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Comparison and Variation of Different Methodologies for the Detection of Autoantibodies to Nuclear Antigens (ANA)

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Interest in the assessment of autoantibody specificity stems from the need for an autoantibody marker capable of predicting clinical events in autoimmune disorders. However, the multiplicity of epitopes present on autoantigenic particles, the quantitative and qualitative heterogeneity of autoantibodies, as well as the nature of the tests, mean that each of the assays used in their determination have different characteristics. The aim of this study was to compare the specificities of different ANAs using four commercial assays. The routine method used for the detection of ANA is indirect immunofluorescence on Hep-2 cells. The assays used were: counterimmunoelectrophoresis (CIE), enzyme-linked immunosorbent assay (ELISA), and two immunoblotting assays. Kappa statistic was applied to evaluate the consistency between tests. Kappa index is a measure of agreement between categorical data. Kappa has a maximum of 1.00 when the agreement is perfect, a value of zero indicates no agreement better than chance, and negative values show worse than chance agreement.

For SS-B antibodies, there was a good concordance between all four methods used (Kappa 0.66–0.74). For anti RNP antibodies, the results for CIE/ELISA (Kappa 0.60) were consistent as were the two immunoblot methods (Kappa 0.69). For anti Scl-70 (topoisomerase I) antibody, results from the ELISA and CIE methods were totally consistent (Kappa 1.00). In spite of the high prevalence of anti SS-A/Ro antibodies, the agreement between the methods was poor, without statistical significance. Finally, for Sm antibodies, more consistent results were obtained between CIE/ELISA (Kappa 0.51) and between one of the immunoblotting methods and ELISA (Kappa 0.54). In conclusion, CIE concurs mostly with ELISA for anti-RNP, Scl-70, Sm and SS-B antibodies, but with some disagreement for SS-A antibodies. J. Clin. Lab. Anal. 11:388–392, 1997. © 1997 Wiley-Liss, Inc.

Key words: antinuclear antibodies; enzyme immunoassay; immunoblotting; counterimmunoelectrophoresis

INTRODUCTION

Antinuclear antibodies (NA) consist of a heterogeneous group of autoantibodies directed against different components of the cell nucleus, such as nucleic acids, nuclear proteins and nucleic acid-protein complexes. They are consistently found in the sera of patients with many types of autoimmune disease manifestations. Indirect immunofluorescence (IF) has for many years been the method of choice for primary screening for ANA (1). However, no molecular information is available about the specific identity of the antigen(s). The term "extractable nuclear antigens" (ENAs) refers to a group of nuclear proteins extractable in salt solution, which include proteins and RNA-protein complex. ENAs were initially defined as two types, Sm and nRNP (2). However, it is currently accepted that there are several; the most frequently detected are Sm, nRNP, SS-A/Ro, SS-B/La, Jo1, and Scl-70.

The determination of antibody specificity of ANAs is interesting because it allows the identification of markers capable of predicting clinical events in autoimmune disorders. Techniques such as gel diffusion/counterimmunoelectrophoresis (CIE), immunoblotting, immunoprecipitation or enzyme immunoassay allow the determination of some autoantibody specificity. However, the multiplicity of epitopes present on autoantigenic particles, the qualitative and quantitative heterogeneity of autoantibodies, as well as the nature of the tests, allow each of the assays used to detect different, but often overlapping, subpopulations of the total autoantibody response to a given autoantigen. This can lead to discordant results when two or more assays are performed. The aim of the present

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study was to assess the concordance between four commercial assays for anti-extractable nuclear antibody determination.

MATERIAL AND METHODS

Serum Samples

From the serum samples sent to our department for determination of ANA, we selected those that were positive (ANA ≥ 80 arb. U. conc.) with an immunofluorescence screening test on Hep-2 cells. The group of patients consisted of 28 women and 3 men (median age = 48 years, interquartilic range age = 27 years, age range = 15–71 years). The patient's diagnoses were: Systemic lupus erythematosus (SLE) (n = 14), rheumatoid arthritis (RA) (n = 3), mixed connective tissue disease (MCTD) (n = 1), Sjörgren's syndrome (SS) (n = 2), scleroderma (n = 1), polymyositis (n = 1), autoimmune cirrhosis (n = 1), and undifferentiated connective syndrome (UCD) (n = 8). The diagnoses were established according to the American Rheumatology Association criteria (3).

Blood samples were obtained after an overnight fast and allowed to clot at room temperature. Serum was obtained by centrifugation and aliquots were frozen at –20°C until assayed.

