificity of our anti-Sm ELISA assay. The antigen utilized was highly purified, and was the same preparations used in our murine anti-Sm ELISA assays (24). In a survey of human sera, only immunodiffusion positive anti-Sm sera gave strong positivity in the ELISA assay (Fig. 1). Some patients with weaker immunodiffusion lines did not give consistent ELISA positivity, and a single patient with SLE that was immunodiffusion-negative gave significant ELISA results.

We also utilized an extensive series of controls to assure the isotype specificity of each assay. All affinity-purified anti-isotype reagents had to be absorbed further for use in the ELISA assay. Their specificity in the ELISA was then guaranteed by testing of the isotype reagent against all other isotypes in the standard curve assay, and in the anti-Sm assay itself, by inhibiting positivity for each isotype preferentially with the appropriate isotype. With the monoclonal reagents, the specificities were guaranteed by testing in standard curves.

Isotype analysis of anti-Sm antibodies. 19 anti-Sm-positive SLE sera gave strong enough results in the ELISA assay for isotype analysis. The overall isotype distributions are compiled in Table I. In every case, the most prominent isotype was IgG1. In many sera, particularly those with high anti-Sm values, over 90% of the antibody was of the IgG1 isotype. In fact, as shown in Fig. 2, there was a direct correlation between the level of IgG1 anti-Sm and the fraction of the antibody that was of this isotype ($r = 0.85$).

All sera also had IgG3 and IgM anti-Sm antibodies detectable. Some sera had IgA, IgG2, or IgG4 antibodies present,

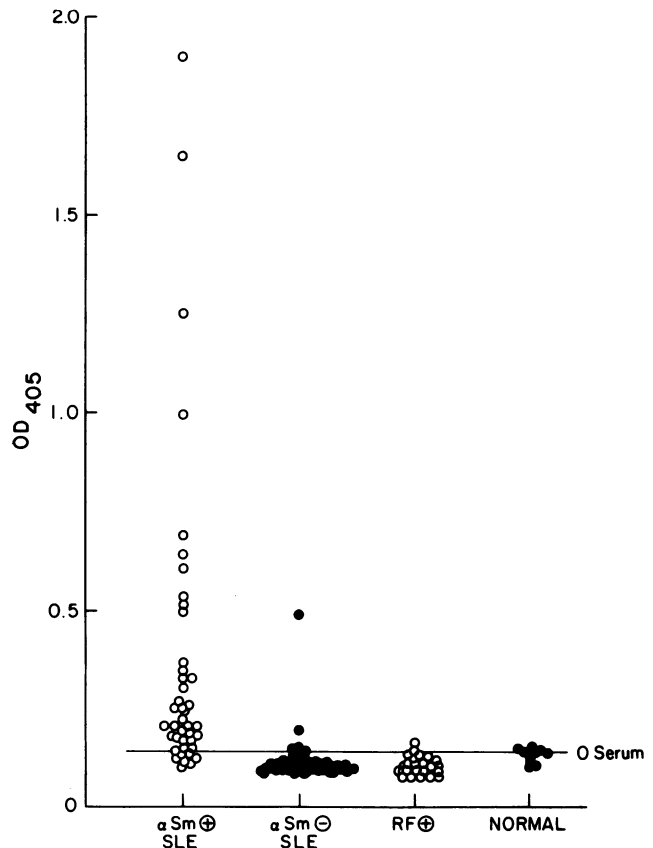


Figure 1. Specificity of anti-Sm ELISA for immunodiffusion-positive anti-Sm sera. 42 anti-Sm-positive, 42 anti-Sm-negative SLE, 29 rheumatoid factor (RF)-positive, and 11 normal sera were tested at a 10^{-4} dilution for IgG1 anti-Sm antibodies.

usually in very low amounts. No IgD or IgE anti-Sm antibodies were seen above the limits of detectability (0.2 and 0.02 $\mu\text{g}/\text{ml}$, respectively).

