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CONCISE REPORT

Assay variation in the detection of antinuclear antibodies in the sera of patients with established SLE

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ABSTRACT

Objective The expression of antinuclear antibodies (ANA) is considered almost constant in systemic lupus erythematosus (SLE), although recent experience has suggested that many subjects with SLE considered for clinical trials are ANA negative at screening. The objective of this study is to determine whether assay variation can influence ANA detection in patients with established SLE.

Methods Sera from 103 patients with established SLE were tested using three different immunofluorescence assays (IFA) for ANA determination. ANA determinations were also performed by an ELISA and bead-based multiplex assay.

Results With IFA kits, the frequency of ANA negativity varied from 5 to 23 of 103 samples (4.9%–22.3%). The ELISA and multiplex assays showed that 12 (11.7%) and 14 (13.6%) samples were negative, respectively. Samples positive in all assays differed from those with discordant assay results in the frequency of historical anti–double-stranded DNA positivity and low complement levels at the time of blood sampling.

Discussion These findings indicate that ANA negativity occurs in patients with established SLE although the frequency varies depending on the assay kit. Given the range of negativity with well-validated assays, these findings raise questions about whether ANA positivity should be employed to determine eligibility for clinical trials.

INTRODUCTION

Antinuclear antibodies (ANA) are important biomarkers for systemic lupus erythematosus (SLE) and represent a criterion for patient classification.[1] While ANAs are not specific for SLE, patients with SLE are thought to be almost invariably positive. ANA testing is usually performed only at the time of diagnosis, however, because of the apparent lack of changes in ANA titres over time. In contrast, repeat testing of anti–double-stranded DNA (dsDNA) antibodies is common since levels of these antibodies are associated with disease activity. Among technologies for ANA determination, the immunofluorescence assay (IFA) is often viewed as the 'gold standard'.[2–4]

In addition to its role in patient evaluation, ANA testing has recently been used to assess the eligibility of patients for entry into clinical trials of new therapeutic agents, deriving from the experience with the development of belimumab. Belimumab is a monoclonal antibody directed against B-cell activating factor/B lymphocyte stimulator and received regulatory approval for the treatment of patients with active, autoantibody-positive SLE receiving standard therapy. After failure of a phase II study, re-analysis of the data showed that patients who were serologically positive (ANA and/or anti-DNA) responded to the agent; in the phase II study, approximately 30% of patients were serologically negative, defined as an ANA with a titre of ≤1:80. Subsequent phase III trials enrolled only serologically positive individuals at screening and met their endpoints.[5–8] Other sponsors conducting clinical trials are now enrolling only patients who are ANA and/or anti-DNA positive.[9] As such, serological testing is being used as a companion diagnostic or theranostic biomarker although existing tests have not been validated for this purpose.

The high frequency of ANA negativity in patients screened for trials is surprising and differs from the usual conceptualisation of the serology of SLE (ie, a frequency of ANA positivity of 95%–99%). One possibility for a discrepancy between historical and screening results may relate to a transition to a serologically negative status, reflecting the natural history of disease or the effects of therapy.[10 11] Alternatively, ANA variability may reflect the performance characteristics of the test kits.[12–14]

In view of the increasing use of ANA for determining trial eligibility, an explanation of these observations is important since it can impact both trial enrolment and eventual utilisation of a product approved for autoantibody-positive patients. To define further the serology of SLE as revealed by different ANA assay platforms and kits, we evaluated the detection of ANAs in patients with established disease rather than at the time of diagnosis.

