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PAPER

Cluster analysis of antinuclear autoantibodies in the prognosis of SLE nephropathy: are anti-extractable nuclear antibodies protective?

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To investigate the possible role of anti-ENA autoantibodies in the pathogenesis of SLE nephropathy, we performed a cross sectional clustering study of 91 SLE patients using 75 clinical and laboratory variables examining the presence of anti-dsDNA and ENA autontibodies by ELISA and Western blot. We applied principal component, hierarchical cluster, multiple correspondence and logistical regression analysis. Two polar forms of SLE nephropathy and five clinical groups were identified: group 1 without overt nephropathy ($n = 37$), group 2 with nephropathy and only proteinuria ($n = 19$), group 3 nephropathy and only hematuria ($n = 11$), group 4 with hematuria and proteinuria ($n = 14$) and group 5 on renal failure ($n = 10$). When analyzed individually, levels of anti-dsDNA and single anti-ENA antibodies did not allow us to differentiate between renal and non-renal groups. However, when the anti-ENA autoantibodies were analyzed as a cluster, a high predictive value for clinical nephropathy was obtained. Thus, the absence of ENA antibodies (ENA ve or Venezuelan cluster) increased eleven-fold the odds ratio to develop SLE nephropathy. We suggested that the ENA ve cluster may predict development of the most severe forms of renal lupus while the ENA Sm/RNP and the ENA Ro/La/Sm/RNP clusters could be associated with the absence and the most benign form of SLE nephropathy. It must be interesting to apply similar cluster methodology in an SLE population with different ethnic background.
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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a remarkable diversity of clinical and immunopathological presentations. Although a number of autoantibodies have been described and related to some of the clinical syndromes, definitive associations have not been clearly and fully established.

Lupus nephropathy is one of the most serious visceral manifestations in SLE. Its ethiopathogenesis has been linked to the presence of complement-fixing anti-dsDNA antibodies.[1] Winn *et al* reported that antibodies to Sm antigen may inhibit DNA-anti-DNA complexes formation.[2] Anti-RNP antibodies have been found in 35% of different SLE series.[3] Kitridou *et al* have pointed out the increasing prevalence of nephropathy in mixed connective tissue disease, a disease in which anti-RNP autoantibodies are present in high titers, whereas anti-dsDNA autoantibodies are usually absent.[4] Several authors have suggested that the detection of anti-RNP/Sm and/or anti-Ro/La antibodies are a good prognostic factor in lupus nephropathy.[5] Reichlin *et al* have concluded that SLE patients with anti-Ro antibodies fall into two subgroups that differ in their prevalence of anti-DNA and serious renal disease, depending on the presence of anti-Ro alone or anti-Ro plus anti-La antibodies.[6] Recently, cross reactivity of autoantibodies as a possible mechanism involved in lupus nephritis has been emphasized.[7,8]

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The present investigation was designed to identify the potential correlation of anti-extractable nuclear antibodies (ENA) and lupus nephropathy, following a cross sectional study by cluster analysis in a horizontally controlled SLE patient population.

Material and methods

Patient population and data collection

Our data was based upon a cross sectional study of 91 patients of Hispano American (a mixture of Spaniard Caucasians, Afro-Caribbeans and Amerindians) descent who fulfilled the 1982 American College of Rheumatology Criteria (ACR)[9] for SLE and who have been followed for the last ten years at our Rheumatology Division. 81 were female and 10 male, mean age 31.2 (range 10–63 y). The mean duration of the disease at the time of inclusion in the study was 6.7 (range 6 months to 24 y). 40 healthy volunteers (36 women and 4 men), mean age 32 (range 22–44 y) were studied as a control group.

The ARAMIS (American Association Measurement Investigative Index System)[10] database using 75 variables was completed at the moment of diagnosis. Clinical and laboratory data were collected on standard forms at the time of each outpatient visit, reviewed and introduced in the SPSS 7.5 program (SPSS Inc., Chicago, IL, USA).[11]

Fluorescent antinuclear antibodies assay

Fluorescent antinuclear antibodies were detected on Hep-2 cells according to Beck.[12] Reference antinuclear antibodies sera AF/CDC (Atlanta, GA, USA)[13] were used for standardization purposes. Sera from 40 healthy controls were also screened. Specific reactions were traced with fluorescent conjugated anti-human immunoglobulin (INSCTAR, Stillwater, MN, USA) diluted 1:30.

