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Research Article

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Clinical and Serological Differences between Systemic Lupus Erythematosus Patients with Antibodies to Ro Versus Patients with Antibodies to Ro and La

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ABSTRACT Among 55 systemic lupus erythematosus patients having antibodies to Ro and/or La, two major groups were distinguished by titration of sera in counterimmunoelectrophoresis. The first group (30 patients) had antibodies to Ro alone. This was associated with a high incidence of antibodies to DNA (77%) and serious renal disease (53%). The second group (23 patients) had antibodies to Ro and La, and this was associated with a lower incidence of antibodies to DNA (30%) and a very low incidence of nephritis (9%). In this group a phenomenon of linkage of anti-Ro and anti-La titers was observed. Additionally two patients with only anti-La were found. Neither had clinically apparent renal disease. Thus, systemic lupus erythematosus patients with anti-Ro fall into two subgroups that differ considerably in their prevalence of anti-DNA and serious renal disease.

INTRODUCTION

Antibodies to the soluble antigens Ro and La are among the several non-DNA antigen-antibody systems that occur in a significant number of patients with connective tissue diseases (1). Ro is now known to be antigenically identical to SSA, and La is known to be antigenically identical to SSB and Ha, respectively (2, 22). The two principal connective tissue diseases in which these antibodies are found are systemic lupus erythematosus (SLE)[1] and Sjogren's syndrome. In SLE patients, several correlations with anti-Ro have been noted. They are the increased incidence of photosensitive skin disease, rheumatoid factor positivity, Sjogren's syndrome (3), and a greatly increased incidence of the DRW_3 haplotype (4, 5). Also a number of these patients tend not to make antibodies to nuclear antigens present in the usual mouse liver or kidney substrates used for the fluorescent antinuclear antibody test (6). Evidence for the participation of Ro/anti-Ro immune complexes in the development of nephritis has been published (7).

Studies using the Ouchterlony technique of double diffusion in agar (8) indicated that antibodies to La were nearly always found in association with antibodies to Ro. A quantitative study of sera from SLE patients with the more sensitive counterimmunoelectrophoresis technique has led to the characterization of two large groups of patients—those having anti-Ro alone and those having both anti-Ro and anti-La. A few patients with only anti-La were identified. This paper examines the clinical and serologic differences between the two main groups of patients.

METHODS

Sera. Patient sera were selected for further testing if adequate clinical information (including follow up) was available and they were found to have either anti-Ro or anti-La by Ouchterlony analysis. Sera containing anti-Sm or anti-nRNP were excluded from this study. Thus sera designated as anti-Ro had only this identifiable precipitin in both double diffusion and counterimmunoelectrophoresis (CIE) tests except for 2 of 30 sera that had unidentified lines. Those determined to contain anti-Ro and anti-La were free of anti-Sm, anti-nRNP, or any other unidentified precipitin lines. All but three patients had four or more of the preliminary American Rheumatism Association criteria for SLE: two of the three were in the anti-Ro and anti-La group and the other in the anti-La alone group. When multiple serum samples were available, the first sample was usually tested.

Normal sera were obtained from laboratory volunteers.

Quantitation of anti-Ro and anti-La titers by CIE. A quantitative titer for anti-Ro and anti-La in each serum was obtained by finding the highest dilution of serum that gave a line in CIE against a source of either Ro or La antigen.

CIE used the following modification of the technique by Johnson et al. (10). Glass plates (8 × 10 cm) were layered

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[1] *Abbreviations used in this paper:* CIE, counterimmunoelectrophoresis; HSE, human spleen extract; PBS, phosphate-buffered saline; SLE, systemic lupus erythematosus.

with 17 cm^3 of 1% agarose in barbital buffer, pH 8.2, and two parallel rows of holes were punched in the agar. Cathodal wells that contained antigen were 3 mm in diameter whereas anodal wells containing dilutions of sera were made 4 mm in diameter to allow for increased sensitivity in detecting antibody titers. 25 μl of diluted serum was placed in the anodal wells and the plates were electrophoresed for 15 min at 25 mA. 15 μl of Ro or La source were placed in the cathodal wells and the plates were electrophoresed for another 40 min. The plates were examined at 24 h for a precipitin reaction. Immersion of the plates in 5% sodium citrate for 20 min was used to remove nonspecific precipitates.

