Antinuclear antibodies: From past to present

In 1957, George J Friou first described an indirect immunofluorescence (IIF) test system for the detection of anti-nuclear antibodies (ANA) – thus beginning a new generation of ANA testing. The test uses HEp-2 cells, a cell line which was established in 1952 by Alice E Moore et al. from tumors that had been produced in irradiated-cortisonized weanling rats after injection with epidermoid carcinoma tissue from the larynx of a 56-year-old male. The HEp-2 cell – a native protein array, with hundreds if not thousands of antigens, provides the ideal substrate for the detection of ANA. Since the inception of utilizing HEp-2 cells for ANA screening, the diagnosis of systemic autoimmune rheumatic diseases (SARDs), has evolved.

The IIF on HEp-2 cells has been replaced in some laboratories with multiplex or ELISA screening methods. Due to concerns over ‘false negative’ results, the lack of transparency to clinicians, and absence of the newer test algorithms, the American College of Rheumatology (ACR) formed a Task Force to recommend the use of the traditional IIF method for ANA screening. This initiated a renaissance of the method which is reflected by entire sessions dedicated to HEp-2 ANA testing at international scientific meetings.

During the last years, the first digital imaging systems have been developed which eliminate major drawbacks of the method – the subjectivity and the lack of automated reading.

In this issue of the INOVA Newsletter we are delighted to present novel insights and updates on ANA detection using IIF on HEp-2 cells, authored by experts in the field.

Enjoy reading!
Detection of autoantibodies is vital in the diagnosis and management of patients with autoimmune diseases (AIDs). The use of new assays (i.e. automated, high-throughput solid phase methods, multi-array systems) and the better knowledge of the physiopathology of AIDs have improved our diagnostic power. As a consequence the impact of AID in the daily practice is increased both from a clinical and a laboratory point of view.\textsuperscript{1, 2}

The spreading of autoimmunity testing from reference to general laboratories raises several practical problems. Among them, the most important are the correct performance and the clinical interpretation of the assays. This is particularly true owing to the increasing need of early diagnosis, a prerequisite for a successful treatment for many AIDs. Besides the problem of the treatment delay, a wrong diagnosis, either through false positive or false negative tests may also be responsible for additional costs due to the repetition of confirmatory tests and/or to consequent unnecessary diagnostic investigations.\textsuperscript{1-4}

The use of new autoantibody assays raises also the problem of the clinical interpretation of their results in comparison with those detectable by “historical” methods. In fact, there are no well planned studies that compared old and new methods in terms of sensitivity/specificity or positive and negative predictive value. Hence, one of the tasks of the international committees for autoantibody standardization in the future will be to draw up specific guidelines regarding how to use and interpret these new assays. These issues are related to any autoantibody assay, and have been reported even for a basic screening test for ANA detection.

The methodology for detection of ANAs has changed over the years from the lupus erythematosus (LE) cell test, to indirect immunofluorescence (IIF) utilizing sections of various rodent organs (e.g. rat or mouse liver or kidney, etc.) to cell lines, in particular HEp-2.

HEp-2 cells contain a large variety of autoantigens (approximately 100 to 150), most of them still undefined. ANAs are detected by IIF, in which both pattern and titer can be described. Although ANA IIF has been used for a long time and does represent classification criteria for several AIDs, still there are problems in its standardization (Table 1).

Over the years, numerous solid phase immunoassays have been developed in order to offer methods for ANA detection much easier, faster, cheaper and better standardized compared to IIF using fixed HEp-2 cells as a substrate. Many commercial laboratories and some hospital labora-

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<thead>
<tr>
<th>Table 1 PROBLEMS IN ANA STANDARDIZATION</th>
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<tbody>
<tr>
<td>Isotype of the fluoresceinated antiserum (IgG vs IgG/IgM/IgA)</td>
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<tr>
<td>Efficiency of the fluorescent microscope</td>
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<tr>
<td>Starting serum dilution (1:80 is the widely suggested starting dilution: with positive results &gt; at 1:160 considered to be pathological)</td>
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<tr>
<td>Recognition of standard patterns (nuclear and cytoplasmic)</td>
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<td>Correct preparation of the slides for reading</td>
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tories have recently switched their ANA screening tests to the new assays. Unfortunately, such a decision was made without solid evidence that the new tests could be able to replace the standard IIF assay. As a result, inaccurate results for ANA tests have been reported and the ACR created an ANA Task Force to evaluate the extent of the problem and to recommend solutions. A review of the literature by the committee indicates that up to 35% of patients with SLE and a positive ANA by IIF were negative on solid phase assays. Accordingly the committee prepared a position report addressing this problem and suggesting specific recommendations (Table 2).

