ORIGINAL ARTICLE

Clinical relevance and frequency of cytoplasmic and nuclear dense fine speckled patterns observed in ANA-HEp-2

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ABSTRACT

Objectives: This study aimed to determine the frequency and antibody titers of nuclear dense fine and cytoplasmic patterns with possible clinical correlation. **Methods**: From 2007 to 2009, the results of 2,788 autoantibody serological tests were assessed by indirect immunofluorescence (IIF) at LAC-HUSM/UFSM, using as substrate HEp-2. **Results**: Among the analyzed samples, 1,998 of them were negative for autoantibodies. Among the positive samples (n = 790), we found 57 (7.2%) showing reactivity pattern described as dense fine speckled (DFS) (3.8%), or cytoplasmic (Cit) fluorescence (3.4%). In samples with standard DFS (n = 29), nine had titers of 1/160, and only one patient had autoimmune disease (AID). Among patients with titers > 1/160, only one patient did not have AID. Among samples with standard Cit (n = 27), 20 had titers of 1/160, and only eight were not associated with AID. The other seven patients with titers > 1/160 reported AID. **Conclusion**: The results confirm the value of 1/160 as the best cut-off point for defining AID presence, for any of the fluorescence assessed patterns. However, attention should be given to lower titers, especially for Cit IIF, since only 40% did not report the presence of AID.

Keywords: antibodies, fluorescent antibody technique, autoimmunity.

INTRODUCTION

The investigation of antinuclear antibodies (ANA) by indirect immunofluorescence (IIF), also known as antinuclear factor (ANF) in serum of patients with suspected autoimmune disease (AID), is an excellent screening test for autoantibodies. In the early 1950s, IIF was performed with cut or imprint of rodent liver as substrate for ANA screening in serum of patients with suspected AID.[1] A positive result indicated the possible presence of some autoantibodies in patients with suspected systemic lupus erythematosus (SLE) and other IDAs, such as systemic sclerosis (SSc) and Sjögren's syndrome. This technique, over the past decades, has been modified to confer a progressive increase in sensitivity.[3]

Currently, IIF using a cell line of human larynx epithelioma – HEp-2 (human epithelioma type 2 – CCl 23ATCC clone) virtually replaced the imprint of rodent cells due to multiple factors, among which the possibility of universal standardization, as various laboratories worldwide are now using the same type of substrate. Currently, several manufacturers provide commercial slides with HEp-2 fixed cells with excellent quality,[2,3] contributing for the implementation of an uniform technique among clinical laboratories.[4] Another important factor in this replacement is the excellent

visibility of multiple fluorescence patterns,[5] and the offering of a diversity of autoantigens inherent to this substrate. As a result, the ANA test also began to show greater sensitivity and lower specificity.[4] Lately; however, laboratory reports with a large number of information, which not always reflected in objective manner or help to determine the health condition of a patient, have been reported. The increase in sensitivity, however, resulted in substantial decrease in specificity, even for autoimmune rheumatic disease diagnosis, as some apparently healthy subjects (2% to 4%) began to show positive results for ANA-IIF technique,[2] which was extremely rare with LE cell tests.

One of the main immunofluorescence patterns present in samples of healthy individuals is the dense fine speckled (SFD) pattern. The high frequency of SFD in individuals with non-autoimmune diseases makes its recognition important for these findings proper assessment of ANA-HEp2 test.[6] It is well demonstrated that the presence of autoantibodies may be transiently triggered by infections, drugs, and malignancies. The cytoplasmic pattern (Cit), now being reported in most cases, has no definite clinical relevance yet.

This study goal was to determine the frequency and antibody titers of nuclear dense fine and cytoplasmic patterns with possible clinical correlation with AID, determining the clinical relevance of trial results.

MATERIAL AND METHODS

Casuistry

The results of ANA-IIF investigation performed at the Laboratório de Análises Clínicas (LAC) of Hospital Universitário de Santa Maria (HUSM) were reviewed and the medical records of patients positive for DFS and Cit patterns from January 2007 to March 2010 were analyzed.