Several points emerge from these data. First of all, in every case, the anti-Sm response was mostly IgG. In particular, the IgG1 isotype was represented far out of proportion to its expected concentration in total serum IgG (37, 38). Table I presents the mean isotype concentration for the 19 sera examined. By this analysis, IgG1 represents 96% of the total population's anti-Sm antibody or 98% of the population's IgG anti-Sm. If a geometric mean is determined for each isotype, then IgG1 is found to represent 91% of the total population anti-Sm or 95% of IgG anti-Sm. Finally, if the percentage of each isotype of anti-Sm is determined for each serum separately and then these percentages are averaged, then IgG1 is found to represent an average of 79% of total serum anti-Sm or 87% of IgG anti-Sm.

In an additional series of experiments, we determined the light chain distribution of anti-Sm antibodies in these same sera. The results, shown in Fig. 3, indicate that in every case, except one weakly reactive serum, both κ - and λ -chains were detectable. In most sera, the κ -chains were somewhat more prominent than the λ -chains and the overall geometric means of κ -anti-Sm and λ -anti-Sm were 101 and 55, respectively. This light chain distribution is quite similar to the expected distribution of light chains in serum IgG.

Further validation of the IgG subclass assays. Although

Table 1. Isotype Distribution of Human Anti-Sm Antibody*

Patient	IgM	IgD	IgG4	IgG2	IgG3	IgG1	IgE	IgA
1	11	<0.2	4	<10	16	15,000	<0.02	<0.4
2	16	<0.2	1	<10	15	1,700	<0.02	<0.4
3	9	<0.2	42	85	32	5,900	<0.02	3
4	11	<0.2	0.1	11	1	1,500	<0.02	<0.4
5	17	<0.2	<0.1	<10	0.4	700	<0.02	1
6	100	<0.2	0.1	11	3	570	<0.02	8
7	6	<0.2	0.1	<10	0.7	190	<0.02	0.8
8	6	<0.2	0.1	52	2	380	<0.02	17
9	40	<0.2	0.8	<10	0.8	480	<0.02	<0.4
10	14	<0.2	0.2	<10	105	510	<0.02	<0.4
11	5	<0.2	<0.1	<10	29	190	<0.02	4
12	14	<0.2	<0.1	<10	13	16	<0.02	<0.4
13	78	<0.2	<0.1	<10	28	123	<0.02	4
14	53	<0.2	<0.1	<10	19	80	<0.02	2
15	27	<0.2	<0.1	<10	45	60	<0.02	<0.4
16	42	<0.2	<0.1	<10	21	155	<0.02	<0.4
17	23	<0.2	<0.1	<10	6	1,300	<0.02	0.6
18	17	<0.2	<0.1	<10	26	190	<0.02	2
19	11	<0.2	<0.1	<10	37	210	<0.02	1
\bar{X}	26	<0.2	2.6	16	20	1,540	<0.02	3.3
SEM	6.0	—	2.2	4.4	5.7	808	—	1.2
Percent of total	1.6	<0.01	0.16	1.0	1.2	96	<0.001	0.2

* Values given in micrograms per milliliter.

every isotype anti-Sm assay was performed simultaneously with a standard curve that had been previously checked for specificity and reliability, it remained possible that the marked skewing of the anti-Sm response to the IgG1 isotype could be an artifact due to a systematic error introduced by our methodology. In order to rule out this possibility, we utilized a set of murine monoclonal antibodies with specificities of the human IgG subclasses. Due to the peculiarities of the monoclonal reagent and their limited supply, we could not develop standard curves with these reagents. Instead, they were used

