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Immunofluorescence microscopy is superior to fluorescent beads for detection of antinuclear antibody reactivity in systemic lupus erythematosus patients

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Abstract

Antinuclear antibodies (ANA) are a hallmark of systemic lupus erythematosus (SLE) and one of its key diagnostic criteria. Recently, new technologies based on antibody binding to fluorescence beads (FB) have been widely employed for ANA screening. We conducted a formal study in 385 consecutive patients who underwent both traditional immunofluorescence (IF) and FB testing and evaluated each patient for the diagnosis of SLE. The distribution of ANA test results was significantly different ($\chi^2=73.12$; $p<0.0001$) due to a marked discordance of double-negative and double-positive results. The concordance of the FB-negative and IF-negative test results was 240/256 (95.6%), while the concordance of double-positive results was 54/129 (41.9%). The sensitivity of IF was markedly higher (48/53; 90.6%) than that of the FB (26/53; 49.1%; $p<0.0001$) for the diagnosis of SLE. IF had a lower specificity (76%) than FB (87%; $p=0.0002$). The present data show that the IF assay has superior sensitivity for detection of ANA and should continue to be used as the primary screening test for the diagnosis of SLE.

Keywords

Antinuclear antibody; Systemic lupus erythematosus; Diagnosis; Immunofluorescence; Flow cytometry

Introduction

The presence of antinuclear antibodies (ANA) is a hallmark of SLE and one of its diagnostic criteria established by the American College of Rheumatology [1]. ANA are seen in 90–95% of patients with SLE. It is traditionally detected by indirect immunofluorescence (IF) assay in which the antibodies of the patients' sera that bind to the nucleus of Hep-2 human epipharynx carcinoma cells are detected by fluor-escein isothiocyanate-conjugated anti-human IgG, using fluorescence microscopy [1]. The IF technique also provides information on the pattern of fluorescence, such as homogeneous, peripheral, nucleolar, or speckled [1]. Such patterns are relevant for antigen specificity and they have been associated with autoimmune disease subsets [1]. Notwithstanding, the detection of ANA by IF is laborious and requires an experienced technician. Flow cytometry with autoantigen-coated fluorescent beads (FB) has been gaining popularity for several years [2]. FB-based techniques, also commonly referred to as Reflex ANA, are claimed to have multiple advantages, such as simultaneous testing for recognition of several antigens, automation, cost effectiveness, and

high sensitivity [2]. This ANA detection technique which was also used in our study, however, has not been systematically validated against IF ANA in patients with SLE and therefore its utility remains unproven. After replacement of the traditional Hep-2 cell-based IF with the FB assay at our Institution, we encountered 11 patients who met the diagnostic criteria for SLE, but tested ANA negative by FB assay. All of these patients were subsequently tested ANA-positive using the IF assay, which was retained by our laboratory for confirmatory testing. This was of concern because the FB technique was being offered as the primary screening method for the diagnosis of SLE. We therefore decided to conduct a retrospective study to analyze how these two methods of ANA detection correlated in SLE and non-SLE patients and compared their sensitivity and specificity for the diagnosis of SLE.

Patients and methods

Based on prior authorization by our Institutional Review Board for the studies of human subjects, we retrospectively analyzed all patients tested for ANA both by IF and FB in the time period of 1/1/03 through 4/30/06. Patients that had an antibody titer of more than 1:50 by IF were considered to have a positive test [1]. The Athena MultiLyte assay employing the Luminex microsphere technology (Zeus Scientific, Raritan, NJ), was used for flow cytometry-based (FB) ANA screening. We then studied the electronic charts of the patients and obtained their clinical diagnosis. All patients charts have been reviewed by two board-certified rheumatologists and the diagnosis of SLE was made based on the presence of 4/11 ACR criteria [3]. IF and FB testing were done on the same sera when ordered simultaneously in a variety of in-patient and out-patient clinical settings by general medicine, rheumatology, nephrology, and neurology services. Sensitivity was calculated by dividing the number of patients that had ANA reactivity (either by IF or FB) over the number of patients diagnosed with SLE. Specificity was calculated by dividing the number of patients with a negative ANA over the number of patients that did not have enough ACR criteria for the diagnosis of SLE [3]. Statistical analyses of the distribution of IF and FB ANA results in all tested sera as well as their sensitivity and specificity in SLE and non-SLE donors were analyzed with two-sided χ^2 testing using the GraphPad Software (San Diego, CA). $p < 0.05$ was considered significant.

