Paradigm shift in antinuclear antibody negative lupus: Current evidence

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Introduction

Systemic lupus erythematosus is an autoimmune multi-system disease most commonly involving skin, joints and vasculature. Owing to its protean clinical manifestations, it could be under-diagnosed or over-diagnosed. To minimize incorrect diagnosis of systemic lupus erythematosus, several clinical and laboratory features are included in the diagnostic and classification criteria. Antinuclear antibody assay, although an old screening test, still plays a pivotal role in diagnosis. But, it has to be correctly performed on properly chosen antigenic substrate and correctly interpreted.

Most laboratories use bead-based multiplex tests, solid phase assays or immunofluorescence technique using liver or kidney tissue substrates of rat/mice for testing for antinuclear antibodies. The reasons for choosing these methods are lower cost, simplicity and easiness in standardization of these tests. But limited antigenicity of the substrate used for testing lead to lower sensitivity of these tests.[1] Consequently, the concept of antinuclear antibody negative lupus emerged and several cases of antinuclear antibody negative lupus were published in the literature around the world. The prevalence of antinuclear antibody negative lupus was even pegged as 5–10%.[2]

Need to Revisit 'Antinuclear Antibody Negative Lupus' and its Causes

Unlike multiplex/solid phase assays or IF-ANA assays using rodent substrates, rapidly dividing human epithelial cell line expresses more nuclear antigens resulting in greater sensitivity. Rodent tissue does not express Ro (SS-A) antigen. Human epithelial cell line expresses other nuclear and nucleolar antigens more readily as well.[3] With increasing use of human epithelial cell line substrates for antinuclear antibody testing during the past decade, cases of systemic lupus erythematosus seronegative for antinuclear factors have become rare. Moreover, many reported cases of systemic lupus erythematosus who were wrongly labelled as "antinuclear antibody negative lupus" became antinuclear antibody positive on serial testing with immunofluorescence utilizing human epithelial cell line substrate.[1] This, coupled with lack of information about the laboratory methods employed for antinuclear antibody assay in a majority of published cases of antinuclear antibody negative lupus raised a question mark on the accuracy of diagnosis of these cases.

In this changed scenario, prevalence of true antinuclear antibody negative lupus seems to be less than 2%.[4] Furthermore, there are certain clinical factors to be kept in mind when interpreting antinuclear antibody test results in suspected cases of systemic lupus erythematosus before jumping to conclusions.[5]

Causes for False Antinuclear Antibody Negative Lupus

Questionable or incorrect diagnosis of 'lupus'

Incorrect diagnosis of systemic lupus erythematosus has led to an increase in the reporting of antinuclear antibody negative cases. Hence, before proceeding to antinuclear antibody assay and interpreting its results, it is necessary to consider the accuracy of provisional diagnosis. No diagnostic method has 100% sensitivity and 100% specificity in systemic lupus erythematosus. Even histopathologic examination or systemic lupus international collaborating clinics 2012 diagnostic criteria cannot rule out the possibility of "false-positive" diagnosis. It has been reported that the diagnosis of "lupus" in approximately 78% cases of "antinuclear antibody negative lupus" reported between 1976 and 2003 around the globe were based on insufficient clinical data and laboratory findings, not even fulfilling the American College of Rheumatology criteria for systemic lupus erythematosus.[1] Hence, before commenting on antinuclear antibody reactivity of suspected cases of lupus, we need to confirm such cases in the best possible way, so that non-lupus or doubtful cases of lupus are not mis- reported as antinuclear antibody negative lupus.

Antigenic deficient substrate and leaching of antigens

The most important and critical factor in antinuclear antibody test is the substrate used for it. The result of the test is based on the availability of

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Access this article online	
Quick Response Code:	Website: www.ijdvl.com
	DOI: 10.4103/ijdvl.IJDVL_204_17

How to cite this article: Tiwary AK, Kumar P. Paradigm shift in antinuclear antibody negative lupus: Current evidence. Indian J Dermatol Venereol Leprol 2018;84:384-7.

Received: March, 2017. **Accepted:** December, 2017.

sufficient substrate antigen to react with the autoantibody. Inadequacy or deficiency of the antigen due to poor choice of the substrate can lead to false-negative antinuclear antibody results. Human epithelial cell line appears to be a more suitable substrate than rodent tissue. It is possible that an antinuclear antibody negative patient may become antinuclear antibody positive if the substrate is changed from rat liver to human epithelial cell line.[1]

Inadequate fixation of the substrate leading to leaching of the antigens may also result in a false-negative antinuclear antibody test.[6]

Concurrent immunosuppressive treatment

Review by Simmons *et al*. identified seven cases which were antinuclear antibody negative at presentation, butlater became seropositive. The interval to develop seropositivity for antinuclear antibody ranged from five months to 10 years, with a median of six years. Of importance, all these patients were on one or more of immunosuppressive drugs.[7] It is now being increasingly recognized that immunosuppressants can alter antinuclear antibody results. Hence, details of previous or concurrent medications should always be reviewed before interpretation of results of antinuclear antibody assay. Most of the earlier published cases of antinuclear antibody negative systemic lupus erythematosus suffered from incomplete documentation of concurrent and previous medications.[1] This raises doubts about the accuracy of diagnosis of antinuclear antibody negative lupus in many reported cases.