The Ro source was a partially purified human spleen extract (HSE) treated with trypsin to destroy La activity. HSE was made by homogenizing spleen tissue obtained at autopsy in a low-speed blender in 0.01 M PO_4, 0.15 M NaCl, 0.01 M Na Azide, pH 7.2 phosphate-buffered saline (PBS) with a tissue to buffer weight to volume ratio of 1:3. All procedures were performed at 4°C. The homogenate was then centrifuged for 3 h at 100,000 *g* (model L, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). The ultracentrifuged supernatant was then added to a DEAE-cellulose (DE52 preswollen, Whatman, Inc., Chemical Separation Div., Clifton, NJ) column equilibrated with PBS. The column was washed with PBS and the Ro activity eluted with phosphate buffer made 0.5 M in NaCl. A 60–80% ammonium sulfate fraction made from the DEAE eluate was dissolved in distilled water, then dialyzed against PBS and concentrated. Trypsin treatment of this concentrated material was done by adding trypsin (Sigma Chemical Co., St. Louis, MO) to a final concentration of 200 μg/ml and incubating at 37° for 1 h. The digestion was stopped by adding soybean trypsin inhibitor (Sigma Chemical Co.) to a concentration of 400 μg/ml.

This trypsin-treated material gave only a single line with widely varying dilutions of both monospecific anti-Ro sera and sera containing both anti-Ro and anti-La. Furthermore, by using a technique of staggered antibody wells (Results) in CIE, a relationship of identity could be demonstrated with the precipitin line of monospecific anti-Ro sera and the single precipitin line with sera known to contain anti-Ro and anti-La.

The La source was calf thymus extract from which Ro antigen had been removed. Fresh calf thymus tissue was homogenized and centrifuged as detailed above. The NaCl concentration of the supernatant from ultracentrifugation was increased to 0.5 M and passed through an anti-Ro affinity column. CIE testing of eluates from the anti-Ro column with anti-Ro serum verified that Ro antigen had been removed. The extract was then dialyzed against PBS and stored at 20°C until needed. The anti-Ro affinity column was prepared by activating 10 cm^3 (packed volume) of sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, NJ) with 1.25 g of cyanogen bromide at pH 11 and at −20°C and adding 4 N NaOH and ice, using a method modified by Cuatrecases and Anfinsen (11). The activated beads were washed with 0.1 M PO_4, pH 6.5, and then coupled with 200 mg of the immunoglobulin (IgG) fraction of a monospecific high titered anti-Ro serum that had been obtained by DEAE chromatography at pH 7 and then dialyzed against 0.1 M PO_4, pH 6.5. Incubation was carried out at 4°C for 24 h. Greater than 90% of the IgG was bound by this method and the coupled beads were then inactivated with 1 M ethanolamine at pH 8. The beads were then alternately washed with 0.1 M sodium bicarbonate, pH 9, and 0.1 M acetate, pH 4, and then equilibrated with 0.01 M PO_4, 0.5 M NaCl, pH 7.2, buffer.

A highly purified La source was prepared for additional testing by affinity chromatography. The La source, devoid of Ro, in 0.01 M PO_4, 0.5 M NaCl, pH 7.2, was applied to an anti-La affinity column (IgG from a serum known to contain high titer of anti-La processed and conjugated as above) until the eluate contained detectable La activity by CIE. The column was extensively washed with 0.1 M PO_4, 0.5 M NaCl, pH 7.2, until the optical density of the eluate at 280 nm was <0.02 OD units. Adsorbed La antigen was eluted with 0.5 M glycine HCl, 0.15 NaCl, pH 3. The eluate was immediately neutralized with 1.0 N NaOH, concentrated and dialyzed against PBS. Antigenic activity was confirmed by double immunodiffusion. Such La preparations did not react with monospecific anti-Ro sera and gave a single precipitin line with human sera containing both anti-Ro and anti-La activities.

