Abnormal Antinuclear Antibody Titers Are Less Common Than Generally Assumed in Established Cases of Systemic Lupus Erythematosus

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ABSTRACT. ***Objective.*** To evaluate antinuclear antibody (ANA) tests in established cases of systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) by indirect immunofluorescence microscopy (F-ANA) and enzyme-immunoassays detecting antinucleosomal antibodies (ANSA-EIA).

Methods. Sera from 50 patients with SLE and 65 patients with RA were analyzed regarding abnormal concentrations of F-ANA (serum dilution ≥ 1:200 = 95th percentile among 300 healthy blood donors). The sera were also analyzed with 2 commercial ANSA-EIA kits.

Results. An abnormal F-ANA titer occurred in 76% of the SLE sera compared to 23% in RA, and was not related to present use of antirheumatic drugs. At dilution 1:50, 84% of the SLE sera were F-ANA-positive compared to 20% of healthy women. Forty percent and 56%, respectively, of the SLE sera tested positive in the 2 ANSA-EIA kits. By the most sensitive assay, 96% of the ANSA-positive SLE sera produced a homogenous (chromosomal) F-ANA staining pattern compared to 18% of the ANSA-negative SLE sera. Ten of the 15 F-ANA-positive RA sera (63%) generated homogenous F-ANA staining and 13 (20%) tested positive in the most sensitive ANSA-EIA, but with no correlation to the F-ANA staining pattern.

Conclusion. The sensitivity of F-ANA at an abnormal titer was surprisingly low (76%) in established cases of SLE. ANSA occurred in 56% of the SLE sera, but also in a fair number (20%) of RA sera. Practically all ANSA-positive SLE sera were identified by chromosomal F-ANA staining. We conclude that the antigen-specific antinucleosomal EIA does not have high enough diagnostic specificity to justify use of this analysis for routine diagnostic purposes. (First Release Sept 1 2008; J Rheumatol 2008;35:1994–2000)

Key Indexing Terms:
SYSTEMIC LUPUS ERYTHEMATOSUS ANTINUCLEAR ANTIBODY
ANTINUCLEOSOMAL ANTIBODY SENSITIVITY SPECIFICITY

Systemic lupus erythematosus (SLE) was once thought of as a rare disease with poor prognosis. Today the 5-year survival rate is well over 90%[1-4] compared to less than 50% in the 1950s[5]. Although the 10-year survival rate is approximating the 5-year figures, the mortality rate in SLE is still a great concern in the long run due to cardiovascular disease[3,4,6]. The improved prognosis is to a great extent due to better treatment strategies, but is also explained by greater awareness of the condition after the introduction of antinuclear antibody (ANA) analyses and other seromarkers in diagnostic routine, resulting in increased numbers of patients diagnosed with SLE. Currently we diagnose not only the severely ill patients with full-blown disease, but also those with mild disease and subtle symptoms. Since the beginning of the 1980s, however, the annual incidence rate of SLE in Sweden has remained stable at about 4.5/100,000 and the prevalence approximately 70/100,000[4].

A positive ANA test by immunofluorescence (IF) microscopy is the serologic hallmark of SLE, and since the 1950s indirect IF microscopy has been the reference method for ANA analysis (F-ANA). But since then the view on ANA has changed[7]; a positive F-ANA test at an abnormal titer is one of the American College of Rheumatology (ACR) classification criteria for SLE[8]. It is often stated that the diagnostic sensitivity of F-ANA in SLE is > 95%[9]. The

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concept of "ANA-negative SLE" was introduced 1976[10], and has been said to be typical of patients with photosensitivity and antibodies to SSA/Ro[11]. With the use of modern microscopes and HEp-2 cells as a source of nuclear antigens, the existence of ANA-negative SLE has been questioned[12]. However, few evaluations have been done recently regarding the frequency of F-ANA at abnormal titers as stipulated by the ACR criteria. Although the diagnostic sensitivity of F-ANA is high in SLE, we know that this test is often positive in other inflammatory conditions as well. Indeed, F-ANA is also common among perfectly healthy individuals, who probably never develop any other signs of systemic disease[13-16]. Another typical feature of SLE is the occurrence of immune complexes in the circulation and in inflamed organs, and immune complexes are believed to account for many of the disease manifestations in SLE, e.g., nephritis[17,18]. Antibodies to double-stranded DNA (dsDNA) are of particular interest, and it has long been believed that immune complexes formed between IgG and dsDNA or DNA-histone complexes (nucleosomes) are of pathogenic importance in lupus nephritis[19,20].