Anti-dsDNA antibodies

Anti-dsDNA antibodies were detected by the indirect immunofluorescent assay using *Crithidia lucillae* as substrate (Kallested, Sanofi Diagnostic Pasteur, Chaska, MN, USA) following the method of Aarden.[14] Positive samples were reported 1 to 4+ according to the intensity of the fluorescence.

Detection of antibodies against extractable nuclear antigens (ENA) by enzyme linked immunosorbent assay (ELISA)

Microwells were properly incubated with the corresponding affinity purified antigen (Sanofi Diagnostic Pasteur, Chaska, MN, USA). 100 µl of patient and control samples and calibrators were diluted and incubated at room temperature during 30 min. Anti-Sm, RNP, SSA/Ro and SSB/La autoantibodies were investigated using the protocol by Engvall and Pellman.[15]

Polyacrylamide gel electrophoresis

Nuclear and cytoplasmic HeLa cells preparations were resuspended in sample buffer (10% glycerol, 5% 2ME: 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCL pH 6.8, and 0.05% bromofenol blue). The stacking gel was prepared in 4% acrylamide/0.1% bisacrylamide and the resolving gel in 10% acrylamide 0.267% bisacrylamide. Cytoplasmic and nuclear components were separated in a preparative set under denaturing conditions (0.1% SDS). Electrophoresis was run at 30 mA constant current during at least 1 h, in a Miniprotean II gel electrophoresis equipment (BIORAD, Berkeley, CA, USA). Proteins were revealed by a mixture of Coomasie brilliant blue and silver staining or transferred to 0.2 pore nitrocellulose sheets for immunoblotting following the method by Towbin[16] and Laemmeli.[17]

Western blot

Nuclear and cytoplasmic proteins were blotted into nitrocelulose sheets at 125 mA in buffer 25 nM tris, 192 nM glycine, 20% methanol. Filters were blocked in buffer containing 0.05 phosphate, 0.85% NaCl, 5% low fat milk and 0.1% Tween-20 (PBS-LFM) and incubated during 1 hour under continuous shaking with sera from controls, patients and AF/CDC reference sera (RS) diluted × 1000 in blocking buffer in a Multiscreen Apparatus device (BIORAD, Berkley, CA). After washing in PBS, appropriate filters were incubated with HRPO-conjugate (dilution 1/2000).

Statistical analysis

Principal component analysis (PCA). PCA was applied as described by Jollife[18] to analyze relationships between renal variables which might indicate possible kidney disease. These variables were proteinuria > 300 mg/24 h, hematuria defined as more than 5 red blood cells per high power field, leukocyturia defined as more than 8 white blood cells per high power field, presence of urinary casts, (80% granular), serum creatinine level higher than 1.5 mg/dl and creatinine clearence lower than 70 ml/min. WHO histopathological classification was used for comparison with renal biopsies analysis.[19]

These clinical variables were used to build indicators (vectors) that can establish differences or similarities among patients according to the degree of renal disease. Eigenvalues (a set of discrete values of a parameter) were used as quantum mechanism to standardize the data. The indicators were interpreted in terms of the address and force of their correlations with the clinical variables as assessed by Pearson's coefficient. Thus, PCA allows finding the addresses in different points of a set of patients picking up relationships at maximum.

Hierarchical cluster analysis (HCA). HCA combines cases into clusters to identify relatively homogeneous groups based on selected characteristics. The methodology suggested by Ward[20] was used in a hierarchical ascending way according to clinical and immunological features. At each step in the analysis, the union of every possible pair of clusters was considered and the two clusters whose fusion resulted in the lowest increase in the sum error of squares were combined. Initially, each of the cases was regarded as a single point cluster and the first fusion clearly involved those points which were closest. At subsequent steps, the fusion of multipoint clusters was considered until a final group in cluster analysis was produced. The Euclidean distance between two points was used to measure the similarity between two individuals. Initially groups of clinical SLE nephropathy clusters were identified.

We defined four possible anti-ENA autoantibody clusters: the ENA ve cluster (ENA venezuelan cluster) when none of these autoantibodies were detected, the ENA-anti-Ro/La cluster when only anti-SSA/Ro and/or SSB/La antibodies were identified, the ENA-anti-RNP/Sm cluster when only these autoantibodies were found and ENA-anti-RNP/Sm/Ro/La cluster when the four autoantibodies were present. We also regrouped these defined ENA clusters in relation to their association with anti-dsDNA titers.