Determination of antibodies to DNA. Antibodies to single-stranded DNA (ssDNA) were assayed by a microcomplement fixation technique (12) using heat-denatured calf thymus DNA. Titers of antibodies to ssDNA were also measured by a radioimmunoassay using the double antibody technique (13). Antibodies to native DNA were detected by indirect immunofluorescence using *Crithidia luciliae* as the substrate (14). Antinuclear antibody was detected by indirect immunofluorescence according to the method of Coons (21). All sera were assayed for ANA at a 1:20 serum dilution using mouse liver sections as the tissue substrate.

Statistical methods. Chi square analysis was performed where appropriate to assess the statistical significance of data between the patient groups.

RESULTS

Patterns of anti-Ro and anti-La titers. Of the 55 sera tested, 30 were found to have anti-Ro alone (Table I) and 23 were found to have both anti-Ro and anti-La (Table II). Two sera were found with anti-La alone. Over an extended period of follow up with predominantly Ouchterlony testing we have found no significant lability of titers to anti-Ro or anti-La that would cause confusion over classification of any patients. Indeed sera containing anti-Ro always contained only anti-Rō in every sample we have tested. In the patient with the longest follow up (I.B., for 15 yr) 10 samples over the period of observation have contained only anti-Ro. Similarly sera containing both anti-Ro and anti-La have always been found to contain both antibodies in all samples tested.

Sera giving a precipitin line with the La source were further evaluated to establish the relationship of the line to the La anti-La precipitin system. This was done in two ways. First, selected sera were found to give a precipitin line in CIE against a highly purified La preparation derived by acid elution from an anti-La affinity column. Second, one of the sera (ET) was used in a staggered well arrangement in CIE to confirm the immunologic identity of other sera giving lines with the standard La source. Fig. 1 shows a line of noni-

TABLE I

Serologic Features in Patients Having Anti-Ro Alone

Patient	Anti-Ro titer	Anti-La titer	ANA	Antibodies to DNA		
				Anti-ss DNA complement fixation	Anti-ss DNA RIA	Antinative DNA crithidia titer
RH	1/4	0	+	+	+	1/1,250
IB	1/32	0	+	−	−	—
LS	1/16	0	+	−	+	—
PW	1/4	0	+	+	−	—
SW	1/128	0	+	−	−	—
RB	1/4	0	+	+	−	—
KG	1/4	0	+	+	−	1/10
CW	1/2	0	+	−	+	—
KL	1/16	0	+	+	+	—
XS	1/4	0	+	+	−	—
KS	1/4	0	+	−	+	—
PC	1/4	0	+	+	−	—
KS	1/64	0	+	+	+	1/1,250
JM	1/256	0	+	+	+	1/1,250
DC	1/32	0	+	+	+	1/6,250
MA	1/8	0	+	−	−	—
DS	1/8	0	+	+	+	1/250
BG	1/8	0	+	+	+	—
CV	1/32	0	+	+	+	1/50
MA	1/2	0	+	+	+	—
WF	1/4	0	+	+	+	1/10
BM	1/4	0	+	−	−	—
KM	1/16	0	−	−	−	—
MG	1/2	0	+	−	−	—
KW	1/64	0	+	+	ND	1/1,250
CC	1/128	0	−	−	−	—
WD	1/4	0	+	+	+	—
WL	1/32	0	+	+	+	—
MT	1/64	0	+	+	−	—
CT	1/32	0	+	+	+	1/6,250

ND—Not determined.

dentity with serum 1 (not anti-La) but clear lines of identity with sera 2, 3, and 4, confirming the presence of anti-La in these sera.

As can be seen in Table II, there is a linkage or consistent relationship of the titers of anti-Ro and anti-La. Specifically, anti-Ro titers are either equal to or higher than anti-La titers. In 12 instances the titers of anti-Ro and anti-La were equal. In 11 instances the anti-Ro titer exceeded the anti-La titer. In several patients these titers were examined in four or five samples taken over a period of 1–5 yr, and the relative titers remained in a constant relationship. This was true despite the fact that the La anti-La precipitates were often thicker than the Ro anti-Ro precipitates (e.g., see this phenomenon in double diffusion in Fig. 2 where the independence of the two systems is demonstrated). In several sera tested by cross absorption with calf thymus extract containing La antigen free of Ro, a significant reduction of anti-La titer was regularly achieved without altering the anti-Ro titer.