Results

FB-based ANA screening was done on 984 patients at our institution in the period from 1/1/03 to 4/30/06. Sera of 385 of these patients were also tested in parallel for the presence of ANA by IF. The electronic charts of these 385 patients were reviewed. The results of ANA testing are shown in Table 1. The distribution of ANA test results was significantly different ($\chi^2 = 73.12$; $p < 0.0001$) due to a marked discordance of double-negative and double-positive results. The concordance of the FB-negative and IF-negative test results was 240/256 (93.8%), while the concordance of double-positive results was 54/129 (41.9%). IF testing had a 90.6% sensitivity and 76% specificity. FB testing only had 49.1% sensitivity while its specificity was 87% (Table 2). Of the 53 patients that met ACR criteria for the diagnosis of SLE, 23 were found to be FB-negative and IF-positive. The specific patterns of IF seen in SLE and non-SLE donors are shown in Table 3. The antigen specificity of FB testing in SLE and non-SLE donors is shown in Table 4. The total number in these tables is higher than the number of patients because multiple samples showed more than one IF pattern or reactivity to different antigens.

A total of 53 patients met the ACR criteria for the diagnosis of SLE. 23 of the 24 discordant lupus cases were positive by IF and negative by FB, while the remaining 1 case was negative by IF and positive by FB. 18 and 5 of the IF+/FB- samples displayed homogenous

and speckled IF pattern, respectively; the IF–/FB+ serum had reactivity to SSB antigen. Out of the 81 non-SLE donors who were found to have a positive ANA by IF, 34 patients had a connective tissue disease: 17 had undifferentiated connective tissue disease, 8 had rheumatoid arthritis, 5 had mixed connective tissue disease, and the four remaining patients had psoriatic arthritis, systemic sclerosis, dermatomyositis, and Sjogren's syndrome, respectively. Table 4 shows that, among the non-SLE patients, 12 had anti-dsDNA antibody and 4 had anti-Sm antibody. Ten of these 16 sera also showed IF ANA positivity. Five of the IF ANA-positive patients had undifferentiated connective tissue disease and have not satisfied the diagnostic criteria for SLE. Out of the five remaining IF ANA-positive patients, two had human immunodeficiency virus infection, one had rheumatoid arthritis, one had psoriatic arthritis, and one had hepatitis C virus infection; none of these patients satisfied the diagnostic criteria for SLE.

Discussion

Antinuclear autoreactivity in patients with SLE was discovered in 1948 with the first detection of the LE (lupus erythematosus) cell [4]. Detection of ANA by indirect IF was introduced in 1957 [5]. In this assay, test sera are incubated with substrate cells, such as Hep-2 cells, at different dilutions and antibody binding is detected by fluorescein isothiocyanate-conjugated IgG by direct visualization under a fluorescent microscope. Because of high sensitivity, it is an excellent screening method for ANA detection [1]. IF ANA testing is laborious and efforts have been made to replace it with more rapid assays. Earlier, the enzyme-linked immuno-sorbent assays (ELISA) did not withstand rigorous testing for utility to diagnose autoimmune rheumatic disease patients when compared head to head with IF ANA [6,7]. The detection of ANA using fluorescent beads (FB) has been recently introduced [2,8]. It offers multiple advantages such as being able to detect many different antibodies simultaneously, automation, rapid results, and cost-effectiveness. Shovman et al. have proposed FB as a good screening test for connective tissue disorders. The sensitivity of FB testing for SLE in a group of 113 patients was 80.5% compared to 82.3% when ANA was detected by ELISA [2], in this study, the Athena MultiLyte FB assay (Zeus Scientific, Raritan, NJ) using the Luminex microsphere technology, the same used in our institution, was utilized. Thus, the difference in sensitivity cannot be explained by using different kits. In another study, FB testing had a concordance rate of 99.1–100% with ELISA [9]. A third study also showed good correlation between FB and ELISA assays. Out of the 56 confirmed SLE patients, the frequency of a positive autoantibody test with the multiplexed FB assay was 21.4% for SSA, 7.1% for SSB, 10.7% for Sm, 32.1% for RNP, and 0% for Scl-70, however, IF ANA test results were not provided for any of the patients and, in particular, for the ENA FB-negative patients. The percentage of ENA positivities correlated well with the ones previously described in the literature [8]. Most importantly, neither sensitivity nor specificity of FB-based ANA detection has been compared to IF in patients with SLE.