Indirect immunofluorescence (IIF)

ANA-IIF investigation on HEp-2 cells (Hemagen Diagnostics, Inc-Virgo® Products Division, Columbia, Maryland 21045 USA) is held at LAC-HUSM, according to the following protocol: cells are incubated with the patients' sera diluted in pH 7.2 phosphate buffered saline (PBS) for 30 minutes in a moist chamber at room temperature. Then, the slides are washed twice for 10 minutes in PBS and incubated for 30 minutes with secondary antibody conjugated with human antigamma-globulin fluorescein isothiocyanate (FITC) in a dark chamber at

room temperature. After incubation, slides are washed in PBS and mounted in buffered glycerin and coverslip. Reading is done with a fluorescence microscope (Olympus BX50 model under 500 magnification). At each routine for testing ANA, negative and positive internal controls are performed.

Data were analyzed using descriptive statistical (mean and frequency [%]). This study was approved by the Research Ethics Committee of UFSM under the number: CAAE 0196.0.243.000-09.

RESULTS

Among the analyzed results of ANA-HEp-2 (n = 2,788) between March/2010 and January/2007 at LAC/HUSM, 1,998 (71.66%) were negative for the presence of autoantibodies and 790 (28.33%) had positive results.

Of the 790 positive results, 734 (92.92%) showed the following patterns of fluorescence: nuclear homogenous, nuclear gross speckled, nuclear fine speckled, nuclear type-nuclear membrane, nucleolar homogeneous, nucleolar speckled, nucleolar speckled isolated spots, nucleolar speckled centromere, nucleolar cluster, and 56 (7.1%) were nuclear DFS or Cit. And 29 samples revealed nuclear pattern DFS and 27 Cit (Figure 1).
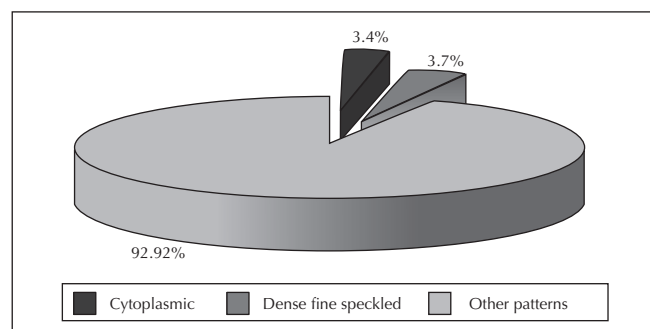

Figure 1
Frequency of fluorescence patterns of ANA-HEp-2 in the studied population.[18]

It was found that 74% of the patients with Cit pattern showed low titers (1/40 and 1/80). On the other hand, approximately half (55.2%) of the patients presenting DFS pattern showed high titers (1/320 and 1/640). As the titles increased, the prevalence of cytoplasmic pattern decreased, unlike the DFS pattern that prevailed in patients with higher titles (Figure 2).

When IIF title is associated with evidence of IDA, it can be observed that the prevalence of AID occurred in
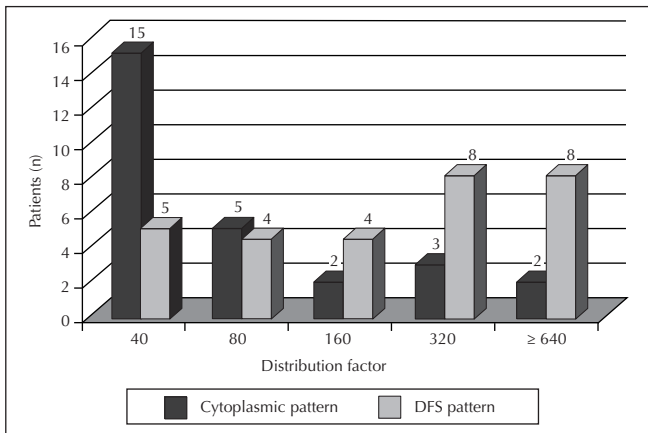
Figure 2
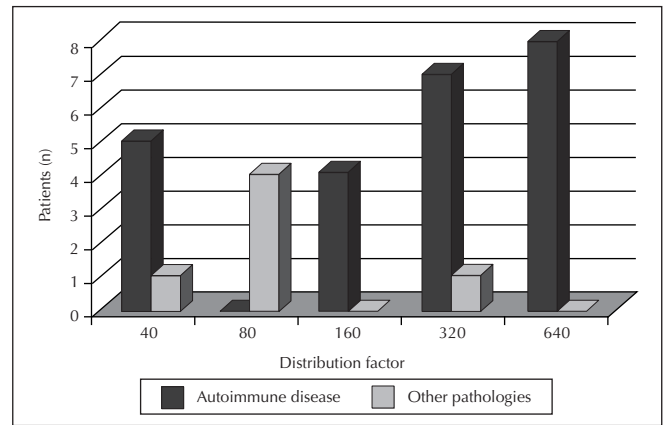Frequency of ANA titers and fluorescence patterns in the studied population.