Correlations with anti-DNA antibodies. As can be seen in Tables I and II, antibodies to DNA determined by any of the methods used were present three times as often (23/30) in the anti-Ro alone group as in the patients with both anti-Ro and anti-La (7/23)($P = 0.001$). Fig. 3 illustrates the differences in anti-ssDNA detected by double antibody radioimmunoassay in the two groups. The complement fixation technique is used routinely in this laboratory and was done on all available samples, some of which were no longer available for testing by radioimmunoassay. Because patients were considered to have antibodies to DNA

TABLE II

Serologic Features in Patients Having Anti-Ro and Anti-La

Patient	Anti-Ro titer	Anti-La titer	ANA	Antibodies to DNA		
				Anti-ss DNA complement fixation	Anti-ss DNA RIA	Antinative DNA Crithidia titer
SH	1/16	1/16	+	−	−	—
LLa	1/128	1/32	+	+	−	—
ERa	1/16	1/2	+	−	−	—
KD	1/8	1/8	−	−	−	—
SD	1/128	1/64	+	−	−	—
DB	1/4	1/4	+	−	−	—
ML	1/2	1/2	+	−	−	—
GB	1/256	1/128	+	−	−	—
LLi	1/32	1/32	+	+	+	1/250
ET	1/16	1/16	+	−	−	—
DT	1/32	1/32	+	+	−	—
MK	1/32	1/4	+	−	−	—
AT	1/4	1/4	+	−	−	—
JL	1/32	1/32	+	−	−	—
KB	1/4	1/1	+	−	−	—
LS	1/4	1/4	+	+	−	—
ERu	1/32	1/32	+	−	−	—
AO	1/256	1/32	+	−	+	—
DH	1/128	1/64	+	−	+	1/250
AP	1/4	1/4	−	+	−	—
SM	1/16	1/4	+	−	ND	—
PR	1/256	1/8	−	−	−	—
EB	1/32	1/16	+	−	−	ND

ND—Not determined.

if they were detected at any time during their clinical course, this probably accounts for the occasional discrepancy in detecting antibodies to ssDNA by complement fixation, but not by radioimmunoassay in a few patients. In almost all instances, the assay for all the DNA antibody tests were done on the earliest available sample when the patients were first seen. However, multiple tests for anti-DNA were only done by the complement fixation technique and were done equally frequently in the anti-Ro group and the anti-Ro and anti-La group. The sera on which the multiple anti-DNA tests were performed were also typed for the presence of anti-Ro vs. anti-Ro and anti-La. Also of note, antibodies to nDNA are present five times as frequently in the anti-Ro alone group and are often in high titer (see Tables I and II). The data probably reflects an underestimate of the frequency of antibodies to DNA because they often disappear rapidly with glucocorticoid therapy. Sera were not invariably obtained for testing before patients were placed on steroid therapy as patients were not infrequently first seen by our group after the initiation of such therapy. Table III shows that more anti-Ro alone patients received glucocorticoid therapy than patients having both anti-Ro and anti-La. The true incidence of anti-DNA from the early sera are very likely underestimated more in the anti-Ro group than the anti-Ro plus anti-La group.

ANA positivity. The vast majority of patients were ANA positive in both groups. The number of ANA-negative patients in the anti-Ro alone group was not significantly different than in the group having both anti-Ro and anti-La ($P > 0.1$).

Clinical correlations. Clinical data on these patients is summarized in Table III. There were significant differences between the two groups ($P < 0.05$) with respect to photosensitivity and discoid rash, as these findings were seen more frequently among the patients having both anti-Ro and anti-La. The apparent differences in polyarthritis and cytopenia between the two groups did not reach significance at the $P = 0.05$ level. Of prime clinical interest is the marked difference in the occurrence of serious renal disease between the two groups ($P < 0.001$). In the anti-Ro