Screening of Antinuclear Antibodies

The presence of ANA in serum was determined by indirect immunofluorescence on Hep-2 cells (Inova Diagnostic Inc., San Diego, CA, USA). Fluorescein isotiocyanate labelled antihuman IgG, IgA and IgM was obtained from Dako (Dako A/S, Glostrup, Denmark). Serum samples were diluted 1:80 with phosphate buffered saline (PBS) solution (0.14 mol/L NaCl, 0.01 mol/L sodium phosphate, pH = 7.4). Briefly, spots of Hep-2 cells were incubated for 30 min at room temperature with the diluted serum samples. After being washed with PBS (twice for five min each), the slides were incubated for 30 min with the antibody diluted 1:200 in PBS. The slides were washed with PBS again (twice for five min each), counterstained with 0.1 mmol/L Evans blue and read using a fluorescence microscope.

Counterimmunoelectrophoresis

Precipitating antibodies were detected by counterimmunoelectrophoresis in 1% agarose in 0.025 mol/L barbital buffer, pH = 8.6 (Beckman Instruments Inc., Fullerton, CA, USA). Antibodies and purified antigens from calf thymus were supplied from Inova Diagnostics Inc. (San Diego, CA, USA). Holes, 4 mm in diameter, were punched, 5 mm apart, and filled with antigen and serum. Electrophoresis was carried out at 6 mA per plate for 30 min. After extensive washing with PBS, the plates were dried under heat and pressure. Precipitation lines were stained with a solution of 5 g/L Coomasie brilliant blue in H_2O:ethanol:acetic (9:9:2). After destaining with 0.9 mol/L acetic acid, plates were read and observed reactivity was classified as antibodies to Ro/SS-A, La/SS-B, RNP, Sm, Scl-70 or Jo-1 by comparison with antibody control samples.

Enzyme Linked Immunosorbent Assays (ELISAs)

ELISAs for the detection of ENAs were carried out as described by the manufacturer (Inova Diagnostics Inc., San Diego, CA, USA). These methods include affinity purified antigens and peroxidase conjugated antihuman IgG. They were automated in the FP 1300 II Autoplate System (Labsystem, Finland). The cut-off in the RNP ELISA assay was increased from 20–25 U. Samples with results higher than the cut-off were considered positive.

Immunoblottings

We adhered strictly to instructions of manufacturers concerning the storage and use of test kits. All incubations and washing steps were performed at room temperature on a rocking platform.

Blotting 1: ANA Western Blot (Bio-Genex, San Ramon, CA, USA) is composed of purified antigens from human cells blotted onto nitrocellulose sheets. An alkaline phosphatase goat anti-human IgG is used.

Blotting 2: Anablot System II (Biolab, Wavre, Limal, Belgium) included an antigen cocktail of isolated mammalian cells nuclear extracts and rabbit antibodies (antihuman IgG, IgM and IgA) associated with peroxidase.

Statistical Method

The results were analyzed by a commercial statistics program (SPSS) on a personal computer. Kappa statistic was applied to evaluate the agreement between tests (4). Kappa is calculated from the observed and expected frequencies on the diagonal of a square table of frequencies. If there are n measures in g categories, then the observed proportional agreement is

$$p_o = \sum_{i=1}^{g} f_{ii}/n$$

where f_{ii} is the number of agreement for category i. The expected proportion of agreement by chance is given by

$$p_e = \sum_{i=1}^{g} r_i c_i / n^2$$

where r_i and c_i are the row and column totals for the ith category. The index of agreement, kappa, is given by

$$\kappa = \frac{p_o - p_e}{1 - p_e}$$

Table 1. Relative Frequencies of Positive Results in the Different Methods studied

	RNP	SCL-70	Sm	Jo-1	SS-A	SS-B	Total
CIE	0.29	0.06	0.22	0.06	0.35	0.29	0.21
	(9)	(2)	(7)	(2)	(11)	(9)	(40)
ELISA	0.42	0.06	0.29	0.06	0.61	0.30	0.28
	(13)	(2)	(9)	(2)	(19)	(8)	(53)
Blotting 1	0.35	0.06	0.19	0.03	0.48	0.30	0.23
	(11)	(2)	(6)	(1)	(15)	(8)	(43)
Blotting 2	0.22	0.03	0.14	0.06	0.30	0.16	0.14
	(7)	(1)	(3)	(2)	(8)	(5)	(26)

In brackets, absolute frequencies of total 31 sera analyzed. CIE, Counterimmunoelectrophoresis; ELISA, Inova enzyme linked immunosorbent assay; Blotting 1, Bio-Genex ANA Western Blot; Blotting 2, Biolab Anablot System II.

The approximate standard error of κ is

$$se\,(\kappa) = \sqrt{\frac{p_o\,(1-p_o)}{n\,(1-p_e)^2}}$$

so that a 95% confidence interval for the population value of k is given by

$$\kappa \pm 1.96\,.\,se\,(\kappa)$$

Kappa has a maximum of 1.00 when the agreement is perfect, a value of zero indicates no agreement better than chance, and negative values show worse than chance agreement.