Figure 3.
Frequency of autoimmune disease (AID) associated with anti-bodies titers found in dense fine speckled (DFS) fluorescence pattern in ANA HEp-2 cells.

two patterns studied, although with different levels of reactivity (Table 1).

The results reported as DSF fluorescence pattern were stratified according to the titer in serum of 29 patients classified according to the presence or absence of autoimmunity evidence (Figure 3).

Regarding Cit, it was verified that in low titers (1/40) a significant number of IDA was found (Table 1). However, a lower specificity was seen in titers below 1/160 present in sera from individuals with no evidence for AID (Figure 4).

DISCUSSION

Detection of autoantibodies in serum has a prognostic role in IDA, as it reflects the presence, nature, and intensity of autoimmune response. There are over 100 different types of
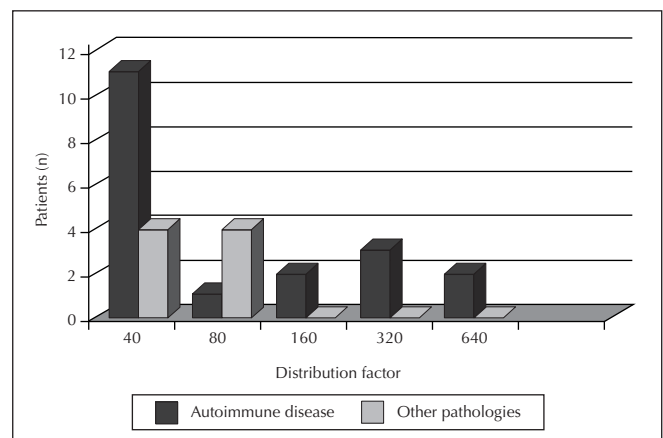

Figure 4
Frequency of autoimmune disease (AID) associated with anti-bodies titers shown by cytoplasmic fluorescence (Cit) pattern in ANA HEp-2 cells.

Table 1

Diseases associated with the fluorescence patterns found in ANA-HEp-2

	Pattern							
	Dense fine speckled				**Cytoplasmic**			
Title	**Autoimmune disease**	**(n)**	**Other condition**	**(n)**	**Autoimmune disease**	**(n)**	**Other condition**	**(n)**
1/40	RA *Pemphigus Vulgaris*	1 1	Stress Polytrauma	1 1	SLE RA Biliary Cirrhosis Polymyositis	5 5 1 1	Cardiopathy Depression Arthralgia	1 1 1
1/80	Hepatitis AI	1	Herpes HIV Psoriasis	1 1 1	Hemolytic anemia Sclerodema	2 1	Depression	1
≥ 1/160	SLE RA Scleroderma	14 4 2	Cancer	1	SLE RA Hepatitis AI	4 2 1		
Total	23		6		23		4	

SLE: systemic lupus erythematosus; RA: rheumatoid arthritis; AI: autoimmune.

rheumatic diseases and there is often a need for differential diagnosis between these conditions.[7]

There was a marked evolution in IIF patterns interpretation for autoantibodies detection on HEp-2 in the last few years. This methodology has the ability to track a wide variety of antibodies, providing the location and probable identity of autoantigen, but shows as a limitation variations in interpretation of fluorescence patterns.[8] Years ago, most studies were focused mainly in ANA tests, but today it is estimated that antibodies against cytoplasm, cell surface, intercellular space, and cell components are also important in the context of IDA.[9]

According to the III National Consensus Standards for ANA Records, more than 25 possible patterns of fluorescence are described. Each one of them may reflect a given antigen expression recognized by its autoantibody.[10,12]

A pioneering study of DFS pattern was performed by Ochs et al.[13] and, as already reported by Laurino et al.,[14] there is a low prevalence of this fluorescence pattern with positive metaphase plate compared to the other existing patterns. Leser et al.[15] demonstrated that DFS pattern showed high rate of association with absence of autoimmunity in low, medium, and high titers.

