Journal of Clinical Laboratory Analysis 24 : 230–236 (2010)

Determination of Cut-Off Titers and Agreement Between Immunofluorescence and Immunoblotting Methods for Detecting Antinuclear Antibodies in Children

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Detection of antinuclear antibodies (ANA) is a diagnostic adjunct in patients with suspected autoimmune connective tissue diseases, and various detection methods are in use. The aim of this study was to analyze the agreement between the ANA immunoflourescence (IF) and immunoblotting (IB) methods and determine cut-off for children subjects in a laboratory setting. We evaluated 729 serum samples that were analyzed by both ANA IF and IB. The results were evaluated by χ^2 test and, for agreement, κ index was used. Frequencies determined for both 1:40–1:100 cut-off titers of ANA IF in relation to IB testing supported the idea that 1:100 starting dilution should be recommended in children subjects for ANA IF method and antigen specific immunoblot testing was needed, especially for some of the ANA IF negative samples. Agreement between the two methods, especially with homogenous, granular, and nucleolar ANA IF patterns, was statistically significant. J. Clin. Lab. Anal. 24:230–236, 2010. © 2010 Wiley-Liss, Inc.

Key words: ANA; immunoflourescence; immunoblotting; cut-off titer; children

INTRODUCTION

Antinuclear antibodies (ANA) are directed against a variety of components of the cell nucleus. Detection of ANA aids in the diagnosis of patients with suspected autoimmune connective tissue diseases, such as systemic lupus erythematosus (SLE), mixed connective tissue diseases (CTD), Sjögren's syndrome, scleroderma, dermatomyositis, and polymyositis, and they are also produced transiently in infectious diseases (1). Measurement of ANA is used for screening, diagnosis, and monitoring of these disease entities.

ANA have been categorized into two groups: autoantibodies to DNA and histones; and autoantibodies to extractable nuclear antigens (ENA; Sm, RNP, SSA/Ro, SSB/La, Scl 70, Jo1, Pm, CNPB, PCNA, AMA-M2, Ku, RNA-polymerase I–III, and topoisomerase-I), so called as they were originally extracted from the nuclei with saline (2).

Various detection methods are in use and there are newer techniques that are continuously put forward to facilitate diagnosis and therapeutic monitoring in CTD patients. Indirect immunofluorescence assay (IF) is the most commonly used routine test for the detection of ANA (3) and also accepted as "gold standard" test for the detection of ANA positivity in serum. The advent of using Hep-2 cell line for detection of ANAs in the past 20 years provided sensitivity and brought more standardization and, therefore, acceptance of this test globally (4). This method is inexpensive, easy to perform, and has high sensitivity and specifity (2). Advances in technology have recently provided a new methodology options for ANA testing, such as immunoblotting (IB), enzyme immunoassays (5–8).

Although very low frequency of autoantibody positivity can be seen in healthy population, a raise in CTD patients is expected while mildly increased positivity in the elderly is observed (2).

IF and other methods are useful for detecting autoantibodies against many nuclear antigens. These are needed for determining ANA subtype and for distinctive diagnosis of connective tissue diseases. These techniques allow determination of some subgroups of

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Received 5 January 2010; Accepted 5 May 2010
DOI 10.1002/jcla.20391
Published online in Wiley InterScience (www.interscience.wiley.com).

autoantibodies, such as Sm, nRNP, SSA/Ro, SSB/La, Jo1, and Scl 70 (6).

The aim of this study was to analyze the agreement between the ANA IF and IB methods and determine cut-off titers in respect to IB results, for the first time in children subjects in a laboratory setting.

METHODS

Our study included all pediatric venous blood samples for the detection of ANA, consecutively sent to Ege University Medical Faculty Pediatric Immunology Laboratory from January 2006 to November 2009. We evaluated 729 serum samples that were analyzed by both ANA IF and IB. These samples belonged to patients: female/male ratio: 404/323 (55.6% F vs. 44.2% M) and mean age being 9.72 ± 4.80 years (this epidemiologic information was extracted from hospital records). These samples were sent by physicians for ANA analysis to our Reference Laboratory. Samples were evaluated randomly and blindly without any clinical information or suspected diagnosis.