In a series of elegant experiments in the 1950s, Kunkel and coworkers demonstrated that the "LE-cell" phenomenon (neutrophil phagocytosis of nuclear material in blood smears from SLE patients) was explained by autoantibodies to DNA-histone complexes, i.e., nucleosomes[21]. It is well known that antinucleosomal antibodies (ANSA) produce a homogenous (chromatin) staining pattern on IF-microscopy, similar to anti-dsDNA and antihistone antibodies[22]. Nucleosomes are the fundamental elements of chromatin, formed as "packages" of double-histone tetramers connected through a linker histone and wrapped up with a 146-base pair sequence of DNA. In unbroken chromatin, neighboring nucleosomes are connected by 15–100 base pairs of DNA, forming a long chain[23-25]. During apoptosis this chain is broken down into mono- and dinucleosomes, which may be released into the circulation within 24 hours[26]. Elevated concentrations of circulating nucleosomes have been found in SLE[27]. Recently, much attention has been drawn to the hypothesis that deficient handling of apoptotic material, including circulating nucleosomes, is an important etiological factor in SLE[28-31] and is reflected by abnormal formation of ANA, including ANSA. It has been suggested that ANSA can serve as a valuable diagnostic marker of SLE or be used to measure lupus disease activity[32-34]. Commercial enzyme immunoassay antinucleosomal antibody (ANSA-EIA) tests are available for this purpose. However, it has been pointed out that the development of new diagnostic antinuclear antibody assays requires careful consideration regarding their clinical utility[35,36]. We analyzed the sensitivity of a positive F-ANA test at abnormal level and of ANSA analyzed by EIA in cases of established SLE, compared to rheumatoid arthritis (RA) and healthy blood donors.

MATERIALS AND METHODS

Subjects. Fifty patients (46 women, 4 men, mean age 42 yrs, range 23–57) diagnosed with SLE at the rheumatology unit, Lund University Hospital, were studied. Serum samples were drawn at routine clinical visits during 2001 to 2003, and kept frozen at –20°C until analyzed. Previously, at the time for diagnosis between 1963 and 2000, sera from all SLE patients had been subjected to LE-cell and/or F-ANA analysis, and were then all judged as positive by the techniques and equipment used at the time. It was not possible to assess retrospectively whether the cutoff limits for positive F-ANA at the different times throughout that period identified only abnormal levels of ANA. Disease activity at the serum sampling in the period 2001-2003 was assessed by the SLE Disease Activity Index (SLEDAI)[37]. Forty-seven of the patients fulfilled the 1982 ACR classification criteria for SLE[8], and the remaining 3 had positive F-ANA and at least 2 typical organ manifestations at the time of diagnosis.

Sera from 65 patients with a clinical diagnosis of RA (49 women, mean age 61 yrs, range 23–80; 16 men, mean age 68 yrs, range 27–85) were drawn consecutively in conjunction with routine followup visits at the rheumatology outpatient clinic in Linköping in 2004, and kept frozen at –20°C until analyzed.

For the F-ANA analyses, sera from 300 healthy blood donors were used as reference material. Sera were collected and kept frozen (–20°C) at the Department of Clinical Immunology and Transfusion Medicine at Linköping University Hospital. Of these, 100 were collected and analyzed in 1997 (50 men, mean age 44 yrs, range 21–66; 50 women, mean age 41 yrs, range 19–67). In 2000, another 200 healthy donor sera drawn from 100 men (mean age 41 yrs, range 20–66) and 100 women (mean age 41 yrs, range 18–68) were analyzed for F-ANA.