Multiple correspondence analysis (MCA). MCA is a geometric method displaying the rows and columns of a matrix as points in a low-dimensional vector space that allow multiple continuous table analysis.[21] MCA was used to obtain indicators of the behavior of the various anti-ENA clusters, as related to the combined behavior of clinical renal features, the levels of anti-dsDNA titers, renal histopathology and clinical disease activity as assessed by the Mex-SLEDAI.[22]

Relative risk (RR) and logistic regression model (LRM). Relative risk (RR) in 2×2 tables was used to determine the association between each single antinuclear antibody and SLE nephropathy. LRM[23] was employed to measure the possible contribution of each specific antinuclear antibody cluster and the score of disease activity in the development of SLE nephropathy.

Analysis of variance (ANOVA). ANOVA was used to compare the average behavior of the autoantibodies results among the nephropathy clusters. Student's t test (two tails) was used to compare lupus ages between clinical nephropathy group 1 (without overt renal disease) and groups 2–5, patients with renal disease. In every test, P values less than 0.05 were considered significant.

Results

Patient population and clinical parameters

The clinical features of our SLE patient population are shown in Table 1. Articular (74.7%), hematological (63%) and renal (60%) manifestations were the most common clinical features. PCA allowed us to show a highly significant correlation among the SLE nephropathy variables ($P < 0.001$). Eigenvalues and percentages of the information captured by the principal

Table 1 Clinical features of the SLE patient population ($n = 91$)

	n	%
Articular	68	74.70
Hematological	58	63.00
Renal	54	60.00
Skin and mucous	45	49.50
Central nervous system	24	26.40
Cardiorespiratory	13	14.30
General manifestations	88	90.00
Fatigue	43	47.30
Fever	36	39.60
Polyadenopathy	09	9.90

components (CP1 and CP2) were representative enough of 72% of the original data. CP1 showed a positive Pearson correlation with proteinuria, hematuria, urinary sediment and creatinine serum levels, coinciding in the same dimensional space ($P < 0.001$). As expected, there was a negative correlation of creatinine serum levels and the creatinine clearance ($P < 0.001$). The second principal component CP2, based on the level of renal function, allowed a better discrimination among renal patients. The presence of two polar groups was established, one of patients showing proteinuria, abnormal urinary sediment and renal failure, and another characterized by normal urinary sediment and renal function.

SLE nephropathy clusters

Lupus renal clusters are depicted in Figure 1A. Creatinine serum level, hematuria and 24 h proteinuria (> 300 mg) showed a slight increment in the median when we moved from group 1 (without clinical nephropathy) to intermediate clinical groups 2, 3 and