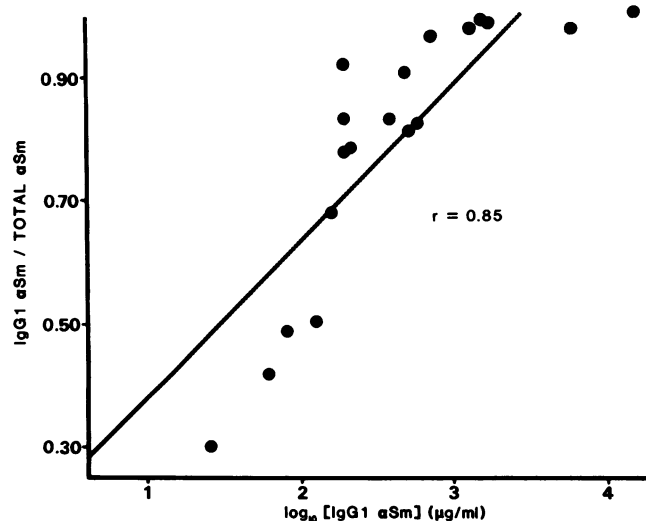


Figure 2. Correlation of serum IgG1 anti-Sm levels and subclass restriction. The horizontal axis depicts the absolute amount of IgG1 anti-Sm. The vertical axis shows this quantity divided by the total serum anti-Sm as determined by summing the absolute quantities of each separate isotype.

at predetermined dilutions that were found to be relatively equipotent in detection of myeloma proteins in ELISA assays. The results of a typical experiment are summarized in Fig. 4. In agreement with the results of polyclonal antisubclass reagents,

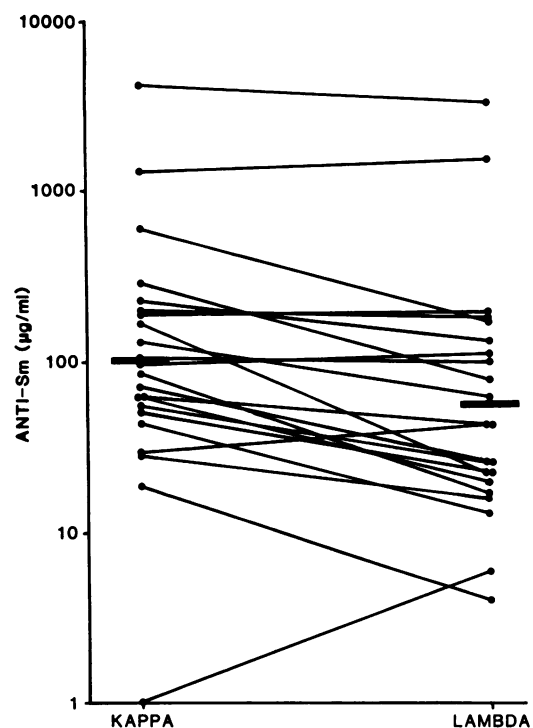


Figure 3. Light chain distribution of anti-Sm antibodies. Absolute amounts of anti-Sm antibodies of the κ - and λ -isotopes were determined as described. κ - and λ -determinations for an individual patient are connected by a straight line. Also shown is the geometric mean levels of the κ - and λ -anti-Sm antibodies.

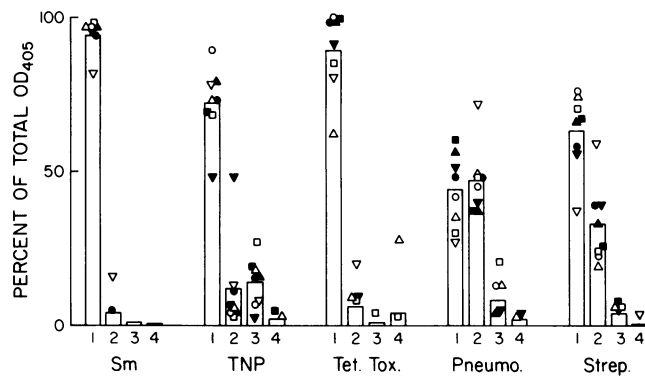


Figure 4. IgG subclass distribution of anti-Sm and other antibodies determined with hybridoma reagents. ELISA assays for the indicated antigen specificities were developed with standardized IgG subclass specific monoclonal antibodies. For each serum, the sum of the ODs obtained in all four assays was divided into the results with each monoclonal to determine the percent contribution of each subclass to the total response. The bars indicate the mean percents for each subclass for all sera. Seven individual anti-Sm sera are shown and correspond to those listed in Table I: ○, patient 1; △, patient 14; ▽, patient 8; □, patient 2; ●, patient 5; ▲, patient 17; ▼, patient 3; □, normal human serum. Points < 3% were omitted from the graph for clarity. All sera were tested in all assays, except that the results of the normal serum in the anti-Sm assay did not give sufficient OD for analysis. Antigens utilized: Sm; TNP-BSA; tetanus toxoid (*Tet. tox.*); Pneumovax (pneumococcal polysaccharides (*Pneumo.*)); group A streptococcal cell wall (*Strep.*).