RESULTS

Frequency analyses of results obtained with the four methods used demonstrate that ELISA (28%) gave the greatest number of positive results, both overall and for each antibody (Table 1). For CIE (21%) and blotting 1 (23%) the frequency of results were similar, while for blotting 2 the number of positive results was lower (14%). The antibodies most frequently detected are those directed against SS-A (61–30%) and RNP (42–22%) nuclear antigens; lower frequencies were obtained for SS-B (30–16%) and for Sm (29–14%), while antibodies to Scl-70 and Jo-1 were detected in less than 6% of the cases (Table 1).

The profile of autoantibodies obtained in each of the 31 sera analyzed is shown in Table 2. This profile has been defined as a function of the results that have been confirmed by at least two of the methods used in the study. When the capacity of each technique is individually analyzed for the detection of the indicated profiles, relative indices of sensitivity and specificity can be obtained (Table 3). The sensitivity for Scl-70 and Jo-1 has not been obtained because of the scarce number of antibodies detected. The method with greatest sensitivity in general is ELISA, although CIE showed the greatest sensitivity for detection of antibodies to Sm. The method with the lowest sensitivity is blotting 2, with results which fluctuate between 33–64%. Specificity was over 90% for all antibodies detected and for the four methods assessed, except for antibodies to SS-A detected by ELISA which was 78%.

Analysis of agreement between the results obtained by the four methods was carried out by the Kappa Index. Table 4 shows the results obtained. There was moderate agreement (Kappa > 0.50) for antibodies to RNP and SS-B detected by the four methods used, and especially for SS-B and RNP detected by ELISA and CIE (Kappa > 0.60). Agreement be-

Table 2. Profile of Autoantibodies Obtained in the Different Sera

Serum	Profile
1 Scleroderme	Scl-70
2. SLE	RNP/Sm/SS-A
3. SLE	RNP/Sm/SS-A
4. SLE	RNP/Sm/SS-A
5. RA	SS-A
6. Polymyositis	Jo-1
7. SLE	Sm/SS-A/SS-B
8. SLE	SS-A/SS-B
9. MCTD	RNP
10. SLE	—
11. Autoimune Cirrhosis	—
12. UCS	RNP
13. SS	SS-A/SS-B
14. UCS	Scl-70/SS-A/SS-B/RNP
15. UCS	SS-A
16. SLE	RNP/Sm/SS-A
17. SLE	SS-A/SS-B
18. SLE	RNP/Sm/SS-A
19. SLE	SS-A/SS-B
20. SS	SS-A/SS-B
21. RA	SS-A/SS-B
22. SLE	RNP/Sm/SS-A
23. UCS	—
24. SLE	—
25. UCS	—
26. SLE	RNP/Sm
27. UCS	SS-A
28. SLE	RNP/Sm
29. RA	—
30. UCS	—
31. UCS	—

SLE, Systemic lupus erythematosus; RA, Rheumatoid arthritis; MCTD, Mixed connective tissue disease; SS, Sjögren's syndrome; UCS, Undifferentiated connective syndrome.

Table 3. Sensitivity and Specificity of the methods used for detection of autoantibodies to nuclear antigens

		RNP	Scl-70	Sm	Jo-1	SS-A	SS-B
CIE	Se[A]	0.67	—	0.89	—	0.71	0.87
	Sp[B]	0.94	1.00	0.91	0.97	0.93	0.96
ELISA	Se	1.00	—	0.78	—	0.94	1.00
	Sp	0.90	1.00	1.00	0.97	0.78	0.96
Blotting 1	Se	0.81	—	0.67	—	0.82	1.00
	Sp	0.89	0.96	0.95	1.00	0.93	0.91
Blotting 2	Se	0.64	—	0.33	—	0.41	0.62
	Sp	1.00	0.96	1.00	0.97	0.93	1.00

[A]Se: Sensitivity
[B]Sp: Specificity
CIE, Counterimmunoelectrophoresis; ELISA, Inova enzyme linked immunosorbent assay; Blotting 1, Bio-Genex ANA Western Blot; Blotting 2, Biolab Anablot System II.

tween results for antibodies to Sm and SS-A was worse, especially for the latter, where the Kappa Index, when significant, was lower than 0.5. The lack of agreement between antibodies either to Scl-70 or Jo-1 can be explained by the scarce number of positive results obtained.