A third collection of 100 healthy donor sera was used as reference material for the ANSA-EIA (50 men, mean age 37 yrs, range 18–60; 50 women, mean age 36 yrs, range 18–65). These sera were collected in 2003 and kept frozen at –20°C until analysis.

Indirect IF microscopy. Swedish laboratories performing routine ANA diagnostics follow national guidelines and are subjected to the national quality assurance program, Equalis (www.equalis.se/). Autoantibody diagnostics at these laboratories are accredited by the Swedish Board for Accreditation and Conformity Assessment (http://www.swedac.se/sdd/System.nsf/(GUIview)/index_english.html), which also scrutinizes the quality of diagnostic laboratories on a regular basis. There is a consensus that a cutoff limit at the 95th percentile should be used in order to identify only F-ANA at abnormal levels, whereas endpoint titration is not mandatory.

In our study, 2 separate analyses were performed using the 2 different serum materials from healthy donors, in order to set the cutoff limit for an abnormal F-ANA titer. In the first evaluation the 100 sera were serially diluted in phosphate-buffered saline (PBS, pH 7.4) in 4 steps: 1:40, 1:80, 1:100, and 1:160. In the second setting, 200 sera were diluted: 1:50, 1:100, 1:150, and 1:200. Female and male sera were analyzed separately in both settings.

Multispot slides with fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA) were incubated with diluted sera in a moist chamber for 30 min at room temperature. After 10 min washing in PBS, fluorescein-isothiocyanate (FITC) conjugated gamma-chain-specific rabbit anti-human-IgG (Dako, Glostrup, Denmark) was applied to the slides (at an optimal dilution decided by checkerboard titration) for 30 min. The slides were washed with PBS, mounted with PBS-buffered glycerine, and inspected under a Nikon fluorescence microscope with halogen lamp (HBO 50) epi-illumination and filters for FITC activation/emission. The results were judged as positive or negative, and positive results were categorized into homogenous, speckled, or other staining patterns. Since F-ANA-negative cases of SLE have been reported to test positive regarding antibodies to SSA/Ro, all SLE serum samples were also analyzed by double radial immunodiffusion in gel (ImmunoConcepts plates) to assess antibodies against SSA/Ro and SSB/La. The specificities of other autoantibodies precipitating extractable nuclear antigens (ENA) were not identified with ref-

erence sera, but simply recorded as a precipitation line of non-identity to the anti-SSA/SSB reference.

Antinucleosome antibody EIA. The 50 SLE sera were analyzed with 2 commercial ANSA-EIA kits: Anti-Nucleo (GA Generic Assays GmbH, Dahlewitz, Germany) with purified avian nucleosome antigen, and Quanta Lite Chromatin (Inova Diagnostics, San Diego, CA, USA) using histone-H1-stripped calf-thymocyte chromatin as antigen. The 65 RA sera were analyzed with Quanta Lite alone. 100 sera from healthy donors were used as controls. Reference sera, wash solutions, horseradish peroxidase (HRP) conjugates, and stop solutions were provided with the kits, and the procedures were done according to instructions by the manufacturers. In both tests the sera were diluted 1:100 with buffers included with the kits, and run in duplicates. The results were achieved by reading optical density (OD) in an automatic plate reader (Anthos Labtec HT3) at dual wavelengths 450/600 nm.

Anti-cyclic citrullinated peptide antibody EIA. Since the occurrence of ANA in RA has been suggested to predict more serious disease with risk of extraarticular manifestations[38], and since the occurrence of antibodies to cyclic citrullinated peptides (CCP) is known to identify RA patients with a more severe disease course[39], the RA material was subdivided into anti-CCP-positive/negative cases. The 65 RA sera were analyzed for anti-CCP antibodies using Immunoscan RA Mark 2 (Euro-Diagnostica, Arnhem, The Netherlands) according to the manufacturer's instructions. An antibody level ≥ 25 units was considered positive.