the anti-Sm response appeared to be markedly skewed to the IgG1 isotype. In contrast, pneumococcal polysaccharide antibodies in these same sera were mainly IgG2 and IgG1, while anti-TNP and anti-group A streptococcal cell wall were of several isotypes. Tetanus toxoid antibodies in these sera were mainly IgG1, as has been previously reported (16). As none of these assays showed significant IgG4 antibodies, we performed an additional control with 12 hyperimmune antiragweed sera (provided by Dr. David Klapper, University of North Carolina, Chapel Hill, NC) in a ragweed-specific ELISA and 7 hyperimmune antihouse dust mite sera (provided by Dr. Thomas Platts-Mill, University of Virginia, Charlottesville, VA) in a mite-specific ELISA. In one antiragweed serum and one antimites serum, the specific antibodies were mainly IgG4, as determined with the monoclonal reagents. Although this assay could not be done with the anti-Sm SLE sera, it did confirm that the monoclonal IgG4 reagent would bind to appropriate antibodies in an ELISA assay similar to the anti-Sm assay.

Isoelectric focusing analysis of anti-Sm antibodies. The light chain distribution of anti-Sm antibodies, as determined by the ELISA technique, suggested that these antibodies were polyclonal, in spite of their subclass restriction. We further analyzed this issue by focusing anti-Sm sera in agarose gels and overlaying the patterns with biotinylated Sm antigen. Positive staining was obtained with 10 of the stronger anti-Sm sera. Multiple anti-Sm negative SLE and normal sera gave no visible staining. Although in some cases anti-Sm bands were present, in all patterns the anti-Sm activity included diffuse staining ranging from an isoelectric point of pH 6 to pH 9.5 (Fig. 5). These patterns were consistent with the involvement of multiple clones in the anti-Sm response in every serum tested.

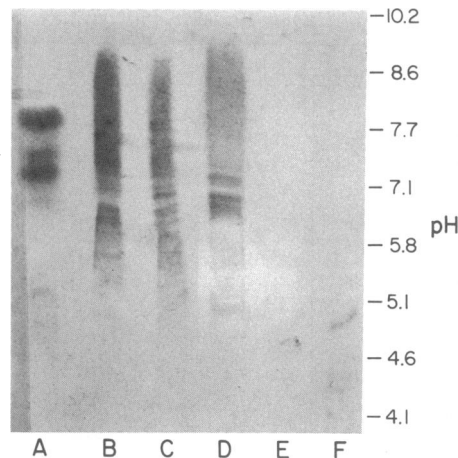


Figure 5. Isoelectric focusing analysis of the heterogeneity of anti-Sm antibodies. Anti-Sm-positive sera (lanes B, C, and D corresponding to patients 1, 3, and 8 from Table I) and normal human serum (lane E) were focused in agarose gels and overlain with Sm antigen in an enzyme technique. Lane A contains myoglobin and cytochrome C standards, while lane F contains ferritin.

IgG subclass of anti-RNP antibodies. Anti-RNP antibodies are related to anti-Sm antibodies because the two specificities are frequently associated in SLE patients and because the antigenic determinants for each specificity are complexed together in nRNP particles (39, 40). We would therefore predict that the immunoregulation of anti-RNP and anti-Sm specificities would be similar and that anti-RNP antibodies should also be restricted to the IgG1 subclass. To test this hypothesis, we used the monoclonal antibodies to determine the IgG subclass distribution of nine sera typed as anti-RNP-positive, anti-Sm-negative by double immunodiffusion. The anti-RNP sera selected showed much higher reactivity with the RNP preparation than they did with (RNase-digested) Sm (Table II). The IgG subclass distribution of the anti-RNP antibodies was similar to what we had found with anti-Sm antibodies, in that the IgG1 isotype accounted for >90% of the reactivity.