Blood samples were drawn after an overnight fasting and allowed to clot at room temperature. Serum was obtained by centrifugation and aliquots were frozen at -20°C until assay. The presence of ANA in serum was determined by IF on mosaic Hep-20–10/LİVER MONKEY CELL (Euroimmun, Lübeck, Germany). FITC labeled antihuman IgG was obtained from Euroimmun. Serum samples were diluted in titers of 1:40, 1:100, 1:1,000, and 1:1,280 with PBS solution. Briefly, spots of Hep-20–10 cells were incubated for 30 min at room temperature with the diluted serum samples. If the reaction is positive, specific antibodies of IgG are attached to the antigens. After washing with PBS (for 5 min each), Biochip slides were incubated for 30 min with the antibody diluted in PBS. Slides were washed with PBS again (for 5 min each). All slides were evaluated under EurostarTM fluorescence microscope (Euroimmun, Luebeck-Germany) by two pediatric immunologists in a double-blind setting (each person evaluated the samples separately, with no clue to the other's results or patient info). Negative and positive controls with known antibody titers for each assay were used for quality control. ANA IF titers of 1:40 and 1:100 taken as cut-off titers were compared with other parameters within the study.

For IB assay, Euroimmun ANA Profile 3 Euroline kits (Lübeck) were used, which provided a qualitative in vitro assay for human autoantibodies of the IgG class to 14 different antigens: nRNP, Sm, SSA (SSA native and Ro52), SSB, Scl70, PM-Scl, Jo1, CENPB, PCNA, dsDNA, nucleosomes, histones, ribosomal P protein, and AMA-M2 in serum. Strips coated with nuclear and cytoplasmic antigens, which were separated according to their molecular weight by polyacrylamide gel electrophoresis, give positive band result if ANA binds, allowing specification of distinct antigens (2). Our results were evaluated with Euroline ScanTM (Lübeck) program. Strength of positivity was marked as +, ++, or +++ ($\geq$ + is taken as cut-off) (Fig. 1).

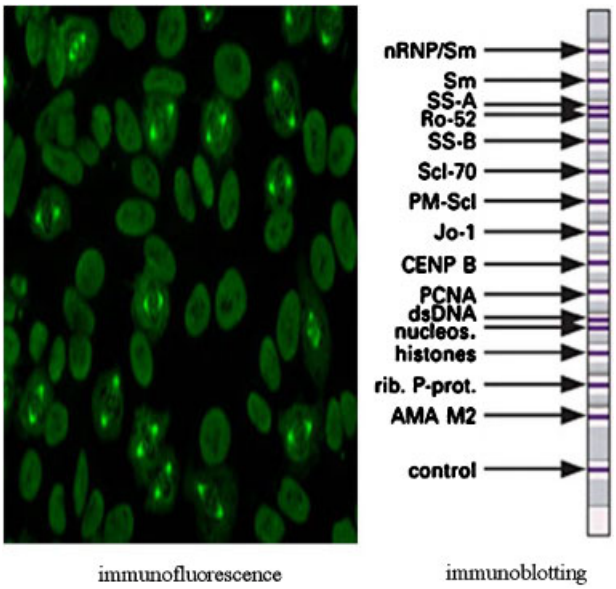


Fig. 1. An example of ANA by immunoblotting and ANA pattern by immunofluorescence.

Statistical analysis was performed using SPSS 17. Data were analyzed using χ^2 test. A P-value <0.05 was considered statistically significant. Cohen–Kappa statistics was used to determine consistency between tests. Kappa index is a measure of agreement between categorical data. Kappa value has a maximum of 1.00 when the agreement is perfect and a value of 0.00 indicates no agreement (6).