Among the patterns found in this study, the frequency of DFS (3.7%) and Cit (3.4%) patterns was lower than that reported in another service, which showed frequency of 6.3% and 9%, respectively.[14] This is probably due to the clinical context of each patient when the examination was requested, especially by the rheumatologists of HUSM, leading to a smaller number of pattern detection linked to specificity. Tampoia et al.[16] observed that rheumatologists ordered ANA test with higher frequency in patients with two or more criteria for AID classification, unlike other medical specialties that ordered this test in patients who had only inflammatory conditions.

All individuals have some degree of physiological autoimmunity, which may vary depending on overloads the immune system is exposed. Transient elevations of ANA titers may occur in individuals with acute infectious conditions and certain drugs, such as hydralazine, carbamazepine, hydantoins, procainamide, isoniazid, methyldopa, among others.[12] Probably due to this factor, some individuals with other non-autoimmune conditions may have presented a positive ANA test as shown in Table 1. It was not possible to consider the low reactivity exclusively as a normal factor, as the sample analyzed was composed of sera from individuals who sought treatment at HUSM and had their examination conducted by this hospital central laboratory, therefore, the presence of a disease can not be excluded. It is known that despite the high sensitivity of ANA testing (almost 100%), particularly for LES, its specificity may be low, especially when the reaction intensity is low

(low titers), making it also detected in a number of different clinical conditions.[17]

The nuclear DFS pattern with positive metaphase plate is described in literature as having a higher prevalence in patients with no clinical evidence of AID.[18] Leser et al.[15] noted that the nuclear DFS patterns, nuclear reticulated speckled and nuclear fine dotted speckled, were predominantly associated with individuals without any evidence of AID. On the other hand, by combining information from fluorescence pattern and title, it was seen that, for the fine speckled and thick reticulated speckled patterns, the association with absence of AID was real only in low titers. The same was not absolutely true for DFS pattern, which showed high rate of association with absence of AID in low, medium, and high, titers.[15]

Watanabe et al.,[6] in a study of frequency and ANA-IIF pattern in healthy workers of a hydroelectric power plant, revealed that the DFS pattern was frequently found in those with positive ANA, appearing in low, medium, and high titers.

These results could also be verified in this study, however, as shown in Figure 3, although there is a predominance of other autoimmune conditions in lower titers than 1/80, the probability can not be totally exclude, as five patients with AID presented title 1/40 with this fluorescence pattern. The patients showed no evidence of AID only in titers greater than or equal to 1/640.

A second point to be considered is the title of ANA-HEp-2, although its value is relative. In general, patients with AID tend to show moderate (1/160 and 1/320) and high ($\geq$ 1/640) titles, while the healthy subjects with ANA-HEp-2 positive tend to have low titers (1/80).[2] However, it is important to remember that exceptions on both sides are not uncommon.[15] Thus, it is relevant to mention a multicenter study of major importance by Tan et al.[2] with people aged 20-60 years. Among healthy individuals, the following percentages of positive tests were found: 1/40 in 31.7% of individuals, 1/80 in 13.3%, 1/160 in 5.0%, and 1/320 in 3.3%.

The clinical relevance of Cit pattern is not yet fully established. According to the researchers Massabki et al.[19] and Eystathioy et al.[20], the cytoplasmic pattern, in low or moderate titers, may have no defined clinical relevance. However, our study found that patients with AID, such as rheumatoid arthritis, SLE, SSc, and polymyositis present this fluorescence pattern in low titers.

In conclusion, the detailed interpretation of IIF patterns plays a key role in the assessment of results. The ANA-HEp-2 title is a parameter of relative value, while the fluorescence pattern can have a more decisive impact. The results found in

this study confirm the titer of 1/160 as the best cut-off point for defining the presence of AID for any of the fluorescence patterns evaluated. However, the low titers should be considered, especially for fluorescence with Cit pattern, as only 40% did no show presence of AID.

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