Persistent renal loss of proteins

Proteinuria is a prominent feature of systemic lupus erythematosus and profound and persistent renal loss of immunoglobulins may result in false antinuclear antibody negative result. This fact also explains those cases of antinuclear antibody negative lupus with profound proteinuria reported by Persellin and Takeuchi, who became antinuclear antibody positive after treatment with prednisolone and chlorambucil.[8] In such cases, detection of antinuclear antibody in pleural fluid and urine may be helpful, as noted by Ferreiro *et al*.[2] Clinicians should be aware of the possibility of false antinuclear antibody negativity in the presence of marked proteinuria. Serial testing of serum samples is warranted in such cases.

National Committee for Clinical Laboratory Standards Guidelines for Antinuclear Antibody Testing by Indirect Immunofluorescence

Variations in antinuclear antibody testing methods may have profound impact on the diagnosis and management of lupus patients. Attempts have been made to standardize laboratory testing for antinuclear antibodies. In December 1996, National Committee for Clinical Laboratory Standards proposed certain recommendations to be followed during antinuclear antibody testing by immunofluorescence technique.[9] These are categorized into two groups for better understanding. [Table 1].

Table 1: National Committee for Clinical Laboratory Standards guidelines for antinuclear antibody testing by indirect immunofluorescence

Practices designed to ensure appropriate interpretation of test results
It includes consistent nomenclature, reporting format and appropriate reference ranges
Nomenclature and report format: It describes whether the test result is negative (no discernible pattern of nuclear fluorescence) or positive at the cut-off dilution and if positive, a description of the fluorescence patterns observed, intensity of fluorescent staining and the end-point titre at which a discernible pattern of fluorescence is observed
Reference ranges: Each laboratory should set its own reference intervals to report and interpret the laboratory results
Practices designed to ensure accurate and reliable test results
These are further divided into the following components
Regulatory requirements for IF-ANA test methods
Personnel: Minimum qualifications required for Laboratory directors-doctoral degree Technical supervisors-doctoral degree, master's degree, or bachelor's degree plus experience Clinical consultants, general supervisors, and testing personnel-associate degree engaged in "high-complexity" testing
Competency assessment: Annual assessment of direct observation of testing and reporting of results
Quality control: Laboratories must have an "on-going mechanism" to identify problems and produce corrective actions
Proficiency testing: ANA is a "regulated analyte;" acceptable performance on proficiency testing is defined by a result equal to the target value ±2 dilutions; acceptable results must be obtained on 4 of 5 challenges in each mailing
Specimen collection and storage: Test specimen should be serum which can be stored at 4°C for up to 72 h/at−20°C or colder (without freezing and thawing) indefinitely
Substrate slides: Use acetone-fixed substrate slides; alcohol fixation is avoided as these may remove Ro (SS-A) antigen. HEp-2 cells are preferable to mouse or rat tissues as already discussed
Anti-Ig conjugate: Anti-Ig conjugates must be having following characteristics
Isotype specificity: IgG specific (preferably not be polyvalent conjugates)
FITC to protein ratio: Approximately 3.0; higher FITC protein ratios may cause increased nonspecific staining
Antibody to protein ratio: ≥0.1
Specific antibody content: 30-60 mcg/mL
Working dilution: Determined by titration using serial dilutions of positive control sera with known patterns and end-point titers
Use of reference sera: Reference sera of defined ANA content and specificity are available from the WHO, ANA international reference preparation 66/233 and ANA reference laboratory at the centers for disease control and prevention. Individual laboratories should identify closely comparable in-house reference sera

ANA: Antinuclear antibody, IF: Immunofluorescence, IgG: Immunoglobulin G, FITC: Fluorescein-isothiocynate

Limitations of immunofluorescence-antinuclear antibody test

1. Inter-method standardization of immunofluorescence-antinuclear antibody substrates and anti-Ig conjugates is still difficult[9]
2. There is no standard protocol for reference ranges in the background of variable prevalence of weakly positive antinuclear antibody results in healthy persons.