Both physicians taking care of AID patients and people working in autoimmunity diagnostic laboratories should be kept informed of such problems and on the possible solutions to avoid misdiagnosis. Comparative studies on the new and the “old” techniques are mandatory to better define their use and limitations.

**ANA screening assay: Take home messages**

- IIF ANA test is still the recommended method for ANA screening
- IIF ANA positivity is required for the classification of several AIDs
- Solid-phase or multiplex assays can detect only the specific autoantibodies directed against the limited number of autoantigens that are displayed

### Table 2

**RECOMMENDATIONS OF THE ACR ANA TASK FORCE***

<table>
<thead>
<tr>
<th>Recommendation</th>
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<tbody>
<tr>
<td>Laboratories using bead-based multiplex platforms or other solid phase assays for detecting ANA must provide data to ordering physicians that their assay has the same or improved sensitivity and specificity compared to the IFA ANA</td>
</tr>
<tr>
<td>In-house assays for detecting ANA as well as anti-DNA, anti-Sm, anti-RNP, anti-Ro/SS-A, anti-La/SS-B, etc. should be standardized according to national (e.g., CDC) and/or international (e.g., WHO, IUIS) standards</td>
</tr>
<tr>
<td>Laboratories should specify the utilized methods for detecting ANA when reporting their results</td>
</tr>
<tr>
<td>IIF ANA test should remain the gold standard for ANA testing</td>
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</table>

*Members of the ACR ANA Task Force: Peter Schur (chair), Donald Bloch, Joe Craft, John A. Goldman, Pier Luigi Meroni, Eileen Moynihan, Morris Reichlin, Westley Reeves, Eng Tan, Dan Wallace, and Mark Wener.

References

Detection of antinuclear antibodies

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Antinuclear antibodies (ANAs) are found in patients with rheumatic diseases, such as SLE, systemic sclerosis (SSc), Sjögren’s syndrome (SjS) and polymyositis-dermatomyositis (PM/DM). Antinuclear antibodies, though, are also found in patients with non-rheumatic diseases such as infectious disease, malignant disease and thyroid disease, and even in individuals with no medical condition, particularly women >40 years old and elderly people.1, 2

Evidence-based guidelines for the use of ANA testing proposed by the American College of Rheumatology (ACR) ad hoc committee on immunologic testing state that ANA testing is useful to varying degrees for the diagnosis and monitoring of certain systemic autoimmune rheumatic diseases (SARDs).

The guidelines also state that ANA testing is not useful for the diagnosis, monitoring, or prognosis of other diseases including rheumatoid arthritis and thyroid disease.1

Antinuclear antibodies are directed against various nuclear antigens

Traditionally, IIF on HEp-2 cells is used for ANA screening followed by more specific second line tests which are performed to identify the target antigen of the antibodies (e.g. dsDNA or extractable nuclear antigens [ENAs]).

The term ENA generally includes, but is not limited to Sm, RNP, Ro (SS-A), La (SS-B), Jo-1 and Scl-70. Anti-dsDNA and anti-ENA antibodies are clinically important in patients with SARDs.

Some major patterns can be discerned by IIF on HEp-2 cells: homogenous, speckled, centromeric, nucleolar, speckled or diffuse cytoplasmic. Although not absolute, there exists a relationship between the pattern observed on HEp-2 cells by IIF and the presence of anti-dsDNA and/or anti-ENA antibodies.2 There is an association between anti-dsDNA/ENA antibodies and specific autoimmune diseases.2

HEp-2 patterns, the target antigens, and their associated disease states:

1. A homogenous pattern is associated with antibodies to dsDNA (SLE) or histones (drug-induced lupus)
2. A speckled pattern is associated with antibodies to U1-RNP (mixed connective tissue disease, SLE), Sm (SLE), and Ro (SS-A)/La (SS-B) (SjS, SLE)
3. A centromere pattern is associated with anti-centromere antibodies (limited cutaneous form of SSc)
4. A nucleolar pattern is associated with antibodies to PM/Scl, RNA-polymerase III, and Scl-70 (SSc)
5. A speckled cytoplasmic pattern is associated with antibodies to mitochondria (primary biliary cirrhosis) or Jo-1 (PM), whereas a diffuse cytoplasmic pattern is associated with anti-ribosome antibodies (SLE)