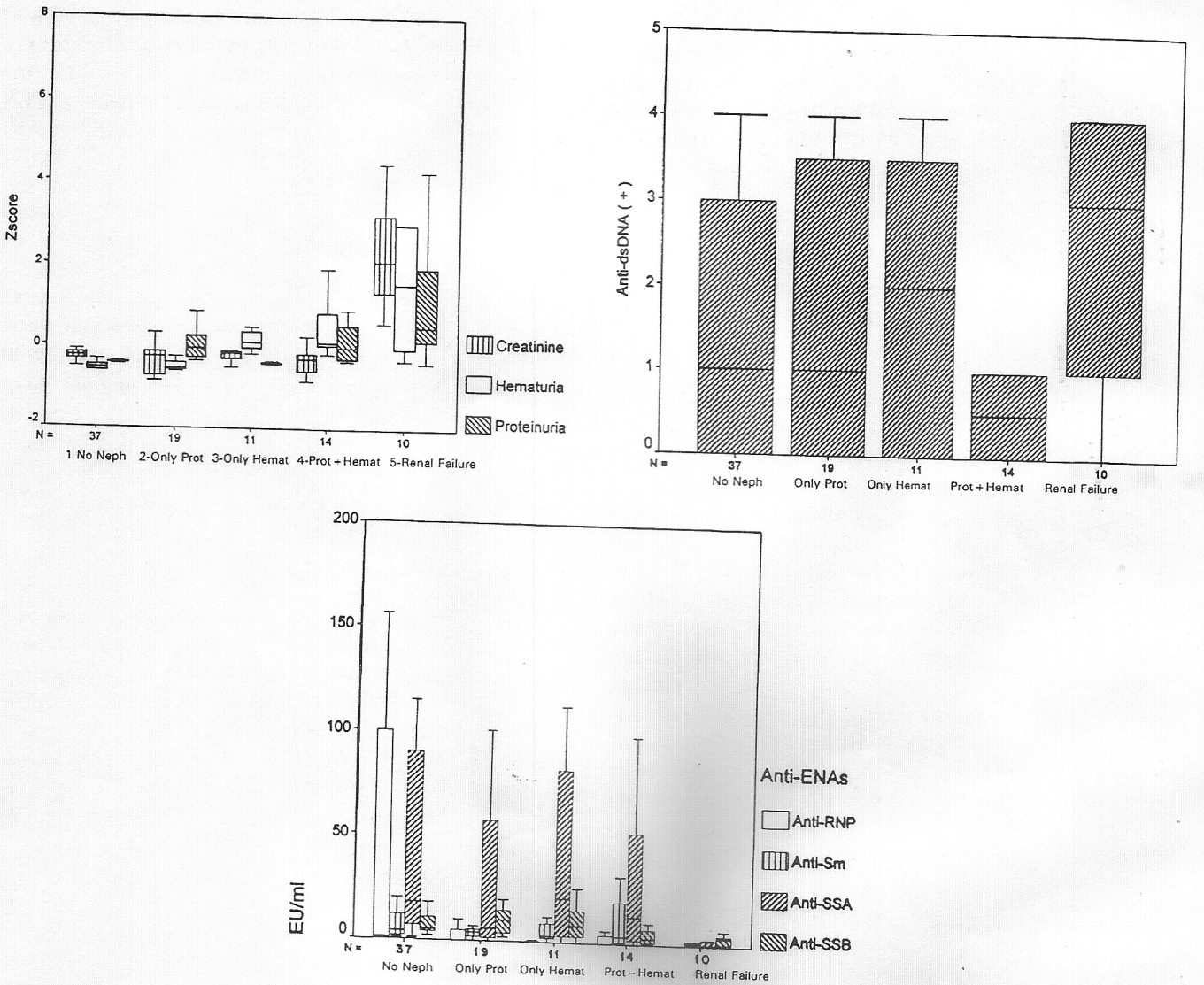

Figure 1 (A) Two polar forms are identified by PCA and five SLE nephropathy groups (1–5) by HCA: Group 1 ($n = 37$) patients with no evidence of clinical nephropathy; Group 2 ($n = 19$) who only developed proteinuria; Group 3 ($n = 11$) with only hematuria; Group 4 ($n = 14$) who showed proteinuria and hematuria; Group 5 ($n = 10$) with the most severe form, on renal failure. A high accumulation of variables is noted in Group 1, which is gradually lost, reaching a very dispersed distribution in Group 5, indicating the great heterogeneity in the latter group. (B) Anti-dsDNA titers for each SLE nephropathy group are depicted in boxplots, using the median and percentile 50 as the central tendency. The median level of anti-dsDNA titers increased from 1+ in Group 1 to 3+ in Group 5. In the intermediate groups, only in group 3 the median rose to 2+, showing a great variability into the different groups. (C) Anti-ENA in the SLE nephropathy groups. High titers of anti-RNP autoantibodies were detected in Group 1, with progressive reduction until no detection when skewing from Group 1 to Group 5. Anti-SSA/Ro and anti-Sm/anti-RNP antibodies were present in every group of clinical nephropathy without any statistically significant difference among them. Anti-ENA antibodies were not detected in Group 5 with most severe renal subset.

4 (only proteinuria, only hematuria or proteinuria plus hematuria respectively) to group 5 (end-stage renal failure). A high accumulation within normal range of nephropathy variables was noted in group 1 (patients without nephropathy), which is lost gradually in the intermediate groups, reaching a very dispersed distribution in group 5, indicating the greatest heterogeneity in the latter group. There was no statistical difference in the mean of SLE duration between group 1, 5.6 y (4.12, 7.08), 95% confidential interval (CI) and the four nephropathy groups 7.5 y (5.52, 9.48), 95% CI ($P = 0.125$).