METHODS

The study involved 103 patients from a cohort of patients with SLE who had historical ANA positivity followed at The Ohio State University. SLE was determined on the basis of four or more American College of Rheumatology criteria for classification. Table 1 presents a description of the patient population. Of the patients, approximately one half had a history of renal disease. Sera from patients were screened by two experienced observers with three commercially available IFA assays at a dilution of 1:40 in a single laboratory. We used results from the 1:40 dilution which is the recommended screening dilution by kit manufacturers and has maximum

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Table 1 Characteristics of patients who were ANA positive in all assays and patients who showed discordance between assays

	ANA positive all assays	Disagreement among assays	P value
Age (years)*	34.2 (20.2–67)	38.4 (21.6–62.5)	NS†
Duration of SLE (years)*	6.3 (0.1–33.4)	5.7 (1.6–19.8)	NS
Male (%)	6.3% male	11.4% male	NS‡
Caucasian (%)	56%	66%	NS‡
African American (%)	38%	31%	NS‡
Lupus nephritis (%)	67%	51%	NS‡
Ever anti-dsDNA positive	59%	23%	0.0006‡
C3§ (SD)	99 (32)	121 (34)	0.0058¶

*Median (range).
†Mann-Whitney test.
‡Fisher's exact test.
§Complement component C3, measured in a serum sample taken at the same time the sample for ANA was obtained.
¶Unpaired t test.
ANA, antinuclear antibody; ds-DNA, double-stranded DNA; SLE, systemic lupus erythematosus.

sensitivity. In addition, the sera were tested by an ELISA as well as a bead-based multiplex assay.

RESULTS

As the results in table 2 indicate, the frequency of ANA positivity varies markedly depending on the assay platform and kit used. Among IFA kits, the frequency of negativity varied from 5 to 23 of the 103 (4.9%–22.3%) samples tested although some samples were considered to have indeterminate results. For the ELISA, 12 (11.7%) had negative values. For the multiplex assays, 14 (13.6%) of the samples were reported as negative; in this assay, limited elevations of anti-dsDNA lead to a result called indeterminate.

To determine any features associated with serological status, the patients were divided into those who were consistently ANA positive in all assays and those who showed discordancy among assays. Using this categorisation, a preliminary analysis indicated that those patients who demonstrated consistent ANA positivity differed from those who had disagreements among assays in the likelihood of historical anti-dsDNA positivity and low levels of C3 complement (table 1). Disease duration and the occurrence of nephritis did not differ significantly in the two patient groups.

Table 2 Antinuclear antibody testing kits

Test result	IFA kit 1	IFA kit 2	IFA kit 3	ELISA	Multiplex
Negative	23 (22.3)	10 (9.7)	5 (4.9)	12 (11.7)	14 (13.6)
Indeterminate	9 (8.7)	10 (9.7)	2 (1.9)	0	8 (7.8)

Data shown in brackets are the percentages of the total number of samples analysed.
IFA, immunofluorescence assay.
The 103 samples were analysed using three commercially available ANA IFA kits, an ELISA and a multiplex assay called the BioPlex 2200. The following IFA kits were used: kit 1, ImmunoConcepts (distributed by GFMD, Novi, Michigan, USA); kit 2, Inova Diagnostics, San Diego, California, USA; and kit 3, Bio-Rad Kallestad (Bio-Rad, Hercules, California, USA). For the ImmunoConcepts kit, the Hep-2000 ANA-Ro kit was used. All assays were performed according to the manufacturer's instructions, using secondary antibodies provided. For the IFA analysis, the serum samples were diluted 1:40 with 1× PBS to allow for the determination of whether the samples were positive or negative for ANA antibodies, as instructed by the manufacturer. Further titration of ANA-positive serum samples was not performed at this point of the studies. IFA was performed in one laboratory by two experienced observers, one of whom read kits 1 and 2; the other read kit 3. ANA-positive samples were defined by positive staining of the nucleus; staining of cytoplasm was not considered in this study in view of studies indicating the uncertain reliability of IFA in detecting antibodies to ribosomal P proteins (anti-P), a specificity that can lead to cytoplasmic staining.[15] Only four sera had anti-P antibodies by the multiplex assays. Since these samples were all consistently ANA positive, our consideration of only nuclear staining appears to reasonably capture antibodies to relevant target antigens.
The IFA slides were examined using the EVOS FL Cell Imaging System (Thermo Fisher, Waltham, Massachusetts, USA). An objective lens of ×20 was used, and the light source was an adjustable intensity LED. The samples also underwent ANA assessment by an ANA EIA as well as the BioPlex 2200 ANA Screen (both products of Bio-Rad); these assays were performed at Bio-Rad. The number (%) of samples identified as negative for each kit is shown. For IFA assays, indeterminate samples showed weak or borderline staining and could not be consistently classified as either negative or positive. Assays with modest elevations of anti-dsDNA are reported as indeterminate by the BioPlex 2200.