The Athena MultiLyte FB assay (Zeus Scientific, Raritan, NJ) using the Luminex microsphere technology, was introduced for ANA screening at our Institution in 2003. It has the ability to detect antibodies to 9 different antigens (SSA, SSB, Sm, RNP, Scl-70, Jo-1, dsDNA, centromere, histone) and the presence of ANA is predicated on detection of antibody to at least one of these antigens. While the present study shows that IF has a lower specificity than FB, detection of ANA by IF is markedly more sensitive than the FB technique and therefore a better screening test for the diagnosis of SLE. The higher sensitivity of IF ANA is attributable to the presence of antibodies to many more than 9 antigens. FB ANA testing is clearly faster and offers more information than IF alone, in terms of antigen specificity. However, conjugating many antigens to fluorescent beads carrying different fluorochromes has potential pitfalls that should not be overlooked.

Covalent binding of proteins to solid phase affects the conformation of the proteins, thus, eliminating some native epitopes and generating new ones. Moreover, the fluorochromes have variable size and charge that also affect binding of autoantibodies. Indeed, recent reports reveal that 0.8% [10] to 5% of human sera show spurious polyreactivity to multiple antigens in the FB assay [11]. Some of these polyreactive sera had no Western blot reactivity to the original antigens coupled with the FB [10,11]. Unfortunately, these studies did not have clinical information, regarding SLE, on the patients whose sera were tested.

The Athena MultiLyte FB assay was previously compared with IF and ELISA for the detection of ANA and ANCA in 1088 serum samples from patients with a possible diagnosis of an autoimmune disorder [12]. Although the FB technology correlated well with ELISA and ENA-screening methods, the FB technology was found to have a low sensitivity for identifying IF-positive cases (58%). In this study sensitivity was calculated utilizing the number of IF-positive patients, different from our study in which sensitivity was calculated using the number of patients with a defined diagnosis, such as SLE. Nevertheless, the sensitivity of FB technology for identifying IF-positive patients was lower, 41.9%, in our population, as compared to 58% [12].

In summary, this study indicates that conventional Hep-2 cell-based IF has superior sensitivity for detection of ANA with respect to FB and should remain the primary screening test in the diagnosis of SLE. Although FB testing has higher specificity, spurious polyreactivity in 0.8–5% of the sera may lead to unnecessary additional testing. This study encourages rheumatologists to be aware of the methods used for detecting of ANA in their patients.

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Table 1

Presence (+) and absence (–) of ANA based on IF and FB testing in 385 blood donors; 53 of these donors satisfied the ACR criteria of SLE

All donors	FB–	FB+	Total
IF ANA–	240	16	256
IF ANA+	75	54	129
$p<0.0001$	315	70	385

Table 2
Sensitivity and specificity of ANA testing by IF and FB for detection of SLE

	ANA		Sensitivity	<i>p</i> <0.0001
	IF	FB		
SLE	48	5	90.6%	
	26	27	49.10%	
			Specificity	<i>p</i> =0.0002
Non-SLE	81	251	75.6%	
	44	288	86.7%	

Table 3

IF pattern of ANA testing in SLE and non-SLE sera

	Homogenous	Speckled	Nucleolar	Peripheral	Polyreactive	Negative	Total
SLE	32	17	2	0	3	5	56 (53)
Non-SLE	62	27	2	0	10	251	342 (332)
Total	94	44	4	0	13	256	398 (385)

The total number of reactivities exceeds the number of sera tested (in parenthesis) due to polyreactivity of 3 SLE and 10 non-SLE sera.

Table 4

Antigen specificity of FB-reactive sera in SLE and non-SLE sera

FB	dsDNA	SSA	SSB	Sm	RNP	Scl-70	Jo-1	Centromere	Histone	Negative	Polyreactive	Total
SLE	4	13	2	2	5	1	0	0	3	27	4	57 (53)
Non-SLE	12	13	6	4	14	6	2	4	12	288	14	361 (332)
Total	16	26	8	6	19	7	2	4	15	315	18	418 (385)

The total number of reactivities exceeds the number of sera tested (in parenthesis) due to polyreactivity of 4 SLE and 14 non-SLE sera.