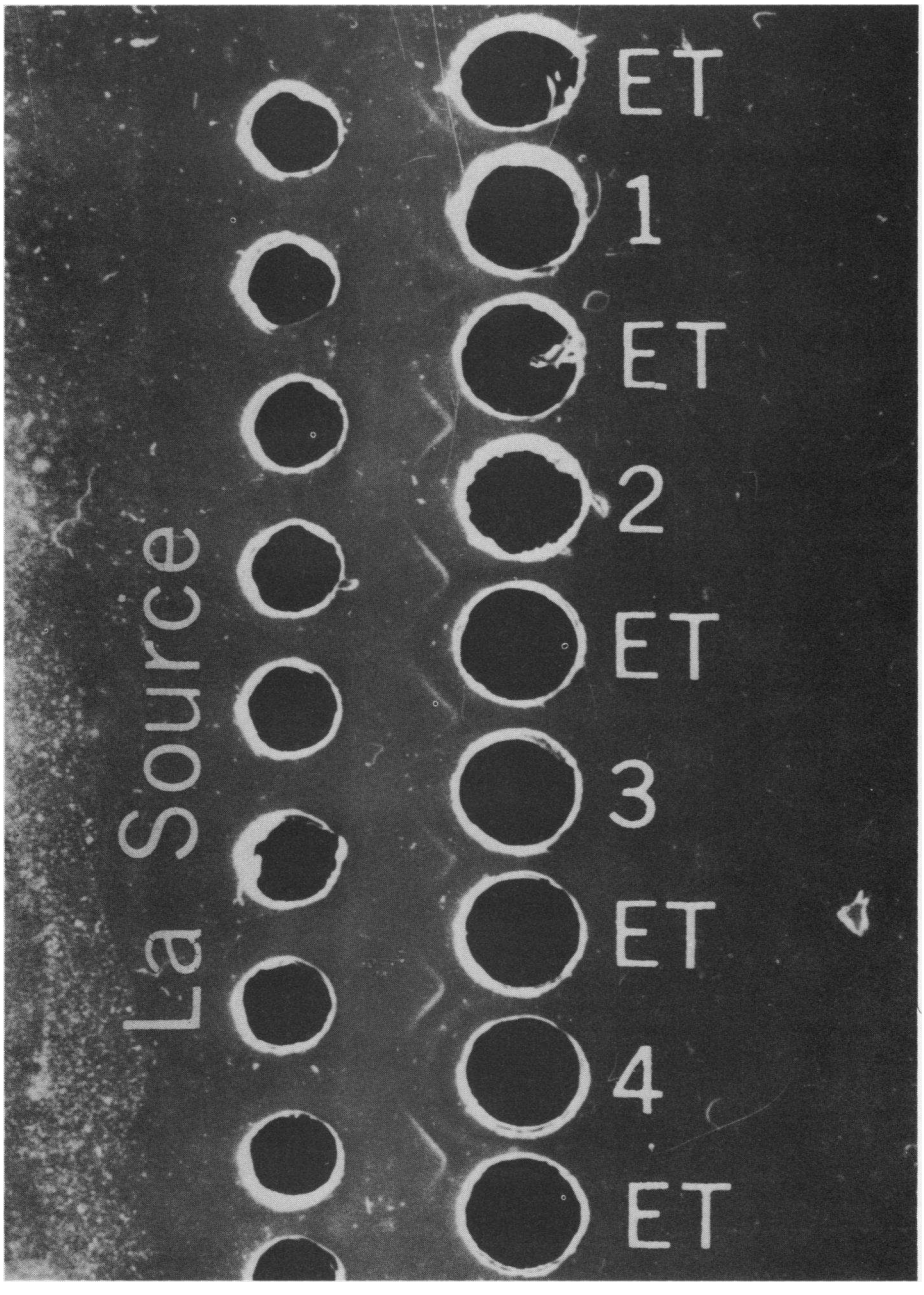

FIGURE 1 Evaluation of immunologic identity of precipitin lines in CIE. ET is the standard known anti-La serum. Serum 1 shows a reaction of nonidentity with ET. The precipitin lines with sera 2 through 4 show identity with ET, confirming them to be anti-La.

alone group 53% had signs of serious renal disease (proteinuria > 2 g or cellular casts) and 37% had significant renal insufficiency. This contrasted with the lower incidence of renal disease in the patients having both anti-Ro and anti-La (9%).