<table>
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<th>Table 1</th>
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<tr>
<td><strong>ANTINUCLEAR ANTIBODY TESTING IS:</strong></td>
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<tr>
<td>Very useful for the diagnosis of SLE and SSc</td>
</tr>
<tr>
<td>Somewhat useful for the diagnosis of SjS and PM/DM</td>
</tr>
<tr>
<td>Very useful for the monitoring or prognosis of juvenile chronic arthritis (to stratify the risk for uveitis)</td>
</tr>
<tr>
<td>A critical part of the diagnosis of drug-associated lupus, mixed connective tissue disease, and autoimmune hepatitis</td>
</tr>
</tbody>
</table>

The guidelines also state that ANA testing is not useful for the diagnosis, monitoring, or prognosis of other diseases including rheumatoid arthritis and thyroid disease.1

Antinuclear antibodies are directed against various nuclear antigens

Traditionally, IIF on HEp-2 cells is used for ANA screening followed by more specific second line
Anti-ENA and anti-dsDNA antibodies occur with the highest prevalence in samples with high ANA titers. The ENA specificities that are most related to high ANA titers are U1-RNP and Sm. Patients with no autoimmune disease and healthy individuals usually have low ANA titers.

Quantitative automated solid phase methods have in some settings replaced IIF methods for the detection of ANA. Because solid phase methods employ only a limited number of autoantigens, they have a lower sensitivity than IIF. For example, the sensitivity of IIF for SLE is reported to be 90-95%, whereas the sensitivity of antibodies to dsDNA, Sm, Ro (SS-A), and La (SS-B) for SLE is reported to be 50-70%, 8-20%, 30-50%, and 20%, respectively. In our own experience, 18 (29%) of 62 SLE patients were negative for anti-dsDNA, anti-Sm, anti-U1-RNP, or anti-Ro (SS-A) antibodies (unpublished data). Of these 18 patients, 15 were ANA positive by IIF on HEp-2 cells. The low sensitivity of solid phase methods has also been illustrated by a case report in which a diagnosis of SLE was delayed because of a false negative ANA result by solid phase method.

The sensitivity of IIF for SSc is reported to be 85-90%, whereas the sensitivity of anti-Scl-70 and anti-centromere antibodies is reported to be 15-20% and 40-60%, respectively. In our own experience, 23 (33%) of 70 SSc patients had no antibodies to centromere, Scl-70, PM-Scl-70 or RNA-Pol-III. Of these 23 patients, 20 were positive by IIF. Anti-Scl-70, anti-centromere, anti-RNA Pol-III, and anti-PM-Scl-100 antibodies were found in 21%, 37%, 7%, and 4% of patients with SSc, respectively.

Given the low sensitivity of solid phase immunoassays as a screening test for detection of ANA, a task force of the ACR has recently concluded that solid phase immunoassays may not be appropriate at present to replace IIF as a screening test for detection of ANA. They recommend that IIF ANA testing should remain the gold standard.

Antinuclear antibodies are not only important for the diagnosis of SARDs, but also for autoimmune hepatitis. It should be mentioned that the target antigens of the ANAs in autoimmune hepatitis are diverse and/or unknown and that no solid phase methods are available to screen for autoimmune hepatitis-related ANAs at present.

**Although indirect immunofluorescence has a high sensitivity, the specificity is low**

Antinuclear antibodies are also found in patients with non-rheumatic diseases and in healthy individuals. Moreover, one should appreciate that anti-ENA antibodies (especially anti-Jo-1, anti-ribosomal P and anti-Ro (SS-A) may be overlooked by IIF on HEp-2 cells. In a prospective study in which we evaluated antinuclear antibodies in 2405 consecutive samples by IIF on HEp-2 cells and by solid phase assays we found the sensitivity of antinuclear antibody testing by IIF for detection of anti-ENA antibodies to be 82.9%. Recently, we reported the prozone phenomenon for the ANA test by IIF in a case of SjS with anti-Ro (SS-A) antibodies. Thus, although IIF on HEp-2 cells has a high sensitivity, it may miss clinically important autoantibodies. When there is a high clinical suspicion, irrespective of the ANA result, focused testing for specific autoantibodies should be performed.
Other challenges facing IIF on HEp-2 include intra- and inter-laboratory variance. Many sources contribute to the variability of indirect immunofluorescence on HEp-2 cells:

- Different sources of HEp-2 cell lines
- Different fixations
- Different reading systems and optics
- Different secondary antibodies, and heterogeneous assays
- Visual evaluation is subjective and requires considerable expertise of the technician

Taken together, IIF on HEp-2 cells remains an important and established laboratory method in a multi-step diagnostic approach to systemic rheumatic diseases and autoimmune hepatitis. The advent of digital IFA systems will undoubtedly result in a more standardized approach to antinuclear antibody testing.