Statistics. Two-tailed Fisher's exact test was used to compare the distribution of autoantibody results in SLE patient subgroups. Correlation of results from the 2 ANSA-EIA was by Spearman's correlation test.

Informed consent was obtained from each patient and the regional ethics committee in Lund approved the study protocol.

RESULTS

F-ANA. Results of F-ANA analyses in the 2 groups of healthy donor materials are summarized in Figure 1. At a serum dilution of 1:40, 45% of the 100 healthy donors tested positive, with no difference between the sexes. At higher dilutions a steep slope was seen for the presence of ANA, and it was found that F-ANA was more prevalent in women compared to men. Defining the 95th percentile as cutoff for positive F-ANA, titers ≥ 1:200 are regarded as abnormal for women. The corresponding level for men was 1:80.

The F-ANA results in the SLE materials are shown in Table 1. In total, 76% of the patients were F-ANA-positive at an abnormal serum level (all 3 patients who did not fulfil the ACR SLE classification criteria were F-ANA-positive). The homogenous staining pattern prevailed and was seen in 62%. At a serum dilution of 1:50, 84% of the SLE patients were F-ANA-positive (Table 1) compared to 20% of healthy female blood donors (Figure 1). The occurrence of ANA was not associated with the use of disease modifying antirheumatic drugs and/or oral glucocorticoids (data not shown). Apart from one case, however, all F-ANA-negative SLE patients had low disease activity, with SLEDAI scores below 4 (Figure 2). The majority (88%) of SLE patients were diagnosed after 1980. Comparing the 24 patients diagnosed with SLE 1963-1990 with the 26 diagnosed 1991-2000, the frequency of positive F-ANA was significantly higher ($p < 0.05$) in the former group: 88.5% versus 62.5%.

Table 1. F-ANA results at different serum dilutions of the 50 SLE sera. Serum dilution 1:200 is the cutoff for an "abnormal level."

F-ANA Pattern	Serum Dilution 1:50, n (%)	1:100, n (%)	1:200, n (%)
Homogenous ± other pattern	33 (66)	33 (66)	31 (62)
Speckled	7 (14)	5 (10)	5 (10)
Other pattern	2 (4)	2 (4)	2 (4)
Total F-ANA-positive	42 (84)	40 (80)	38 (76)
F-ANA-negative	8 (16)	10 (20)	12 (24)

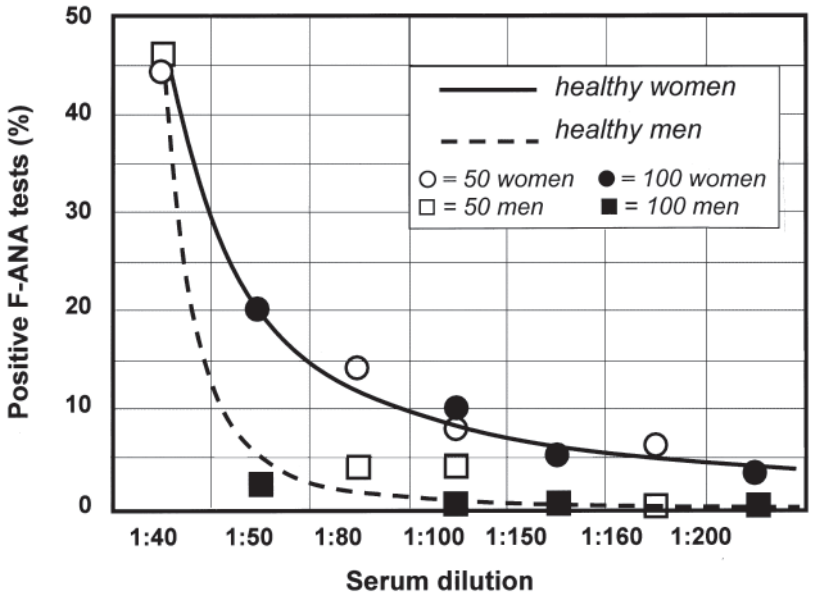

Figure 1. F-ANA results in serial dilutions of 300 healthy donor sera (150 women, 150 men); 2 separate reference materials were run on 2 different occasions.