DISCUSSION

Two main phenomena are identified in this study. The first is the presence of a higher frequency of nephritis in SLE patients making anti-Ro than in SLE patients

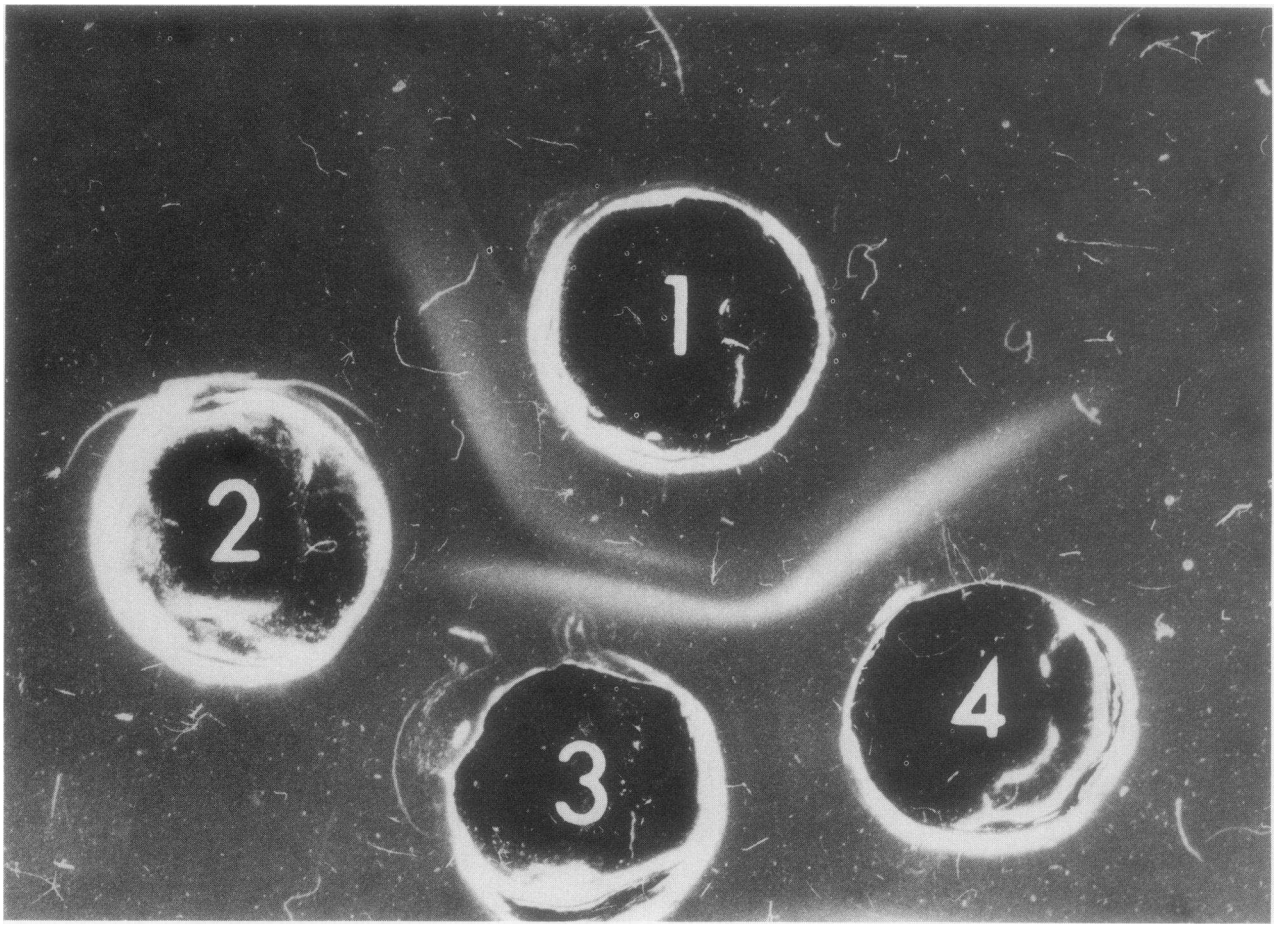

FIGURE 2 Antigenic independence of Ro and La antigens. Well 1 is a source of both Ro and La antigens. Well 2 is a monospecific anti-Ro serum. Wells 3 and 4 are different dilutions of the same serum containing both anti-Ro and anti-La. The anti-Ro and anti-La lines in wells 3 and 4 are clearly independent.

making both anti-Ro and anti-La and the second is the linkage of anti-Ro and anti-La titers in SLE patients making both antibodies.