References

Digital image analysis results show high reproducibility and agreement with human interpretation on HEp-2 cells

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The term antinuclear antibody (ANA) describes a variety of autoantibodies that react with constituents of cell nuclei including DNA, RNA, proteins and riboproins.\(^1\) The detection of ANA in human serum is an important tool for diagnosing connective tissue diseases, especially systemic lupus erythematosus (SLE).\(^1-3\) Indirect immunofluorescence (IIF) is the reference method for ANA testing which detects a wide range of autoantibodies to nuclear and cytoplasmic antigens.\(^1,2\) A negative test virtually rules out SLE.\(^3\) Currently, the American College of Rheumatology recommends IIF on HEp-2 as the method of choice for ANA screening. In conjunction with the patient history and physical condition, IIF on HEp-2 offers excellent sensitivity (95%) for SLE.\(^3\)

Lack of standardization for IIF ANA testing still remains a concern.\(^4\) Sources of variability include, but are not limited to, the microscope and the interpretation by the operator. The introduction of automation can eliminate these sources of variability as it provides an objective output.\(^5,6\) NOVA View\textsuperscript{®} digital IFA system contains a microscope with an automated stage, a CCD (charge-coupled device) digital camera, a LED light source and software that controls the motorized stage. This system takes digital images, archives the images, preliminarily categorizes the samples as positive or negative and provides pattern recognition for positive samples. The automated reading is followed by human visual interpretation of the archived images that allows review and user confirmation of the automated results. The archived images facilitate training and allow for the exchange of results between labs and clinicians.\(^6\) NOVA View reduces variability and provides an approach to standardize ANA interpretation.

Benefits of an Automated Digital Image Analysis Analyzer

**Reduces hands-on time**
- Automated scanning and digital imaging of HEp-2 slides increases productivity by reducing hands on time

**Supports standardization**
- Proprietary algorithms provide objective and consistent output

**Prompts appropriate analysis**
- Helps recognize samples requiring additional review

**Facilitates case review**
- Creates digital image database that allows review, follow-up and consultation
In order to assess the performance of NOVA View, studies were conducted to evaluate precision based on light intensity units and endpoint titration data. In addition, agreement was determined by comparing results obtained by NOVA View on clinical samples to visual human interpretation of the captured images.

**Methodology**
- Intra-assay variability was determined by running five sera with five different patterns 36 times each (Table 1a).
- Total variability was determined by running five sera 45 times. The 45 individually run assays integrated two lots of HEp-2 slides, two lots of conjugates and three operators (Table 1b).
- Endpoint titration studies were performed by diluting five sera 1:40 to 1:81,920 in PBS for 25 separate runs. (Fig. 1).
- 204 clinically defined sera were used for comparison. The output of NOVA View was compared to the visual human interpretation of the archived images (Fig. 2).
- The study was conducted using NOVA View digital IFA system (INOVA Diagnostics, Inc.) with pre-production software version 1.0.1.**

### Table 1

**Assay Variability**

#### a)

<table>
<thead>
<tr>
<th>Intra-assay Variability</th>
<th>Pattern</th>
<th>Average LIU*</th>
<th>% CV</th>
</tr>
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<tbody>
<tr>
<td>Homogeneous</td>
<td>486.2</td>
<td>12.3</td>
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</tr>
<tr>
<td>Speckled</td>
<td>421.6</td>
<td>16.9</td>
<td></td>
</tr>
<tr>
<td>Centromere</td>
<td>343.1</td>
<td>14.5</td>
<td></td>
</tr>
<tr>
<td>Nucleolar</td>
<td>451.6</td>
<td>9.0</td>
<td></td>
</tr>
<tr>
<td>Nuclear Dots</td>
<td>422.1</td>
<td>13.1</td>
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</table>