DISCUSSION

Measurement of antibodies to SS-A, SS-B, nRNP, Sm, Jo-1, and Scl-70 has been performed routinely by Ouchterlony gel diffusion and by CIE (5,6). However, recently ELISA and immunoblotting methods have been introduced in the market for the determination of ENAs (7,8). ELISA involves enzymatic amplification of the interaction between coated antigen on the microtiter plate and the primary antibody. This allows detection of antibodies with both low affinity and low avidity, together with a high sensitivity. On the other hand, a low specificity is ascribed (9). In our results, ELISA was actually the method with greater sensitivity, and at the same time showed a great specificity, except for SS-A. Moreover, ELISA can be automated, a factor that makes its use very attractive. However, we must consider that a high sensitivity can modify clinical associations and the classic profiles obtained by less sensitive methods, such as Ouchterlony gel diffusion and CIE, in non-selected patients (10). In spite of this fact, and if we consider CIE as the reference method, ELISA is the method which showed the greater consistency in our study, with significant results for all of the antibodies measured, except for those directed to either Scl-70 or Jo-1. The small number of positive results does not permit testing for significant agreement. In spite of this fact the results obtained for antibodies to Scl-70 were the same.

A greater sensitivity of blotting methods for antibodies to SS-A, SS-B, RNP and Sm when compared with gel-immunoprecipitation techniques has been shown (11,12). In our study, one of the blotting methods, which uses purified proteins as antigen, showed greater sensitivity than CIE and a good concordance with both CIE and ELISA for antibodies to RNP, SS-A and SS-B. However, the other blotting method, which uses nuclear extract of mammalian cells as a substrate, showed lower sensitivity than the other methods used.

The antigen preparation in blotting methods includes a protein denaturation step prior to electrophoretic separation of proteins. Theoretically, autoantibodies directed against antigenic determinants that depend on protein superstructure may

Table 4. Concordance of the Methods Used for the Detection of Autoantibodies to Nuclear Antigens

	Kappa Coefficient and I.C. 95%					
	RNP	Scl-70	Sm	Jo-1	SS-A	SS-B
ELISA-CIE	0.60	1.00	0.51		0.40	0.74
	(0.31–0.89)		(0.16–0.86)	*N.S.*	(0.09–0.71)	(0.46–1.00)
ELISA-Blotting 1	0.53				0.48	0.74
	(0.24–0.82)	*N.S.*	*N.S.*	*N.S.*	(0.17–0.79)	(0.46–1.00)
ELISA-Blotting 2	0.51		0.54			0.64
	(0.19–0.83)	*N.S.*	(0.12–0.96)	*N.S.*	*N.S.*	(0.32–0.96)
CIE-Blotting 1	0.41				0.36	0.66
	(0.06–0.76)	*N.S.*	*N.S.*	*N.S.*	(0.03–0.97)	(0.35–0.97)
CIE-Blotting 2	0.51					0.71
	(0.31–0.71)	*N.S.*	*N.S.*	*N.S.*	*N.S.*	(0.40–1.00)
Blotting 1-Blotting 2	0.69					0.71
	(0.55–0.83)	*N.S.*	*N.S.*	*N.S.*	*N.S.*	(0.40–1.00)

N.S.: No statistic significance.
CIE, Counterimmunoelectrophoresis; ELISA, Inova enzyme linked immunosorbent assay; Blotting 1, Bio-Genex ANA Western Blot; Blotting 2, Biolab Anablot System II.

become loose. Autoantibodies directed against SS-A are especially susceptible to this phenomenon (13). Thus, autoantibodies directed against SS-A are poorly detected by blotting methods because the antigen may not demonstrate the proper structure for recognition. In our study, we have considered autoantibodies to SS-A, both directed to T2 peptide and to Q 60 KD. In spite of this fact, the agreement between the results has been low, either due to alterations of 60 KD protein tertiary structure, with absence of reactivity, or because 60 KD and 50 KD peptides do not show uniform behaviour in immunofluorescence, immunoblotting and immunoprecipitation methods (14).

Recently, antibodies to SS-B directed against a conformational epitope present in the intact ribonucleoprotein particle SS-A/SS-B that include RNA motif has been described (15). However, due to the high degree of agreement between the methods used, the antibody recognizes this antigen both in the intact molecule and after denaturation in immunoblotting methods. Previously, it has been observed that anti-SS-B is readily detected by blotting methods (16,17).

Immunoblotting methods exhibit difficulties in the detection of antibodies to determinants susceptible of proteic degradation (SS-A, Scl-70, centromere). Also, they show lower sensitivity than ELISA and they require molecular-weight band controls in order not to confuse similar bands. On the other hand, CIE adds a subjective interpretation to a lesser sensitivity than ELISA. Thus, as was noted by the European Consensus Study Group, there is in the clinical laboratory a growing tendency to introduce ELISA methodology for the study of autoantibodies to nuclear antigens (16,17). Efforts should be made for method standardization in the clinical laboratory and for the introduction of analytical quality control programs with the use of reference standard sera (18).

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