Because a quarter of SLE patients have anti-Ro with or without anti-La, a considerable number of patients are involved in these two anti-Ro subgroups. This study identifies the group of patients making both anti-Ro and anti-La as a SLE subset with a paucity of serious renal disease. This correlates well with the lesser frequency and lower titer of anti-DNA seen in these patients. This situation of a low frequency of anti-DNA and low prevalence of renal disease is found among another SLE subgroup—those patients having high titer anti-nRNP alone (12, 15). The mechanism for this negative relationship between antibodies to these antigens (La or nRNP) and the antibodies to the DNA antigens is unknown. None of the sera in this study contained ānRNP so that the low frequency of anti-DNA in the anti-Ro and anti-La groups is not due to the presence of this antibody.

Given, however, the well described participation of DNA anti-DNA complexes in immune complex nephritis (16–18) and the evidence that the Ro anti-Ro complexes also participate in renal inflammation (7), it is reasonable to speculate that a multiplicity of antigen-antibody reactions that are potentially phlogistic increases the probability of immune complex nephritis in a given patient. One mechanism that might relate to the different capacity of SLE patients with anti-Ro to produce anti-DNA as compared with SLE patients who produce both anti-Ro and anti-La is a genetic difference between these two groups. Several observations support the notion that genetic factors may play a role in the production of these antibodies. First

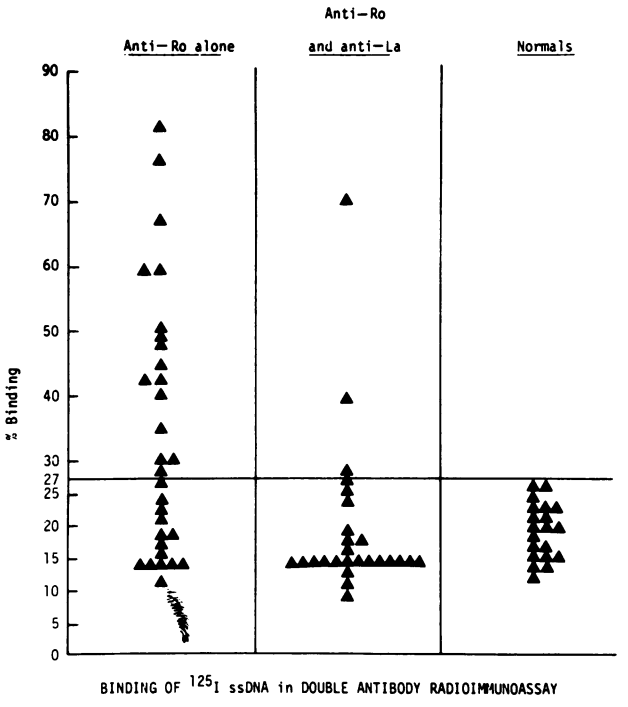

FIGURE 3 Difference in binding of ^{125}I ssDNA between anti-Ro alone and anti-Ro and anti-La together subgroups. The anti-Ro alone group is on the left, the anti-Ro and anti-La together group is in the center panel and the normals are represented in the third panel. The horizontal line across the figure represents the mean+2 SD for the normal group.

as already mentioned, published data reveal a great enrichment of the DRW_3 haplotype in patients with anti-Ro. 10/10 anti-Ro patients with SLE were found to carry this haplotype (4) whereas among subacute lupus erythematosus patients 67% of this group produce anti-Ro and/or anti-La and also carry the DRW_3 haplotype (5). These figures greatly exceed the 25% incidence of the DRW_3 haplotype noted in the relevant control populations. The fact that anti-Ro production on the one hand and anti-Ro and anti-La production on the other are stable properties of individual patients may also reflect a genetic difference between these two groups of patients but other explanations are possible. More compelling data suggesting a genetic difference between these two groups of patients comes from a study of homozygous C_2-deficient patients (M. Reichlin and T. T. Provost, unpublished data). In this study 9 of 17 such patients have been found to produce anti-Ro alone; none were found to produce both anti-Ro and anti-La. Similarly in a study of 63 patients with primary biliary cirrhosis (E. V. Penner, P. J. Maddison, E. R. Dickson, M. M. Weiser, F. Milgrom, and M. Reichlin) 12 patients were found who produced anti-Ro alone and none produced both anti-Ro and anti-La. Such data suggest independent mechanisms for the production of anti-Ro alone vs. the production of both anti-Ro and anti-La and a genetic basis for this mechanism receives support from the haplotype association data as well as the linkage of anti-Ro production with