N=36

*light intensity units

**Upgraded software version is available at time of print

#### b)

<table>
<thead>
<tr>
<th>Total Assay Variability</th>
<th>Pattern</th>
<th>Average LIU*</th>
<th>% CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogeneous</td>
<td>1054.3</td>
<td>19.1</td>
<td></td>
</tr>
<tr>
<td>Speckled</td>
<td>1859.4</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>Centromere</td>
<td>672.2</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>Nucleolar</td>
<td>625.6</td>
<td>16.7</td>
<td></td>
</tr>
<tr>
<td>Nuclear Dots</td>
<td>877.3</td>
<td>14.8</td>
<td></td>
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</table>

N=45
In Summary

- NOVA View results are highly reproducible and precise
- Results obtained using clinical samples demonstrate the capability of NOVA View to correctly discriminate between positive and negative
- Archived images can be stored, reviewed, and shared at any time

References

High impact of the dense fine speckled pattern on HEp-2 cells on the diagnosis of systemic autoimmune diseases

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INOVA Diagnostics, San Diego, CA, USA

History of ANA antibodies

The presence of autoantibodies against intracellular antigens, especially antinuclear antibodies (ANAs), is a hallmark of systemic autoimmune rheumatic diseases (SARD). The indirect immunofluorescence (IIF) assay is one of the most commonly used routine tests for the detection of ANA and was recently recommended by a task force of the American College of Rheumatology (ACR). However, approximately 20% of serum samples from healthy individuals (HI) have been reported to yield a positive ANA test, the majority of which are caused by autoantibodies to dense fine speckles 70 (DFS70) antigen. Anti-DFS70 antibodies were initially identified in a patient with interstitial cystitis, but were later associated with various disease conditions and especially atopic dermatitis.

Clinical association of anti-DFS70 antibodies

Since the first description, anti-DFS70 antibodies have been found in the sera of patients with a variety of chronic inflammatory conditions, cancer, and even in HI. Dellavance, et al. evaluated over 10,000 ANA positive samples by IIF and then by immunoblot, reporting that anti-DFS70 antibodies were common among ANA-positive individuals with no evidence of SARD and that among autoimmune patients with this autoantibody, over half had evidence of autoimmune thyroiditis. Although the clinical association and the root cause of anti-DFS70 antibodies are still unclear, it has been confirmed by different research teams that anti-DFS70 antibodies are more prevalent in apparently HI vs. patients with SARDs. Considering the prognostic and long term outcome of individuals that have anti-DFS70 antibodies, it was recently reported that, out of 40 anti-DFS70 positive HI, none had developed SARD over an average 4-year clinical follow-up. Based on this observation, it has been suggested that the presence of isolated anti-DFS70 antibodies could be used to exclude the diagnosis of SARD, such as systemic lupus erythematosus (SLE). The decreased prevalence of anti-DFS70 autoantibodies in SARD patients is interesting, and the reasons underlying this observation are unclear, but may include demographic, genetic, racial and/or technologies used to detect this autoantibody.

IIF pattern and cellular function

The typical IIF staining pattern has been described as dense fine speckles that are distributed throughout the nucleus and on metaphase chromatin. Since a 70-kDa protein was recognized by immunoblotting, the antigen was initially termed DFS70 but, the primary target autoantigen was later identified as the lens epithelium–derived growth factor (LEDGF) or DNA binding transcription coactivator p75. This protein has a number of physiological functions including serving as a cofactor for human immunodeficiency virus replication through an interaction with the viral integrase and it is highly expressed in prostate tumor tissue.

Consequences for ANA testing – a new algorithm

In a previous study, 172/21,512 (0.8%) of consecutive samples tested for ANA showed the typical DFS pattern by IIF. This pattern was one of the most common in the routine laboratory setting. Since the presence of ANA are considered a reliable screening biomarker for SARD and are included in the classification criteria for SLE, ANA–HEp-2 testing outside a proper clinical framework may yield a sizable portion of ANA-positive individuals with no consistent evidence of SARD, potentially causing some concern and anxiety in patients and physicians alike. This becomes even more crucial with the now compelling evidence that autoantibodies may precede the clinical onset of SARD by many years.
pointed out by Fritzler MJ, not all sera demonstrating the DFS pattern are from HI and it remains unclear whether this staining pattern is universally recognized in clinical diagnostic laboratories. In particular, the discrimination between DFS and the so-called ‘quasihomogeneous pattern’ might be a challenging task for routine diagnostic laboratories. This underlines the importance of a better understanding of anti-DFS70 antibodies and the inclusion of testing for anti-DFS70 antibodies into the diagnostic algorithm for ANA testing (Fig. 1).