Isotype of anti-DNA antibodies. In a final set of experiments, we determined the isotype distribution of anti-DNA antibodies by the *Crithidia lucilia* assay. The polyclonal isotype reagents were utilized at a dilution four times greater than their endpoint titer, as determined by immunodiffusion. The results are shown in Table III. In accord with previous work by others (41, 42), the anti-DNA response was generally restricted to IgM and the IgG1 and IgG3 isotypes. In most cases, the titer with the IgG1 reagent was higher than with the IgG3 reagent. Occasional sera had IgG4, IgG2, or even IgE anti-DNA antibodies. Specificity for each isotype was checked by inhibition with appropriate myeloma proteins. Light chain analysis indicated the presence of both κ - and λ -anti-DNA antibodies in the great majority of sera. Complement fixation by the *Crithidia* assay was also determined on these same sera and was found not to correlate with any obvious aspects of the isotype distribution.

Discussion

We have determined that the anti-Sm autoantibodies in sera of patients with SLE were relatively restricted to the IgG1

Table II. IgG Subclass Distribution of Anti-RNP Antibodies

Patient	Diagnosis	Percent of total OD				Total OD for four subclasses	
		IgG1	IgG2	IgG3	IgG4	$\Sigma(\text{OD}_{\text{RNP}})$	$\Sigma(\text{OD}_{\text{Sm}})$
1	PSS/DM	95	4	1	0	1.420	0.136
2	SLE	74	3	1	21	0.775	0.063
3	PSS	97	2	1	1	1.143	0.039
4	PSS/DM	93	2	4	0	0.461	0.044
5	MCTD	86	12	2	0	0.367	0.087
6	SLE	98	1	1	0	0.627	0.446
7	SLE	94	4	1	0	1.063	0.599
8	MCTD	96	1	2	1	0.353	0.131
9	Cervical dysplasia	92	1	2	4	0.752	0.305
\bar{X}		92±8	3±3	2±1	3±7	0.773±0.371	0.206±0.200

Abbreviations used in this table: DM, dermatomyositis; MCTD, mixed connective tissue disease; PSS, progressive systemic sclerosis.

isotype. In spite of this subclass restriction, these antibodies were polyclonal, as judged by the presence of both κ - and λ -light chains and by the diffuse patterns on isoelectric focusing. The related specificity anti-RNP was also nearly entirely IgG1. Anti-DNA antibodies were mainly of the IgM, IgG1, and IgG3 isotypes, and like the anti-Sm antibodies, contained both κ - and λ -light chains.

An essential aspect of studies of this sort is an assurance of specificity of the assays employed and the avoidance of systematic methodological idiosyncrasies which would skew the results toward a particular isotype. The specificity of

isotype detection was monitored by initial preliminary studies with standard curves of inappropriate myelomas. In addition, the actual anti-Sm and anti-DNA tests were further controlled by showing that only the appropriate myeloma would inhibit the assay positivity. To rule out further any possibility of systematic errors, the IgG subclass distribution was confirmed using monoclonal reagents, which showed the same striking predominance of the IgG1 subclass in the anti-Sm response, while other responses had different subclass distributions (Fig. 5).

The subclass distribution of human autoantibodies has

Table III. Ig Isotypes in Human Systemic Lupus Erythematosus ds DNA Antibodies as Determined by Indirect Immunofluorescence Using Crithidia Luciliae (liter/titer)