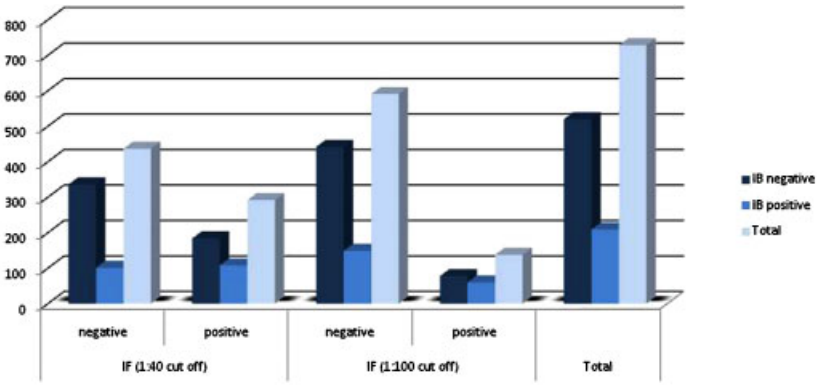
RESULTS

Total number of negative and positive samples for both methods, IF and IB, is listed in Figure 2.

ImmunofluorescenceIn our study group, if "ANA negative-1: 40 titer" was accepted as "negative," the frequency of positive ANA testing was 40.1% in all serum samples, whereas it would decrease to 18.9% if 1:100 was taken as cut-off.

In the samples examined, the highest frequency was 21.1% for positivity with 1:100 titers. Positive ANA titers of 1:160–1: 320 were seen in approximately 14% of samples.

Figure 3 shows the distribution of fluorescence patterns in these ANA positive samples within 1:40 cut-off. The most prevalent pattern was fine granular (26.9%) followed by homogenous (23.4%) and nucleolar



		IF								Total	
		cut off 1:40				cutoff 1:100					
		negative		positive		negative		positive			
		n	%	n	%	n	%	n	%	n	%
IB	negative	336	77	184	63	442	75	78	56.5	520	71.3
	positive	101	23	108	37	149	25	60	43.5	209	28.7
Totalstudy group		437	59.9	292	40.1	591	81	138	18.9	729	100

Fig. 2. Number and percentage of cases in ANA IF and IB groups according to 1:40 and 1:100 cut-off values.

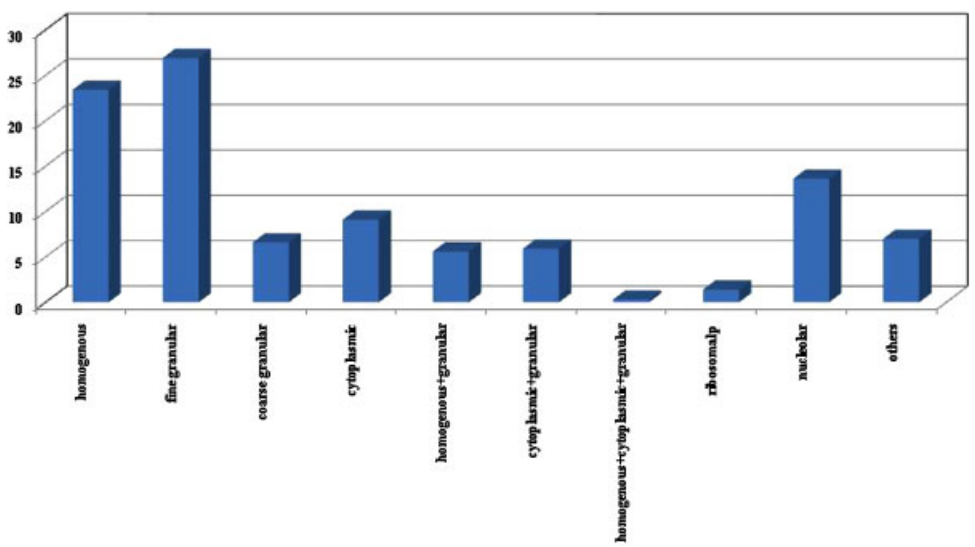


Fig. 3. Distribution of ANA patterns by IF within study group.