Fig. 1

Fig. 1 Characteristic staining pattern and proposed test algorithm considering anti-DFS70 antibodies (modified from Mahler et al.)

The characteristic dense fine speckled (DFS) staining pattern of interphase cells is indicated by the red arrow and the strong chromatin staining of mitotic cells by the blue arrow. Samples with a DFS pattern could be tested for anti-DFS70 antibodies by a confirmatory test and by ANA Screen ELISA (QUANTA Lite® ANA Screen) containing various SARD associated autoantigens. In this context, it is important to mention that the majority of monospecific DFS70 samples are negative on the QUANTA Lite® ANA Screen. Patients with negative ANA Screen ELISA and positive DFS70 test result have a lower likelihood for having SARD. Patients with a positive ANA Screen ELISA result, identified ENA specificity and negative DFS70 test result have an increased likelihood of having SARD. The likelihood for SARD in patients with a DFS pattern and a positive ANA Screen ELISA is less understood. However, the data presented by Mariz et al., indicates that SARD is unlikely in those patients since a DFS pattern with confirmed DFS70 reactivity (indicating mono-specific DFS70 reactivity) is negatively associated with SARD. Further studies performed in routine settings are required to analyze the likelihood ratios of this proposed algorithm.

It is suggested that samples with a DFS staining pattern identified by IIF should be tested for anti-DFS70 antibodies using a specific immunoassay. The test results need to be reported and clearly explained to clinicians.
**Immunoadsorption of anti-DFS70 antibodies**

In a recent study, it has been shown that DFS pattern is found in 33.1% of ANA positive HI compared to 0.0% of ANA positive patients with SARD \((p<0.0001)\), which significantly affects the diagnostic power and efficiency of the IIF assay.\(^3\) Thus, accurate pattern recognition, interpretation and reporting of results to clinicians are of high importance because it could decrease the referral of patients with a positive ANA for unnecessary consultation and evaluation. Since the identification of the DFS pattern might be challenging for routine diagnostic laboratories and inaccurate interpretation can have significant consequences, a method that can prevent anti-DFS70 antibodies from binding to their cognate target and producing the DFS pattern would significantly improve the performance characteristics of ANA by IIF.\(^7\) This led us to develop a method, allowing for the immunoadsorption of anti-DFS70 antibodies (Fig. 2), which offers considerable cost-savings in both the laboratory and the medical care system.

**Fig. 2**

**Immunoadsorption of anti-DFS70 antibodies.** Immunoadsorption of autoantibodies associated with systemic autoimmune rheumatic diseases (SARD) are shown in a). No significant change can be observed in samples with anti-RNP, anti-Centromere and anti-Scl-70 antibodies. In contrast, as shown in panel b), anti-DFS70 antibodies are blocked by immunoadsorption with recombinant DFS70 antigen [DFS70 (1)-DFS70 (3)]. In DFS70 (3) a different pattern becomes identifiable after immunoadsorption.
References

17. Bizzaro N, Tonutti E, Villalta D. Recognizing the dense fine speckled/lens epithelium-derived growth factor/p75 pattern on HEp-2 cells: not an easy task! Arth Rheum 2011; 63(12):4036-4037
Autoantibodies that cannot be identified on HEp-2 cell need tissue substrate

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Aesculabor-Hamburg, Germany

Analysis of autoantibodies (AABs) by indirect immunofluorescence (IIF) is an important first step in the diagnosis of autoimmune diseases. Non-organ specific AABs, important in the diagnosis of connective tissue disease, are routinely screened by IIF on HEp-2 cells.

Organ specific AABs are directed against highly conserved antigens. Diagnostically relevant target structures are located in organ specific cells such as parietal cells in stomach epithelium, or actin in stomach epithelium and kidney tissue. In contrast to the HEp-2 testing where the focus lies on the cell organelles that can be stained in the cell and allow a correlation to specific antigens, the focus in tissue testing lies more on the fluorescence pattern of stained cells in the tissue. AABs that can be detected on tissues are often named after the structure in the tissue that is stained by the AABs (Table 1). This is because the antigen was unknown when the AAB was detected. For example, parietal cell antibodies (PCA) are directed against, H+K+ATPase, and endomysium antibodies are directed against tissue transglutaminase.