Patient	Polyvalent	IgM	IgD	IgG4	IgG2	IgG3	IgG1	IgE	IgA	κ	λ	C3 fixation
Ba	2,560	640	—	—	—	40	160	—	—	640	640	8
McK	1,280	320	—	20	4	20	80	—	—	320	320	128
Th	1,280	320	—	—	—	80	640	—	—	320	640	—
Va	1,280	10	—	—	—	40	320	—	—	80	160	ND
Gr	640	160	—	—	—	80	160	—	—	320	640	512
He	640	10	—	4	—	40	10	—	—	20	20	—
Mu	640	80	—	20	—	80	40	—	—	80	80	32
Gi	640	80	—	—	—	4	10	—	—	80	—	—
Ed	512	640	—	—	—	40	320	—	—	80	160	—
Ra	320	4	—	—	—	10	4	—	—	20	4	—
Hal	320	4	—	—	—	10	80	—	—	80	80	—
Br	320	4	—	—	—	4	—	4	—	—	80	—
Fi	320	80	—	—	—	—	80	—	—	—	—	—
Har	320	640	—	—	—	—	40	—	—	80	320	—
Wi	320	40	—	—	—	80	640	—	—	320	320	ND
Pe	160	20	—	—	—	20	20	—	—	40	20	—
Br	160	—	—	—	—	20	—	4	—	40	40	—
Mo	160	40	—	—	—	—	—	—	—	—	—	—
Les	160	4	—	—	—	1	40	—	—	80	80	ND
McP	80	—	—	—	—	4	4	4	—	4	4	ND
Oa	80	40	—	—	—	—	—	—	—	—	—	4
Pe	80	—	—	—	—	4	80	—	—	—	—	—
Ph	40	10	—	—	—	—	—	—	—	—	—	—
Al	40	80	—	—	—	1	10	4	—	40	10	2
Lea	40	4	—	—	—	10	4	4	—	4	4	—
McD	20	—	—	—	—	—	—	—	—	—	80	—

ND, not determined.

been examined by several investigators previously. Sontheimer and Gilliam (41) found that anti-DNA antibodies are mainly of the IgG1 and IgG3 subclass, in accord with our results. Zouali et al. (42) also confirmed the distribution of anti-DNA antibodies. However, they reported that anti-RNP antibodies were of the IgG2 subclass, in contrast to our finding of an IgG1 restriction similar to that of anti-Sm. Antinuclear antibodies detected by immunofluorescence have been characterized as unrestricted (35, 43), or as mainly IgG1 (44) or IgG1 and IgG3 (45). Antithyroglobulin has been found to be unrestricted (46), while the acetylcholine receptor antibodies in certain myasthenic patients were restricted to the IgG3 subclass (47). In our studies of anti-La (anti-SS-B) antibodies, we have found that they are mainly of the IgG1 subclass, in accord with our results with anti-Sm (Pearce, D. C., W. J. Yount, and R. A. Eisenberg, submitted for publication). Finally, in our earlier work with the anti-Sm response in mice, we found a relative restriction to the IgG2a subclass (24).

The isotype restriction and polyclonality of the anti-Sm response suggests certain conclusions regarding their production *in vivo*. First, since the anti-Sm, anti-RNP, anti-La, and anti-DNA responses are all relatively restricted to the same T cell-dependent isotype (IgG1), it is probable that T cells play an important role in the production of these antibodies *in vivo* and their immunoregulation may be similar. Second, the polyclonality of the anti-Sm response, like that of the anti-La response, suggests that it is not the result of a small number of mutations creating "forbidden clones" (48). Rather, it is more probable that the response is antigen-driven. This is consistent with interpretations based on the antigen specificity of these responses (49, 50). The current work is also consistent with previous studies which showed that anti-Sm was mainly IgG and could fix complement (51).

The present results may also explain the old observation of restricted electrophoretic mobility of anti-Sm antibodies (28). This is probably due to their subclass restriction, rather than to oligoclonality. In addition, the relatively normal distribution of κ - and λ -light chains in the anti-Sm response indicates that neither chain is uniquely essential for the anti-Sm specificity. This is in distinction to the rheumatoid factor response, which either in monoclonal rheumatoid factors or in the polyclonal response, is relatively κ restricted (52).

At this point, it is essential to further define the heterogeneity and specificity of the anti-Sm response. This is complicated by the fact that the Sm antigen contains multiple polypeptides, although our preparation may be somewhat simpler (29, 50). Therefore, it is possible that certain populations of anti-Sm antibodies may see one peptide in favor of another. We have recently developed a polyclonal anti-idiotypic reagent which identifies a cross-reactive idio-type(s) in anti-Sm antibodies from some of the same sera tested in the present report (53). Future investigations will involve the definition of anti-Sm idiotypes by these reagents, as well as with monoclonal reagents, and the determination of the relationship of these idiotypes to specificity of anti-Sm antibodies for different Sm polypeptides.

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