(13.6%) patterns. Cytoplasmic pattern was 9.1% within total samples. When 1:100 was taken as cut-off value, the most prevalent patterns were again homogenous (35.6%), fine granular (19.7%), and nucleolar (10.6%). Fine granular and nucleolar patterns were mostly identified patterns at 1:100 titer, whereas homogenous and fine granular patterns were identified at 1:160–1:320 titers (Fig. 4). Observed patterns were 28.6% homogenous, 20.0% fine granular, and 17.1% homogenous+granular pattern, above the titers of 1:320.

In 37 of 292 (12.7%) and 25 of 138 (18.1%) ANA positive samples (cut-offs 1:40–1:100, respectively), a second pattern was identified. The most common second

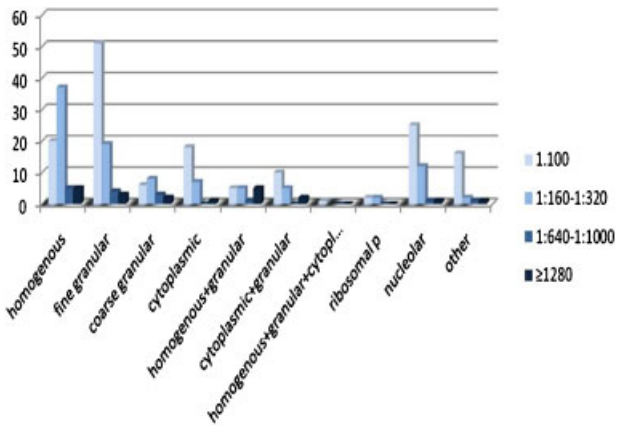


Fig. 4. Frequency of ANA patterns within ANA IF titer-groups.

patterns within these groups were nucleolar (1:40) and nucleolar and homogenous (1:100).

Immunoblotting

IB positive sample ratio within the whole study group was about 28.7% ($\geq +$), and this would decrease to 7.4% if strong positivity ($\geq ++$) was taken. In the IB method, the most commonly identified antinuclear autoreactivity was directed toward dsDNA (21%), Ro52 (11.6%), and histones (10%). The strongly positive ($\geq ++$) autoantibodies were mostly against anti-Ro52 (26.8%) and anti-SSA (21.9%).

Immunofluorescence+Immunoblotting

In the whole sera (729 samples), 108 (14.8%) and 60 (8.2%) were positive by both IF and IB methods, with cut-offs 1:40–1:100, respectively. Relationship between ANA IF and IB positivity was statistically significant ($P<0.001$) at 1:40 and ($P=0.000$) 1:100 cut-offs. IB positivity was 43.5%, if 1:100 was taken as cut-off value, whereas it was 37% in 1:40 within positive ANA groups (Fig. 2).

In the group with only ANA IF positive samples (1:40 cut-off), antibodies were directed mostly to dsDNA, Ro52, histones, SSA, and ribosomal p. If 1:100 was taken as cut-off, antibodies were directed mostly to dsDNA, Ro52, histones, and nucleosomes. A 13.7% showed strong IB positivity.

In ANA IF negative samples within 1:40 cut-off, IB positivity was 23.1% and antibodies were directed mostly to dsDNA, Ro52, nucleosomes, SSA, and SSB. In ANA negative samples, only 14 sera (3.2%) showed strong ($\geq ++$) IB positivity. In ANA IF (1:100 cut-off) negative samples, IB positivity was 29.7%, which was directed mostly to dsDNA, ribosomal p, histones, and Ro52.