Relationship between anti-dsDNA and anti-ENA antibodies titers with the SLE nephropathy groups

A device of multiple boxplots in Figure 1B shows a comparative analysis of the behavior of the anti-dsDNA antibodies within SLE clinical nephropathy groups, indicating that the median level (percentile 50) of anti-dsDNA levels rose from 1+ titer in group 1 to 3+titer in group 5. However, an ANOVA test indicated that there were no statistically significant differences in the comparison groups ($P = 0.239$). In the intermediate groups, only in groups 3 and 5 the median increased anti-dsDNA antibody levels to 2+ and 3+ respectively, showing a great range of variability into the different groups.

In contrast, among the anti-ENA antibodies, the distributions are dispersed in group 1 with a predominance of anti-RNP and anti-SSA/Ro antibodies (Figure 1C). While anti-RNP antibodies significantly decreased, antibodies to SSA/Ro remained almost unaltered among 2, 3 and 4 SLE nephropathy groups. Remarkably, patients in group 5 showed no detectable ENA antibodies. There was a high correlation (80%) between ELISA and Western blot in the anti-ENA antibodies determination.

When we calculated RR to develop SLE nephropathy, the presence of anti-RNP antibodies reduced the risk in 66% (RR = 0.44, $P < 0.002$ (Table 2).

Table 2 Relative risk (RR) for SLE nephropathy to each autoantibody

Autoantibody	*RR*	*P value*	*95% CI*
Anti-dsDNA	1.112	0.678	(0.676, 1.830)
Anti-SSA/Ro	0.792	0.355	(0.483, 1.296)
Anti-SSB/La	1.067	0.862	(0.510, 2.230)
Anti-Sm	0.664	0.162	(0.394, 1.190)
Anti-RNP	0.444	0.002	(0.290, 0.681)

95% CI = confidence interval.

Antinuclear antibody clusters: predictive value for development of SLE nephropathy

In Table 3 we display the prevalence of anti-ENA clusters, the renal histopathology and the presence of anti-dsDNA antibodies. ENA ve and ENA anti-Ro/La clusters (72% of the sample) showed the most severe forms of renal lesions (100%); remarkably, anti-dsDNA antibodies did not establish the difference among the ENA clusters.

Multiple correspondence analysis among antinuclear antibody clusters, SLE nephropathy clusters, renal biopsies and SLE clinical activity

In Figure 2 (left upper quadrant) sera contained within the ENA ve autoantibody cluster (64%) were associated with the highest anti-dsDNA titers (88%), presence of renal failure (90%), IV–VI WHO histopathologic categories (82%) and the highest Mex-SLEDAI scores (44%). Those with ENA anti-RNP/Sm or anti RNP/Sm/Ro/La clusters had less anti-dsDNA titers, absence of clinical renal disease, kidney biopsies of milder severity (I to III) and lesser scores of clinical activity (Figure 3, right upper quadrant). In the two lower quadrants corresponding to the ENA anti-Ro/La antibody cluster, there was association with absence of anti-dsDNA antibodies, presence of membranous glomerulonephritis (class IV) and an intermediate index of Mex-SLEDAI activity.

Using a LRM to calculate the OR to develop SLE nephropathy for each of these autoantibody clusters, the ENA ve cluster increased this probability eleven fold when the ENA anti-RNP/Sm cluster was taken as a reference (Table 4). Therefore, this cluster contributed with the highest predictive value for renal disease. Also in Table 4, we show that for each score point of increment of SLE clinical activity the probability for SLE nephropathy rose 1.15-fold. Finally, the presence of ENA anti-Ro/La and ENA

Table 3 Prevalence of anti-dsDNA and histopathological features in the anti-ENA clusters

	Sera		*Anti-dsDNA*		*WHO classes biopsies (n = 37)*		
Anti-ENA clusters	*n*	*%*	*n*	*%*	*I–III*	*IV–VI*	*V*
ENA ve	35	39	21	60	2	12	3
ENA Ro/La	30	33	20	66	5	8	4
ENA Sm/RNP	14	15	08	57	0	0	2
ENA Ro/La/Sm/RNP	12	13	05	42	0	0	1
Total	91	100	54	59	07	20	10