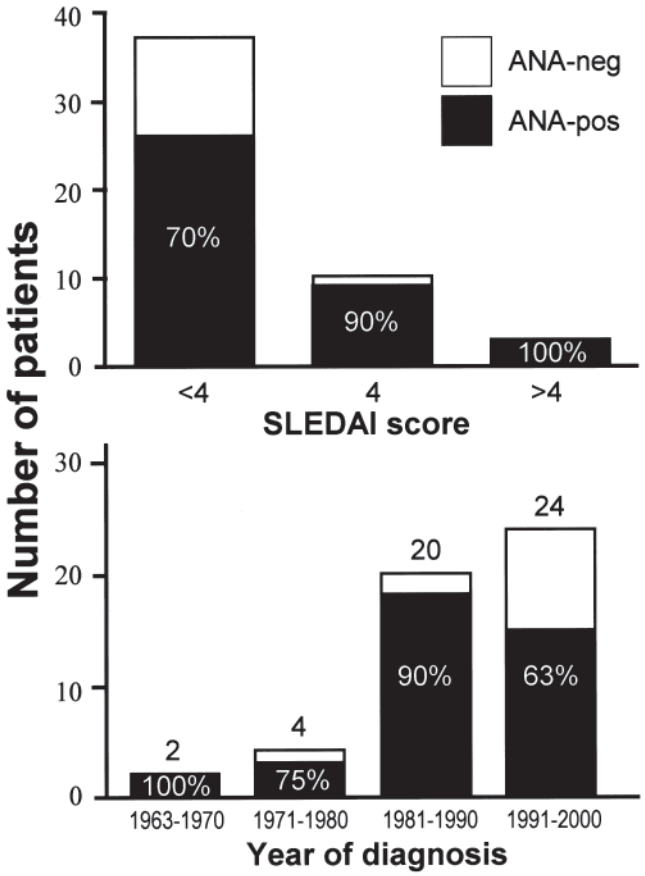

Figure 2. Abnormal levels of F-ANA in relation to SLEDAI scores and to the time period when SLE was diagnosed.

Twenty percent (10/50) of the SLE sera were anti-SSA antibody-positive on immunodiffusion, all but one being F-ANA-positive ≥ 1:200. In addition, 7 SLE sera had anti-ENA antibodies reacting with antigen(s) other than SSA(Ro)/SSB(La).

In the RA material, 23% (15/65) of the sera tested positive for F-ANA at an abnormal level. Here too, the homogenous staining pattern prevailed, with an overall frequency of 15%. 75% (49/65) of the RA sera were anti-CCP antibody-positive, and of these, 24% (12/49) were F-ANA-positive, compared to 19% (3/16) of the patients who were anti-CCP-negative (not significant).

ANSA versus F-ANA. Using the cutoff limits suggested by the manufacturers, ANSA was detected in 20/50 SLE sera (40%) by the Anti-Nucleo test and in 28 (56%) with Quanta Lite (Table 2). The corresponding results in 100 healthy donor sera were 6% and 2%, respectively. The results of these 2 tests correlated significantly testing the SLE serum material ($r = 0.62$, $p < 0.0001$).

Of the 28 ANSA-positive sera identified by Quanta Lite, 27 (96%) were found to have F-ANA of the homogenous staining pattern (Table 2). Four additional sera (4/31, 13%) produced homogenous F-ANA, but were negative in the Quanta Lite assay. Thus, 87% of the sera producing a homogenous F-ANA pattern were found to be positive with this assay. Of the 20 sera positive in the Anti-Nucleo tests, 19 (95%) produced a homogenous F-ANA, whereas the remaining 12/31 (39%) homogenous F-ANA-positive sera were negative in the Anti-Nucleo test (Table 2). The occurrence of ANSA in SLE did not correlate to disease activity as measured by SLEDAI (data not shown).