TABLE 1. Increase of IB Positivity in Relation to Titer

IF titer	IB positivity %
Negative–1:40	23.1
1:100	31.2
1:160–1:1,320	32.4
1:640–1:1,000	66.7
≥1,280	81.0

TABLE 2. Agreement Between Two Methods; IF and IB (Statistically Significant*; *n*: Positive samples; κ Value: Agreement)

IB positivity↔IF pattern	*n*	*P* value (χ^2)	κ value
DsDNA/nuc/Hi↔homogenous	25	0.009*	0.150
SSA/SSB/RNP/Ro52↔fine granular	21	0.006*	0.143
Sm↔coarse granular	4	0.771	0.008
Sc170↔nucleolar	14	0.021*	0.143
AMAM2/Jol↔cytoplasmic	8	0.338	0.055

When we evaluated ANA IF titers with IB positivity, the frequent titer that showed IB positivity was 1:100 titer (22.9%) in the whole group. When titer groups were analyzed individually, IB positivity showed an increase in relation to titer (Table 1). Prevalence of positive ANA IB profile testing in strong ANA positive ($\geq$1:320 titer) population was 75% and 50% if samples with $\geq +$ and $\geq ++$ antibodies were selected, respectively.

In IB analysis of samples that were positive (75%) by IF above 1:320 titer, positivities for anti-dsDNA were estimated as 20% and 18.7% for anti-Ro52, 12.5% for anti-histones, and 11.2% for anti-nucleosomes, respectively.

For evaluation of the concordance of the antigens that were detected by IB and the classical IF patterns, IB dsDNA/histones/nucleosomes positivities were compared with IF homogenous pattern positivity, IB SSA/SSB/nRNP/Ro52 with IF fine granular pattern positivity, IB AMA-M2/Jo1 with IF cytoplasmic pattern positivity, IB Sm with IF coarse granular pattern positivity, IB Scl70 with IF nucleolar pattern positivity, and IB Ribosomal P protein with IF ribosomal P pattern positivity were compared.

In samples with homogenous pattern, dsDNA positivity was about 20% and, in samples with granular pattern, SSA/SSB/nRNP/Ro52 positivity was about 13%.

Analysis of agreement between the results obtained by the two methods, carried out by κ statistics, is listed in Table 2.

Relationship between Scl70 positivity and nucleolar pattern was statistically significant ($P=0.021$) and showed moderate agreement (κ value: 0.14). Relation-

ship between SSA/SSB/nRNP/Ro52 positivity and fine granular pattern was statistically significant ($P = 0.006$) and showed moderate agreement (κ value: 0.14). Relationship between dsDNA/histones/nucleosomes positivity and homogenous pattern was statistically significant ($P = 0.009$) and showed moderate agreement (κ value: 0.15). Other results showed no statistically significant relationship, but there was weak agreement between the two methods (Table 2).

DISCUSSION

In this study, we compared cut-off titers of 1:40 and 1:100 in ANA IF testing and agreement between ANA IF and IB, for the first time in children.

Usefulness of ANA test results depends on clinical situation. The likelihood that a positive test will provide useful information is proportional to the probability that a connective tissue disease is present (9). In our laboratory setting, we did not have any certain or suspected diagnoses of the patients. It is also well known that diagnosis of a connective tissue disorder in a child requires close follow-up in terms of laboratory and positive pathologic findings; hence, it is impossible to predict the diagnosis with one-time samples.

In our study group, prevalence of positive ANA testing was 40.1% at 1:40 titer cut-off value, but if 1:100 was taken as cut-off this would decrease to 18.9%. Peene et al. determined ANA positivity to about 23.5% in the adult group (1:40 cut-off) (10).

The ANA titer provides useful information and is directly proportional to antibody concentration. As a general rule, the higher the titer, the more likely that a connective tissue disease is present (9,11,12). In adults, a titer of 1:160 is taken as significant for the diagnosis of CTD in majority of laboratories (12). Bonaguri et al. (11) recommended that if ANA titer was $<1:80$, no second level test was needed to perform and that, especially, titer $>1:160$ was meaningful in adults. Sack et al. also suggested to begin serum dilution of 1:80, because in the majority of cases with this titer no diagnostically relevant ANA specifities were found (13). In the samples that were assayed, the highest frequency was 21.1% with 1:100 titer in our study and positive ANA titers of 1:160–1:320 was seen in approximately 14% independent from diagnosis.