Different AABs can stain the same cells in tissue sections if both antigens are expressed in the cells. This leads to a similar fluorescence pattern in the tissue. An expert combination of different organ tissue helps to distinguish different AABs from each other. For instance, antimitochondrial antibodies (AMA) and PCA both react with parietal cells in the stomach epithelium.1 To differentiate these AABs an additional liver tissue testing is very helpful because anti mitochondrial antibodies (AMA) lead to a clear fluorescence in the liver. This is in contrast to PCA antigen, which do not, due to the fact that the H+K+ATPase is not expressed in the liver.

A common combination of organ tissue sections is stomach, liver and kidney. This slide allows a screening for gastrointestinal antibodies such as PCA, AMA, smooth muscle antibodies (SMA)/Actin, liver kidney microsomal (LKM) and liver cytosol 1 (LC1).

Table 1

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Detectable antibody</th>
<th>Further differentiation/confirmation</th>
<th>Clinical Association</th>
</tr>
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<tbody>
<tr>
<td>Stomach</td>
<td>PCA</td>
<td>H+K+ATPase</td>
<td>Pernicious Anaemia</td>
</tr>
<tr>
<td>Kinney</td>
<td>AMA</td>
<td>AMA-M2 (MIT3)</td>
<td>Primary Biliary Cirrosis</td>
</tr>
<tr>
<td>Liver</td>
<td>SMA</td>
<td>Actin</td>
<td>Autoimmune Hepatitis</td>
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<tr>
<td></td>
<td>LKM</td>
<td>LKM-1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LC</td>
<td>LC-1</td>
<td></td>
</tr>
<tr>
<td>Pancreas</td>
<td>ICA</td>
<td>GAD</td>
<td>Type 1 Diabetes Mellitus</td>
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<tr>
<td></td>
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<td>IA-2</td>
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<tr>
<td>Cerebellum</td>
<td>ANNA 1</td>
<td>Hu</td>
<td>Paraneoplastic neurological syndrome</td>
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<tr>
<td></td>
<td>ANNA 2</td>
<td>Ri</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ma</td>
<td>Ma2</td>
<td></td>
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<tr>
<td></td>
<td>Yo</td>
<td>Yo (Blot)</td>
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PCA=parietal cell antibodies; AMA=anti mitochondrial antibodies; SMA=smooth muscle antibodies; LKM=liver-kidney-microsomal antibodies; GAD=glutamic acid decarboxylase; LC=liver cytosol; ICA=islet cell antibody; ANNA=anti-neuronal nuclear antibodies.
PCA antibodies can be detected in stomach tissue. The H⁺/K⁺ATPase in the parietal cells are stained by PCA, all other cells in the stomach epithelium, in the liver and kidney are negative. The test is capable to exclude PCA, due to the fact that other unspecific AABs can stain parietal cells on rodent tissue that is commonly used in the test as well. Positive results should be confirmed with H⁺/K⁺ATPase ELISA. PCA can be found frequently in patients without pernicious anaemia because of the long latency of 20-30 or more years before clinical manifestation.¹

AMA stain the cytoplasm of hepatocytes, kidney cells and stomach tissue with an enhancement of parietal cells. A titer of 1:40 or higher is considered as positive.² AMA can be detected also as a granular fluorescence in the cytoplasm of HEp-2 cells. AMA positive samples should be tested additionally with AMA-M2 ELISA. To obtain optimal sensitivity, the M2-ELISA should contain PDC-E2, OGDC-E2, BCOADC-E2 as antigen.² Recombinant mitochondrial antigens have been made available, in the case of pMIT3, the three main autoepitopes are conjugated in one molecule. My personal observation is that AMA testing on HEp-2 cells is more sensitive than triple tissue testing and correlates better to the AMA-M2-MIT3 ELISA results.

“**For detection of organ specific antigens that are not expressed in HEp-2 cells such as PCA, ASMA or LKM, additional tests on tissue sections are required.”**

AMA on HEp-2 Cell

AMA on mouse kidney/stomach tissue

AMA on mouse liver/stomach tissue
**SMA** are directed against structures of the cytoskeleton such as actin, troponin or tropomyosin. F-actin is the blank antigen associated with autoimmune hepatitis. SMA reacts with the wall of small arteries present in all three tissues, the stomach muscular layer and interglandular fibres of the stomach. If additional fluorescence is detected peritubularly in the kidney, the pattern is specific for F-actin. Due to the fact that F-actin positive SMA antibodies are more specific for autoimmune hepatitis than SMA alone, the result is reported as F-actin positive SMA if the peritubular fluorescence can be detected.