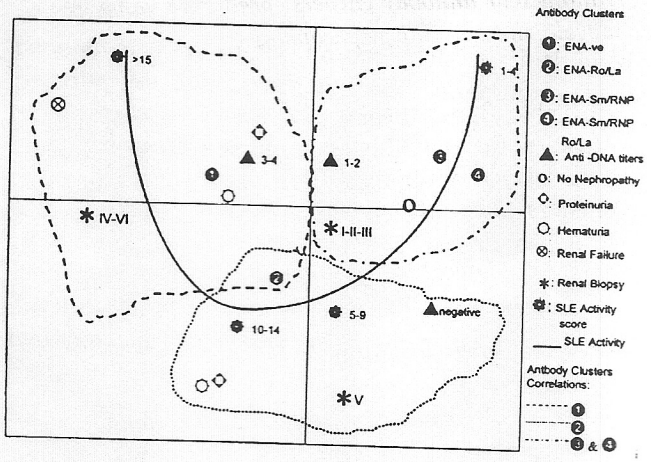

Figure 2 Multiple correspondence analysis among antinuclear antibody clusters, SLE nephropathy clusters, renal biopsies and SLE clinical activity. The MCA depicts the highest predictive value of the ENA ve autoantibody cluster. In the left superior quadrant ENA ve associates with the highest anti-dsDNA titers, the presence of renal failure, IV–VI WHO histopathologic category and the highest Mex-SLEDAI score. In contrast, ENA Sm/RNP and Ro/La/Sm/RNP clusters (right superior quadrant) associates with the lowest anti-dsDNA titers, absence of renal disease, biopsies of mild severity and lesser scores of clinical activity. In the two lower quadrants ENA anti-Ro/La cluster absence of anti-dsDNA and intermediate histopathological classes and SLE activity are shown.

Table 4 Odds ratio to develop SLE nephropathy and clinical activity for each anti-ENA cluster

Variable	*Odds ratio*	*P value*
ENA ve	10.897	0.002
ENA Ro/La	5.181	0.343
ENA Ro/La/Sm/RNP	2.285	0.371
SLE index activity*	1.150	0.002

*Each added point in the SLEDAI score increased the OR for SLE nephropathy to 1.15.

anti-RNP/Sm/Ro/La autoantibody clusters increased the OR for lupus nephropathy by 5- and 2-fold, respectively, without statistical significance.

Discussion

In this study two polar forms of SLE nephropathy were identified. At one end of the spectrum were patients on renal failure and, at the other, patients without clinical evidence of nephropathy. Among them, there were intermediate clinical groups. Interestingly, we found that the presence of anti-dsDNA antibodies alone did not differentiate between the five SLE nephropathy groups. In spite of some trend in relation to anti-DNA titers among the two polar forms, no statistical significance was demonstrated, because of a great range of variability in these autoantibodies in both groups. However, when the activity of the clinical disease was evaluated by the Mexican SLEDAI, an association with high anti-dsDNA antibodies was shown by MCA.

Although anti-dsDNA antibodies have been accepted as a serologic hallmark for SLE nephropathy,[24] in our study its presence was mainly related with the level of clinical activity of the disease. Furthermore, several authors have pointed out that the severity of SLE nephropathy is not exclusively related to the presence of anti-dsDNA antibodies but rather with different specificities of these autoantibodies.[24–26] The studies of chemical properties of anti-DNA antibodies have permitted us to advance the idea that idiotypically related anti-DNA antibodies in tissue lesions, lupus serum and normal serum from different individuals differ on their cross-reactivity and antigen-binding properties, and that the cross reactivity of lupus autoantibodies may influence their capacity to form glomerular immune deposits.[27]

On the basis of the organization of the human V locus, different authors have reported that the VH gene repertoire in the aggressive anti-DNA response has the molecular characteristic of diversification in pathogenic lupus autoantibodies and heterogeneous rearrangements encoding for nephritogenic lupus antibodies arise from different germline genes.[28,29]

On the other hand, when analyzing IgG anti-DNA clonotypes associated with lupus nephritis in humans, Hatakeyama *et al* found that specific clonotypes of serum anti-DNA antibodies were not associated with renal lesions in lupus patients and no differences in anti-DNA antibody patterns existed between patients with lupus nephritis and those with no evidence of renal disease.[30]