McGhee et al. claimed that the diagnostic utility of ANA tests is limited because of the large number of healthy children who have low titer positive tests. They showed that age and ANA titer assist in discriminating children with SLE than from children with other conditions, and ANA tests are of no diagnostic utility in either making or excluding the diagnosis of juvenile idiopathic arthritis (14). Hilario et al. reported ANA positivity frequency as 12.6% (cut-off 1:80) in a group of healthy children with no significant difference between genders (15). At a serum dilution of 1:160, 1 in 20 (5%) healthy people have a positive ANA, and this number increases to 1 in 3 when screening is performed at a 1:40 dilution of serum (16) in adults.

Frequently seen IF ANA patterns are fine and coarse granular, cytoplasmic, homogenous, nucleolar, and centromere, as reported before (2,13). In our study, in the ANA IF positive group, the most prevalent pattern was fine granular (26.9%), which was followed by homogenous (23.4 %) and nucleolar (13.6%), if 1:40 titer was taken as cut-off, whereas these changed to homogenous (35.6%), fine granular (19.7%), and nucleolar (10.6%) when 1:100 titer was taken. Similarly, the most frequent pattern was granular followed by homogenous in a study by Kern et al. (17). Fine granular and nucleolar patterns were mostly identified at 1:100 titer, whereas homogenous and fine granular patterns were identified at 1:160–1:320 titers (Fig. 4). Observed patterns were 28.6% homogenous, 20.0% fine granular, and 17.1% homogenous+granular pattern, above the titers of 1:320.

Though ANA are subdivided into a few basic patterns, these are often present as mixed patterns. These can be differentiated by means of observation at different dilutions (13). In our study, 37 (12.7%) of a total 292 and 25 (18.1%) of a total 138 ANA positive samples (cut-off 1:40–1:100), second pattern was identified and the most common patterns identified in these groups were nucleolar (1:40), and nucleolar and homogenous (1:100).

Different autoantibodies are often measured simultaneously; this typically occurs when using IF on tissue sections or multiplex detection systems and may generate clinically "unexpected" positivities (18).

IB method has advantages, such as being too sensitive, automated, and giving the opportunity to define autoantibodies to distinct antigens. But, on the other hand, it is much more expensive and lacks the ability to detect different patterns available by IF. In our country, it costs twice the IF for each patient.

In a study by Bizzaro et al., ANA is determined by IF, immunoenzymatic assay, counterimmunoelectrophoresis, IB, western blotting, and analytical variability between laboratories was tested (19). On the whole, IB was the most sensitive procedure in detecting these autoantibodies, even though EIA and CIE gave similar performances. But for Scl 70 in particular, the sensitivity of the IB method was inadequate, as Gonzalez et al. reported that IB methods exhibit difficulties in the detection of antibodies to determinants susceptible of protein degradation (SSA, Scl 70, and centromere) (6). In our study group, positive IB testing was about 28.7%

and this would decrease to 7.4% if strong positivity ($\geq ++$) was accepted as definitive.

In the IB method, the most commonly identified antinuclear autoreactivity was directed toward dsDNA (21%), Ro52 (11.6%), and histones (10%) within our whole group, consecutively. The strongly positive ($\geq ++$) autoantibodies were anti-Ro52 (26.8%) and anti-SSA (21.9%). In a study by Peene et al. (10),) the most frequent autoantibody detected was SSA by line assay.

It is possible that some antigens may not be identified on Hep2 tissues, as Hoffman et al. suggested. In such cases, an antigen specific ELISA is strongly recommended. Hoffman et al. showed that some patients with negative IF showed reactivity on line immunoassay (20). They suggest that if there is clinical suspicion for connective tissue disease, testing for ENAs should be performed even if IF is negative. Otherwise, there is a risk of missing anti-SSA, SSB, and Jo1 antibodies. In our study, there was 23.1 and 29.7% IB positivity in respect to 1:40–1:100 cut-offs in ANA IF negative sample group. This supported the opinion that antigen specific ELISA assay was necessary for ANA IF negative, but IB positive samples.

