

Review Article

The history and recent advancements in antinuclear antibody testing

Dimitra Moschou¹, Elena Pipi², Alexandra Tsirogianni²¹Rheumatology Department, KAT General Hospital of Athens, Greece²Immunology-Histocompatibility Department, Evangelismos General Hospital of Athens, Greece**Abstract**

The antinuclear antibody (ANA) testing is an indispensable diagnostic tool for the management of systemic autoimmune rheumatic diseases (SARD). From the initial discovery of ANA identification until today there has been considerable technical progress in ANA testing and standardisation which has allowed the development of commercial ANA assay kits that are widespread used in clinical and research practice. In this mini-review we explore the history and recent advancements in ANA testing and provide guidelines to the laboratory interpretation and clinical assessment of ANA results.

Keywords: Antinuclear antibody, Indirect immunofluorescence pattern, ANA results

ANA history-From discovery to laboratory practice

In 1948, Malcolm Hargraves, Helen Richmond, and Robert Morton during evaluation of bone marrow examinations at the Mayo Clinic (Minnesota, USA) observed exclusively in patients with systemic lupus erythematosus (SLE) the presence of abnormal cells with specific morphology (the nucleus is phagocytosed by mature leucocytes) and introduced the term “LE cell”¹. Although LE cells are usually found in the bone marrow of these patients, it has been shown that after a period of incubation they can be formed in buffy coats of peripheral blood² indicating that the LE phenomenon is rather a secondary response to a blood circulating factor and not a developmental cellular defect.

One year later, two research laboratories independently confirmed the inducible nature of LE phenomenon³. Incubation of bone marrow cells from healthy subject with plasma from SLE patients lead to the formation of LE cells. It took almost a decade and a series of ingenious research to discover that the phenomenon LE cells are autoantibodies that bind to nuclear elements. The molecular nature of the LE factor was determined when it was discovered that this factor lies in the gamma globulin fraction⁴. The nuclear specificity has been shown later by at least two different research groups at the same time. Holborow et al, showed that in all tissues examined (skin, heart muscle, kidney, thyroid and spleen) exposure of the section to LE cells positive sera resulted in

marked specific fluorescence of the nuclei on subsequent staining with the anti-globulin conjugate⁵.

In the same year, Holman and Kunkel published a seminal work in Science where they show that the LE serum factor possesses affinity for cell nuclei and nucleoproteins⁶. One year later, Friou and colleagues introduced an indirect immunofluorescence (IIF) assay with end-point titres for the detection of anti-nuclear antibodies^{7,8} (ANA). Subsequently, three distinct classic patterns (homogeneous, speckled and nucleolar) of ANA fluorescence were described by Swanson Beck during the staining of rat liver sections with patients' serum⁹, introducing the importance of ANA pattern in clinical practice. Since then significant progress has been made on the identification of ANA specificity in SLE and other systemic autoimmune rheumatic diseases, including Rheumatoid Arthritis (RA), Sjögren's Syndrome (SjS), Systemic Sclerosis (SSc), Mixed Connective Tissue Disease (MCTD), and Idiopathic Inflammatory Myopathies (IIM).

The authors have no conflict of interest.

Corresponding author: Dimitra Moschou, Rheumatology Department KAT General Hospital of Athens, Greece

E-mail: dimitramoschou@hotmail.com

Edited by: George Lyritis

Accepted 24 July 2017

ANA testing-IIF on Hep-2

The ANA IIF test initially was performed on rodent tissue substrates but these were later substituted by Human epidermoid-2 (HEp-2) cells, a cell line established from epidermoid carcinoma tissue from the larynx of a 56-year-old male¹⁰. Nowadays, to improve the performance of ANA testing, this cell line has been transfected with the human 60 kDa Ro/SS-A protein, a common nuclear antigen found in different autoimmune systemic diseases^{11,12}. Although the IIF assay on HEp-2 cells has been replaced in some laboratories by ELISAs or multiplex assays using addressable laser beads, the IIF on Hep-2 substrate remains the “gold standard” for ANA screening as per American College of Rheumatology (ACR) recommendations¹³. These recommendations are based on the idea that the HEp-2 cell substrate comprises an “antigenic array” presenting >100 autoantibody targets whereas multiplex screening assays are usually more limited in autoantibody target composition¹⁴.