![Positive interglandular fibers on mouse stomach tissue](image1)

![SMA with a specificity for actin on monkey kidney tissue (red arrow denotes actin)](image2)

**LKM** antibodies are directed against cytochromes in the endoplasmatic reticulum. In IIF, this leads to a fluorescent staining of the cytoplasm in the liver and the proximal tubuli in the kidney. The distal tubuli and stomach tissue are negative.

![Fine granular fluorescence in the cytoplasm of proximal renal tubules but not in distal tubules](image3)

LKM antibodies can be subclassified in LKM-1, LKM-2 and LKM-3 by ELISA or line immunoassay. LKM-1 autoantibodies recognize a major linear epitope between amino acid 263 and 270 of the CYP 2D6 protein. These autoantibodies inhibit CYP 2D6 activity in-vitro and are capable of activating liver infiltrating T-lymphocytes indicating autoimmune hepatitis. LKM-2 autoantibodies are found in ticrynafen induced hepatitis; however, it is less frequent since the diuretic is not longer in use. LKM-3 antibodies can be found in patients with viral hepatitis infection, especially in hepatitis D.
Liver cytosolic autoantibodies (LC-1) recognize formiminotransferase cycloamidase as antigen, a metabolic enzyme that is highly expressed in the liver. The antibody is found in patients with autoimmune hepatitis and hepatitis C infection. LC-1 antibodies alone lead to a clear fluorescence pattern of the liver in IIF. Since LC-1 antibodies are frequently present together with LKM-1 antibodies, which also appear in the liver, it is very easy to overlook the LC1 antibody. However, IIF allows the exclusion of the presence of the antibody if no liver staining is observed.

Islet cell antibodies (ICA) can be found in patients with type1 diabetes mellitus (T1DM) on pancreas tissue sections. The antibodies react against antigens in the pancreatic β cells. In 80% of cases, the antibodies are directed against glutamic acid decarboxylase (GAD). ICA are a powerful predictor of islet cell autoimmunity. Another common antigen is islet cell antigen 512 (IA-2). GAD and IA-2 antibodies can be found alone or in combination in patients with T1DM. In pre-diabetic patients the risk of clinical manifestation increases if both antibodies can be detected. In patients with late onset autoimmunity diabetes in the adult (LADA) with ICA, the antibody cannot be confirmed by GAD or IA-2 ELISA in every case. This suggests that a further antigen plays a role in this cohort. Insulin autoantibodies (IAA) can be found in children, the concentration of which, at the time of diagnosis, is inversely related to the age of the patient, being highest in those less than 5 years of age.

Screening for T1DM antibodies with rodent pancreatic tissue sections is an inexpensive and simple procedure to exclude T1DM antibodies, because the majority of the tested samples are negative for these antibodies. Especially in patients with LADA, ICA is an important marker because GAD and IA-2 testing does not identify all antibodies. In children younger than 5 years of age, additional IAA testing should be performed.
Paraneoplastic neuronal antibodies (PNA) are markers for paraneoplastic neurological syndromes. Only IgG antibodies are considered to be clinically relevant. Indirect immunofluorescence on cerebellar tissue is the initial screen for antineuronal antibodies. Western blots with cerebellar extracts can be used to confirm the specificities. Antibodies that target neuronal nuclei of the central nervous system are Hu-Ab (also called anti-neuronal nuclear antibody 1 (ANNA-1)), Ri-Ab (ANNA-2), ANNA-3 and Ma-Ab.

The most common and widely investigated PNA is Hu-Ab.

An antibody that targets purkinje cell cytoplasm is Yo-Ab. This antibody is the second most common onconeuronal antibody. Three proteins are recognized by the antibodies and are known as cerebellar degeneration-related proteins. The Yo-antibody is associated with neoplasms of the breast and ovaries.

If Yo-Ab cannot be confirmed on Western blot, the PCA2-Ab associated with small cell lung cancer (SCLC) and Tr-Ab associated with Hodgkin’s disease should be excluded because these rare antibodies lead to a similar IIF pattern on cerebellar tissue sections.

References
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