TABLE III
Clinical Data on Patient Subgroups

Clinical features	Anti-Ro alone	Anti-Ro & anti-La	
	n = 30	*n* = 23	*P*
Facial rash	19 (63%)	15 (52%)	NS
Discoid	0 (0%)	6 (26%)	<0.05
Raynaud's	5 (17%)	3 (13%)	NS
Alopecia	6 (20%)	3 (13%)	NS
Photosensitivity	11 (37%)	15 (65%)	<0.05
Oral/nasal ulcers	7 (23%)	2 (9%)	NS
Polyarthritis	21 (70%)	10 (45%)	NS
Pleuritis/pericarditis	9 (30%)	8 (35%)	NS
Neuropsychiatric	5 (17%)	2 (9%)	NS
Cytopenia	22 (77%)	11 (48%)	NS
Diffuse proteinuria or cellular casts	16 (53%)	2 (9%)	<0.001
Renal insufficiency (CR >3.0)*	11 (37%)	1 (4%)	
Glucocorticoid therapy	21 (70%)	11 (48%)	NS
Sicca syndrome	2 (7%)	3 (13%)	NS

* This creatinine level of 3 mg/100 g or greater was sustained for at least 3 mo.

C_2 deficiency, a patently genetically determined condition. That a genetic difference determines the presence and/or quantity of anti-DNA occurring in these patients is an attractive but unproven hypothesis.

The phenomenon of the linked titers of anti-Ro and anti-La in patients producing both antibodies is worthy of comment. No serum sample has yet been found in which the anti-La titer exceeds the anti-Ro titer.

A trivial explanation for such linkage would be the cross reaction of antibody to shared determinants on both antigens. This possibility has been excluded by two experiments. First it is possible to prepare La free of Ro (by affinity chromatography) and Ro free of La (by trypsinolysis). Such antigens show complete independence in precipitin reactions and absorption experiments with purified La antigen do not affect anti-Ro titers at all.

Several possibilities may explain the pattern of linked titers of anti-Ro and anti-La observed in these patients. The most attractive hypothesis is that the structural genes controlling anti-Ro and anti-La production are linked and jointly regulated in analogy to the structural genes, for example, for human Hbs A_1 and A_2. Another genetic possibility is that the genes for the Ro and La antigens are alleles. In this scheme patients producing anti-Ro are homozygous for the Ro genes, those producing both anti-Ro and anti-La are heterozygous, and those producing only anti-La are homozygous for the La gene. It has recently been shown that the Ro and La antigens are RNA protein molecules with an unknown number of polypeptides and distinctive multiple RNA moieties (19). The antigenic differences then may reflect the differences either in the protein or the RNA moieties that would be coded by an unknown number of genes. Analysis of such possibilities awaits the discovery of animal models that produce antibodies to such specificities to see if antibody production behaves as a genetically defined property in such animals and if so, what pattern of inheritance determines the expression of these properties. Similarly, detailed analysis of both the protein and RNA components of the Ro and La antigens should reveal shared and/or distinctive protein components as well as whether their RNA components that are distinctive electrophoretically may also share some sequences. Alternatively, careful family studies with an aggregation of these antibody specificities may reveal clues as to genetic mechanisms underlying their production. Such "Ro positive" families exist and currently are under study.

Finally, intriguing recent molecular biology studies suggest a possible link of the La-immune response to viral infection because anti-La antibodies bind VA RNA of influenza virus-infected HeLa cells (19) as well as two EB virus-encoded RNA; $EBER_1$ and $EBER_2$, which are produced by cells carrying the EB virus genome (20). Antibodies to La also bind a distinctive set of RNA proteins found in uninfected cells as described (19). The role of virus infection in SLE has certainly been the subject of extensive discussion, but data such as these provide, at minimum, a biochemical linkage between viral infection and the disease process of patients producing anti-La.

Further study should place these speculations into their proper perspective and dictate which hypotheses are deserving of intensive investigative effort.

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