In ANA IF negative samples within 1:40 cut-off, the negativity would be 60% in all serum samples if we had accepted that "ANA negative-1:40 titer" as "negative." Higher ratio in low titers and IB negativity in some of the ANA positive group may be related to healthy children showing ANA positivity.

In ANA negative samples in our study group, only 3.2–1% (1:40–1:100 cut-offs) sera showed strong IB ($\geq ++$) positivity. As 60–75% (1:40–1:100 cut-offs) of the total sera were negative and there was a statistically significant relationship between ANA IF and IB positivity in samples equal or more than 1:100 titer, we can presume that results below 1:100 titer may not be meaningful.

In ANA IF positive samples, IB positivity was 36.9 and 43.5% in respect to 1:40–1:100 cut-off, and antibodies were directed mostly to dsDNA, Ro52, histones, and SSA. When we evaluated ANA IF titers with IB positivity, the frequent titer that showed IB positivity was 1:100 (22.9%) in the whole group. When the titer groups were analyzed individually, IB positivity showed an increase in relation to titer group (Table 1).

Kang et al. reported that ANA positivity below $<1{:}160$ titer did not require profile testing, and if there was homogenous pattern, single specific anti-dsDNA should be determined (21). In our study, 75% of serum specimens that showed positivity $\geq 1{:}320$ titer in IF method was significantly IB positive and that dsDNA was the most frequent antibody (20%) in this group.

Perilloux et al. reported that a positive ANA profile strongly correlated with an ANA titre of $\geq 1{:}640$ ($\chi^2 = 5.7$, $P < 0.02$) and suggested not to perform profile testing with all ANA positive samples (22). But Hauksdottır et al. indicated that an ANA cut-off titer of 1:512 (or 1:640) is too high, as many clinically important ANA specificities, such as dsDNA, would not be identified (23).

It has been suggested that some ENAs can be missed on IF, such as anti-SSA/Ro (24) and anti-SSB/La antibodies (25). This may explain our high IB positivity percentage in ANA IF negative samples.

Analysis of agreement between results obtained by the two methods was noteworthy when we compared IF patterns with IB. There are typical nuclear and cytoplasmic fluorescence patterns of autoantibodies: fine granular (SSA/Ro, SSB/La, Jo1), coarse granular (nRNP, sm), cytoplasmic (ribosomal P), homogenous (dsDNA, nucleosomes, histones), nucleolar (scl 70), and centromere (CENP A-E) (2,13). In our study, relationship between Scl70 positivity and nucleolar pattern and relationship between SSA/SSB/nRNP/Ro52 positivity and granular pattern was statistically significant showing moderate agreement. Similarly, relationship between dsDNA/histones/nucleosomes positivity and homogenous pattern was statistically significant, too, and showed moderate agreement (Table 2). Gonzalez et al. compared ANA, using four commercial assays (IF, ELISA, CIE, and IB) and for SSB antibodies; they detected good concordance between these methods (k: 0.66–0.74). But, in spite of high prevalence of anti-SSA antibodies, the agreement between methods were poor, without statistical significance (6).

All of these data have great importance if they are evaluated with the clinical information of the patients. But, in this study, there was no knowledge about suspected diagnosis by the time of samples' arrival to the laboratory. In childhood, diagnosis of CTD should not depend on laboratory data only; much more observation time is required on adults. So, it is difficult to relate these data with diseases.

In conclusion, ANA IF method is inexpensive, reliable, and the first step routine screening method, and IB assay has great importance in determining distinctive autoantibody positivities. In this study, agreement of two ANA testing methods, IF and IB, was examined for the first time in childhood. Identification of strong agreement between the two methods helped us to estimate cut-off values for ANA determination by IF. It has been observed that starting assays with 1:100 dilution is more suitable in children.

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