As depicted in Figures 1B and C, neither anti-dsDNA antibodies nor anti-ENA antibodies when used singly, were useful in predicting overt renal disease. On the contrary, anti-RNP antibodies were predominantly present in patients without clinical renal disease. Our results seem to suggest that this autoantibody may be protective because its presence was associated with a decrease of SLE nephropathy risk in 66% ($P=0.002$) (Table 2). This possible protective nature of anti-RNP autoantibodies was initially emphasized in patients with mixed connective tissue disease, who show high anti-RNP titers and low incidence of renal involvement.[31] Nevertheless, subsequent studies have reported renal disease in at least 26% of patients.[3,32]

SLE is a multifactorial autoimmune disease and tends to occur in clusters.[33] Clustering is an important data analysis tool for discovering structure in data sets. Although cluster analysis rarely proves fruitful in identifying causation, it may have the potential to generate new knowledge.[34] Remarkably, when the

four ENA antibodies were analyzed as clusters, patients of the ENA ve cluster showed the most severe histopathological renal lesion. As the number of ENA autoantibodies increased (RNP/Sm and RNP/Sm/Ro/La clusters), the prevalence of renal lesions diminished (Table 3).

Additional evidence in favor of the predictive power of the ENA ve cluster for the development of SLE nephropathy was obtained by MCA, showing that patients on renal failure (group 5) correlated with the ENA ve cluster, the highest Mex-SLEDAI score, presence of the highest titers of anti-dsDNA antibodies and the most severe renal changes.

By applying LRM to examine the potential association of ENA clusters and the development of the SLE nephropathy groups (Table 4), again the ENA ve cluster contributed with the highest predictive value, increasing this chance 11 fold. In turn, patients of group 1 (no clinical evidences of renal involvement) correlated with ENA RNP/Sm and RNP/Sm/Ro/LA clusters, the lowest SLEDAI score and the lowest anti-dsDNA antibodies titers with minor histopathological renal lesions.

The ratio of anti-U1RNP/Sm antibodies and the presence of antibodies to 70 kD RNP protector have been inversely related to the frequency of lupus nephritis.[35] Alarcon-Segovia and co-workers have demonstrated that anti RNP antibodies are able to penetrate living T lymphocytes, interact with nuclear components and probably alter cell functions linked to T cell regulatory mechanisms. For instance, anti-snRNP plays an important role in the processing of RNA premessenger.[36] Vlahakos *et al*[37] have also shown that murine monoclonal anti-DNA antibodies penetrate renal cells and induce glomerular proliferation and proteinuria in vivo. On the other hand, Zhang *et al*[38] have suggested the anti-Ro and anti-La antibodies may function as anti-dsDNA anti-idiotype.

Several pathogenic hypotheses could be considered to explain our results. A given genetic background may predispose to both a particular autoantibody cluster and susceptibility to nephropathy. Other possible underlying immunological mechanisms different to immune complex deposition may participate in the development of SLE nephropathy. Thus, the cross-reactive antibody and the planted antigen hypothesis are very attractive postulating that anti-dsDNA antibodies are broadly reactive and can bind to a wide array of molecules such as glomerular base components.

In summary, our results suggest a possible significant relationship between clusters of antinuclear antibodies rather than single antibodies and the presence or severity of SLE nephropathy. We suggested that the ENA ve cluster could predict development of the most severe forms of renal lupus while the ENA RNP/Sm and the ENA RNP/Sm/Ro/La clusters may be associated with the absence and the most benign form of SLE nephropathy, respectively.

This ENA ve cluster could be mentioned as a marker of a very severe form of SLE clinical presentation which might identify a different SLE clinical—immunological subset. This possibility raises the question as to whether a similar cluster might be applicable to other ethnic backgrounds. Several reports have emphasized the complexity of genetic susceptibility to SLE. Moser *et al*,[39] Gulko *et al*,[40] Tikly *et al*[41] and García *et al*[42] identified differences in ethnic and racial groups and the prevalence of anti-RNP and anti-Sm antibodies in African-American patients compared with Caucasian-European stock. These data are in favor of the different genetic components of antibody production but do not rule out the possible influence of environmental factors. Our data, in a Hispanic-Afro-Caribbean population, and the contribution of some particular environmental conditions might have influenced in our results. Probably in groups of patients with similar characteristics in close geographic areas it would be possible to identify similar SLE nephropathy clusters. It must be very interesting to apply a similar cluster methodology in an SLE population with a different ethnic background to investigate which mechanism could explain these types of associations, currently addressed at molecular and biochemical levels, with more specific clinical and immunological subsets.

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