DISCUSSION

Our findings provide new insights into ANA expression in SLE and indicate differences among ANA assay kits in the detection of ANA reactivity in sera of patients with established disease. These differences are likely related to technical features of the assays which may differ in variables such as conditions for cell fixation, reagents and ambient assay conditions[12–14]; the array of ANAs in patient sera can also impact on detection. In the routine clinical setting, these findings indicate that the serological evaluation of lupus could be misleading depending on the kit used, an issue not well appreciated by clinicians despite reports in the literature.[13]

In general, ANA testing is performed at initial evaluation; if positive, repeat testing is usually not considered necessary since the criterion for classification or diagnosis has been met. A re-evaluation of ANA status could occur if a patient seeks care from a new provider or undergoes screening for a clinical trial.[9 16] Although the relationship between the patients we studied and those in belimumab trials is speculative, the data clearly show that, depending on the kit, ANA negativity can occur in established lupus not infrequently.

The use of certain ANA assays could affect the frequency of screen failures in the trial setting as well as the eventual utilisation of an agent if approved for serologically active patients.[9] Since the ANA assay used for screening is often not specified in protocols, the selection of a kit could lead to as much as a 17% change in the number of screen failures. Correspondingly, for products approved for serologically active SLE, the use of certain assays could determine whether a patient meets criteria for its use.

Often, the identity of the kit used in published studies is not available since it is generally believed that each has similar performance characteristics. In this regard, we have performed another study on 181 patients enrolled in a clinical trial for a new agent of SLE and found a wide variation in levels of ANA negativity using five kits (0.6%–27.6%).[17] Another study has reported that, while samples from patients with SLE with a high titre ANA are consistently detected by different testing laboratories, those with lower titres are more likely to be identified as negative or equivocal. That study also reported significant variation in the detection of staining patterns.[18] In this regard, in the current study, we found that sera with variable detection by IFA had, in general, low values in the multiplex assay and infrequently expressed antibodies to RNA-binding proteins; these findings suggest that consistent ANA detection depends on ANA titre as well as specificity.

Because of the growing use of ANA as a theranostic and the absence of guidance on which kits can be used for this purpose, our findings suggest that clinical trials using ANA assays for

screening should specify the kit used and its performance characteristics, especially with patients with established disease. We further suggest exploration of different assays as theranostics since our studies suggest that patients who are consistently ANA positive may differ immunologically from those whose ANA responses are variably detected; these differences could relate to past or current disease activity as reflected in anti-DNA or complement levels.

Our findings, along with those obtained during the development of belimumab, suggest that ANA status may identify features that are associated with either disease activity or the likelihood of responding to a therapeutic. This situation can create an uncertainty in the selection of the ANA test for use in screening. An assay that produces a low frequency of negativity can reduce screen failures but could allow entry of patients who are immunologically distinct and display less disease activity. Indeed, the use of such a test could be similar to a reliance on historical ANA positivity.

While the serological changes preceding the onset of SLE have been an area of extensive investigation of 'pre-autoimmunity',[19 20] few studies have addressed events after diagnosis and treatment, a phase of disease that can be called 'post-autoimmunity'. The setting of clinical trials may thus reveal an immunological feature of SLE (ie, frequency of seronegativity in established disease) that has been previously underappreciated. Whether this seronegativity reflects a response to prolonged therapy or natural exhaustion of autoimmune clones is unknown but merits further study. Future studies are therefore needed to determine the ANA assays most informative as theranostic biomarkers. Closely related issues are whether ANA positivity should be a criterion for trial entry in subjects with long-standing SLE and, indeed, whether subjects with long-standing seronegative disease should be studied separately.

Contributors DSP, PEL and BHR designed the study. DMS performed the assays and prepared the data for publication. DSP, PEL and BHR reviewed and interpreted the data and wrote the manuscript.

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Competing interests None declared.

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