Considering the higher frequency of positive ANSA tests using the histone-H1-stripped calf-thymus chromatin kit from Quanta Lite compared to avian nucleosomes in the Anti-Nucleo kit (56% vs 40% in the SLE material), and yet a lower frequency of positive tests in the healthy donor material (2% vs 6%), and the higher correspondence of Quanta Lite results with homogenous F-ANA staining compared to Anti-Nucleo results (87% vs 61%), the former was chosen to analyze the 65 RA sera. Of these, 13 (20%) tested positive for ANSA, 10 of which produced a homogenous F-ANA staining pattern (77%). In contrast to the SLE material, there was no significant correlation between ANSA and a homogenous F-ANA pattern. Eleven of the 13 ANSA-positive RA samples (85%) were anti-CCP-positive.

DISCUSSION

After more than 50 years, IF microscopy is still the reference method for ANA analysis in clinical routine. Referring to the 1982 ACR classification criteria for SLE[8] and because no other available ANA test produces equivalent results[40], F-ANA must remain the method of choice. It is often said that at least 95% of SLE patients are F-ANA-positive[9]. The switch from rat tissue cryostat sections to the use of HEp-2 cells may have decreased the proportion of F-ANA-negative cases of SLE further, but by no means excludes the possibility that F-ANA-negative sera contain antibodies against SSA(Ro)[40]. In our study, 20% of all SLE patients had observable anti-SSA(Ro) antibodies, but only one of 12 F-ANA-negative SLE sera (8%) were anti-SSA(Ro) positive. Thus, it can be concluded that F-ANA-negative SLE is not restricted to a subgroup of anti-Ro-positive SLE patients. ANA have a significant influence in the classification of SLE, since 2 of the 11 ACR criteria are related to ANA, i.e., F-ANA at an abnormal level in serum and any of the following ANA-associated variables: positive LE-cell test, anti-dsDNA antibodies, or anti-Sm antibodies[8]. The inevitable inclusion of ANA tests for the classification/diagnosis of SLE has also introduced a diagnostic bias. However, the requirement of an abnormal serum level of F-ANA has often, regrettably, been neglected. Although the performance of IF microscopy techniques has gradually improved, the cutoff levels for "positive ANA" have many times not been adjusted accordingly. At serum dilutions of 1:50 or more in this study, F-ANA was more common in healthy women compared to men, but at a serum dilution of 1:40 F-ANA was present in 45% of both sexes. This is sim-

Table 2. Antinucleosomal antibody results (ANSA-EIA) from 2 different diagnostic kits compared to homogenous F-ANA in the 50 SLE sera.

ANSA-EIA	Homogenous F-ANA ≥ 1:200 (%)	Homogenous F-ANA Negative (< 1:200) (%)	Total
Quanta Lite-positive	27 (87)	1 (5)	28
Quanta Lite-negative	4 (13)	18 (95)	22
Total	31	19	50
Anti-Nucleo-positive	19 (61)	1 (5)	20
Anti-Nucleo-negative	12 (39)	18 (95)	30
Total	31	19	50

ilar to what Tan, *et al* have reported[41], but in contrast to their conclusions we maintain that this emphasizes the necessity to apply adequately high serum dilutions for F-ANA screening in clinical routine diagnostics in order to identify F-ANA positivity only at abnormal levels. Since diagnostic equipment and procedures vary between laboratories, F-ANA titers cannot be compared directly between different laboratories, which was also evident in the study by Tan, *et al*[41]. Thus, it is imperative that all laboratories providing the service of ANA diagnostics calibrate their cutoff levels based upon defined reference materials.