Current Established Cases of True Antinuclear Antibody Negative Lupus

On reviewing evidence from literature, it becomes apparent that there are only some case reports or series which followed proper technical guidelines to diagnose antinuclear antibody negative lupus. The features that establish the diagnosis of systemic lupus erythematosus unequivocally are high-titre anti-double stranded

Table 2: Review of antinuclear antibody negative lupus reported in past 15 years

Reference	Year	Method used	Substrate used	Old ACR criteria	Massive proteinuria (≥3 +)	Previous Immuno-suppressive drugs	Follow up (>1 year)	Comments on ANA negativity
Locham *et al*.[11]	2000	ELISA*	Not mentioned*	5	No	No	No[‖]	Questionable
Maraina *et al*.[12]	2002	IIF	Hep-2 cells	7	Yes[‡]	No	Yes	Questionable
Sugisaki *et al*.[13]	2002	IIF	Hep-2 cells	4	No	No	Yes	True**
Pratap *et al*.[14]	2004	ELISA*	Not mentioned*	5	No	No	No[‖]	Questionable
Eilertsen and Nossent[15]	2007	Not mentioned*	Not mentioned*	5	No	No	Yes	Questionable
Kim *et al*.[16]	2009	Not mentioned*	Not mentioned*	6	No	No	No[‖]	Questionable
Akhoondian *et al*.[17]	2009	Not mentioned*	Not mentioned*	3[†]	No	No	No[‖]	Questionable
Xie *et al*.[19]	2012	Not mentioned*	Not mentioned*	5	No	No	Yes	Questionable
Caltik *et al*.[18]	2013	Not mentioned*	Not mentioned*	4	Yes[‡]	No	Yes	Questionable
Chaubey and Chhabra[20]	2013	Not mentioned*	Not mentioned*	4	No	Yes[§]	No[‖]	Questionable
Yang *et al*.[21]	2013	Not mentioned*	Not mentioned*	3[†]	No	No	No[‖]	Questionable
Elcioglu *et al*.[22]	2014	Not mentioned*	Not mentioned*	3[†]	No	Yes[§]	No[‖]	Questionable
Hoang *et al*.[23]	2015	Not mentioned*	Not mentioned*	3[†]	No	No	No[‖]	Questionable
Simmons *et al*.[7]	2015	IIF	Hep-2 cells	4	No	No	Yes	True**
Chikkalingaiah[24]	2016	Not mentioned*	Not mentioned*	5	Yes[‡]	No	No[‖]	Questionable
Zhao[25]	2016	Not mentioned*	Not mentioned*	5	No	No	No[‖]	Questionable
Tiwary and Mishra[26]	2016	ELISA*	Not mentioned*	5	No	No	No[‖]	Questionable
Changal *et al*.[27]	2016	IIF	Hep-2 cells	4	No	No	No[‖]	Questionable
Cerqueira *et al*.[28]	2017	Not mentioned*	Not mentioned*	2[†]	Yes[‡]	No	No[‖]	Questionable
Cerqueira *et al*.[28]	2017	Not mentioned*	Not mentioned*	2[†]	Yes[‡]	No	No[‖]	Questionable

Diagnosis of cases with any of the following 5 features have been considered questionable and marked by following symbols. *If other than IIF using Hep-2 cells is done, [†]Where <4 criteria is fulfilled, [‡]If massive proteinuria (≥3 +) is present, [§]If history of previous immunosuppressive drugs is present, [‖]If follow up is for <1 year, **If diagnosis of true ANA negative lupus is established based on the absence of confounding factors taken into account in this study. ANA: Antinuclear antibody, IIF: Indirect IF, ACR: American College of Rheumatology, IF: Immunofluorescence

DNA antibody, anti-Sm (Smith) antibody, biopsy-proven kidney disease or biopsy-proven skin disease. One can make a diagnosis of antinuclear antibody negative lupus if at least one of these features has been documented and due care (as described earlier) has been taken in interpreting the laboratory findings of antinuclear antibody.[10] Searching through the PUBMED database, an attempt has been made to review the cases of antinuclear antibody negative lupus reported in the last 15 years [Table 2].[7,11-28] Based on the confounding factors taken into consideration in this review, out of 19 previously diagnosed cases of antinuclear antibody negative lupus, only two cases deserved to be called as 'true antinuclear antibody negative lupus'. On scrutinizing, true antinuclear antibody negative lupus appears to constitute less than 2% of all systemic lupus erythematosus patients.

Look Before You Leap to Antinuclear Antibody Negative Lupus!!

Based on the use of human cell line derived substrates current NCCLS guidelines for testing and interpretation and considering various clinical and technical factors which can affect the results, it becomes clear that the term 'antinuclear antibody negative lupus' should not be used for labelling any suspicious case of systemic lupus erythematosus. True antinuclear antibody negative lupus appears to be extremely rare. Serial antinuclear antibody assay has to be done in such cases.

Limitations

As we have reviewed antinuclear antibody negative cases of lupus reported in the past 15 years using PUBMED database, it is possible that we may have missed cases of lupus enlisted under other databases. Second limitation of this review is the doubtful reliability of old American College of Rheumatology criteria to label a case as true or questionable lupus. Considering the complex and unpredictable clinical presentations of systemic lupus erythematosus and low specificity of old American College of Rheumatology criteria, it may not be correct to label cases which do not fulfil four of the American College of Rheumatology criteria as non-lupus. Third limitation is the lack of sufficient clinical and laboratory information in many cases. Conclusions made on such incomplete data may not be valid.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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