The frequency of positive F-ANA in SLE does not depend solely upon the cutoff level, but of course also depends on how the patient material is selected. For instance, in a European prospective multicenter study on 289 patients diagnosed with SLE, of which 81% fulfilled the ACR classification criteria, F-ANA was found in 96% of the cases using HEp-2 or HEp-2000 cells (the latter overexpressing SSA/Ro60) as source of nuclear antigens, but the cutoff levels for positive tests were not specified[42] and thus it is not clear how many actually had abnormal levels of ANA. In a study from Finland, with 305 patients fulfilling the ACR criteria, 76% of the cases were F-ANA-positive at a level above the 95th percentile in samples of healthy reference material[43]. A similar frequency of abnormal F-ANA titers in SLE was reported by Nordmark, *et al*[44]. In a recent study of African American patients with SLE, > 93% tested positive for F-ANA at a serum dilution of 1:120, but since 20% of unrelated control persons also tested positive, this did not reflect an abnormal titer of F-ANA. However, at a dilution of 1:1000, where 3.3% of unrelated female controls tested positive (i.e., similar to the abnormal level as defined in our study), only 60% of the SLE patients were found to be F-ANA-positive[45]. Although the main finding in our study has also been reported by others, i.e., that an abnormal titer of F-ANA occurs in only three-quarters of SLE patients with established disease, this finding has not been highlighted before. It may be argued that inclusion of patients with very longstanding disease in this study could have instituted a selection bias, causing an overrepresentation of F-ANA-negative survivors with mild disease, whereas the more serious cases with positive F-ANA and high mortality escaped detection. However, this did not seem to be the case, since a significantly lower proportion of ANA-positive sera was found among the most recently diagnosed cases: 63% in the group diagnosed in the period 1991-2000 (i.e., similar to the results of Kamen, *et al*[45]) compared to 89% of the patients diagnosed 1963-1990. This may indicate that modern diagnostic methods and increased awareness has resulted in identification of milder cases of SLE, perhaps more prone to lose F-ANA positivity with time. Indeed, the majority of SLE patients in our study had low disease activity, and only those with SLEDAI scores ≤ 4 were F-ANA-negative. However, with the exception of anti-dsDNA antibodies, quantification of ANA is not useful to assess disease activity in SLE. Longitudinal studies are warranted to evaluate whether the occurrence or absence of F-ANA in SLE is related to disease activity and/or disease severity. Nevertheless, a clear conclusion from our study is that abnormal titers of F-ANA are considerably less prevalent in manifest SLE than usually stated. Even at a cutoff titer at the 80th percentile (i.e., 20% positive in a healthy female blood donor population) only 84% of the SLE patients were F-ANA-positive in this study. Only one of the remaining 16% ANA-negative sera at this serum dilution was found to be positive regarding anti-Ro/SSA antibodies.

Since the 1950s it has been known that ANA in SLE can be directed against DNA-histone complexes (nucleosomes) and that this is reflected by a positive LE cell test[46]. In recent years, specific antinucleosomal antibody tests have become widely used, and are suggested to be of diagnostic value in SLE[32,33]. We evaluated 2 commercial ANSA ELISA kits. We found that 96% of the ANSA-positive sera were identifiable as F-ANA-positive with a homogenous (chromatin) staining pattern. Since a considerable proportion of patients with RA also proved to be ANSA-positive, we conclude that this test does not add any important diagnostic information to traditional F-ANA analysis. Regarding RA, 23% of the 65 patients were F-ANA-positive at an abnormal serum level. In some studies, F-ANA has been found to be associated with a more severe outcome of RA with extraarticular manifestations, although this was not confirmed in a recent study using an appropriate cutoff limit for F-ANA[47]. Anti-CCP is another marker with well established predictive potentials in

early RA, and a significant association has been reported regarding occurrence of anti-CCP and extraarticular RA[47]. We found no significant association between anti-CCP antibodies and F-ANA in RA.

To conclude, abnormal serum levels of F-ANA are far less prevalent than generally assumed in patients with an established diagnosis of SLE. Our results also suggest that conventional F-ANA is a satisfactory and sensitive means to detect antinucleosomal antibodies in SLE. Further, since the antigen-specific tests for antinucleosomal antibodies did not have high enough diagnostic specificity, and did not add information of significant importance regarding disease activity, we do not encourage their use in clinical routine diagnostic investigation.

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