To promote laboratory quality control of ANA and other autoantibody testing, the Autoantibody Standardization Committee has collaborated with the Centres for Disease Control and Prevention (CDC) and other agencies to provide autoantibody reference standards and provides 17 reference standards available free of charge to all qualified clinical or commercial laboratories and research investigators¹⁵.

Positive autoantibody titres at a dilution equal to or greater than 1:160 are usually considered as clinically significant. Positive titres of less than 1:160 are present in up to 20% of the healthy population, especially the elderly. Although positive titres of 1:160 or higher are strongly associated with systemic autoimmune rheumatic disorders, they are also found in 5% of healthy individuals¹⁶.

Interestingly, there is relationship between the Hep-2 IIF pattern and the presence of a specific antibody. For example, a high titre homogeneous pattern is usually associated with antibodies to dsDNA, whereas a high titre speckled pattern is indicative for antibodies to extractable nuclear antigens¹⁷. A nuclear Dense Fine Speckled pattern has been associated with the presence of autoantibody to DFS70 and is seen in very low frequency in systemic autoimmune diseases but in high frequencies in healthy individuals¹⁸. Notably, a novel immunoadsorption method has been developed that can remove the anti-DFS70 antibodies, thus significantly increases the specificity of ANA testing for systemic autoimmune rheumatic diseases¹⁹.

Apart from nuclear patterns, cytoplasmic and mitotic cell patterns are also recognized. Thus, the term “anti-cellular antibodies” has been suggested to describe the wider spectrum of these autoantibodies. However, because the use of the ANA acronym is firmly established and universally used, the ANA term is maintained for historical reasons and for laboratory coding and invoicing²⁰.

What the IIF patterns on HEp-2 can tell us?

Two separate workshops were dedicated to standardization and harmonization of ANA testing and nomenclature: International Autoantibody Standardization (IAS) and International Consensus on ANA Patterns (ICAP)²¹⁻²³.

Nuclear patterns

Nuclear homogeneous or diffused: It can be described as homogeneous and regular fluorescence across all nucleoplasm. The nucleoli maybe stained or not stained depending on cell substrate. Mitotic cells (metaphase, anaphase, and telophase) have the chromatin mass intensely stained in a homogeneous hyaline fashion. This pattern indicates that the antigenic target is dsDNA, nucleosomes and/or histones and it is clinically associated with SLE, drug-induced lupus or juvenile idiopathic arthritis.

Nuclear dense fine speckled: It can be described as speckled pattern distributed throughout the interphase nucleus with characteristic heterogeneity in the size, brightness and distribution of the speckles. Throughout the interphase nucleus, there are some denser and looser areas of speckles (very characteristic feature). The metaphase plate depicts strong speckled pattern with some coarse speckles standing out and indicates that the antigenic target is DFS70/LEDGF which is rare in SjS, SSc and SLE and more often in healthy individuals.

Centromere or kinetochore: It can be described as discrete coarse speckles (40-80/cell) scattered in interphase cells and aligned at the chromatin mass on mitotic cells. This pattern indicates that the antigenic target is CENP-A/B (C) and it is clinically associated with limited cutaneous SSc and Primary Biliary Cirrhosis (PBC).

Nuclear fine speckled or fine granular: Can be described as fine tiny speckles across all nucleoplasm. The nucleoli may be stained or not stained. Mitotic cells (metaphase, anaphase, and telophase) have the chromatin mass not stained. This pattern indicates that the antigenic target is SS-A/Ro and/or SS-B/La among others. It is clinically associated with SjS, SLE and Dermatomyositis (DM).

Nuclear large/coarse speckled or spliceosome/nuclear matrix: Can be described as coarse speckles across all nucleoplasm. The nucleoli may be stained or not stained. Mitotic cells (metaphase, anaphase, and telophase) have the chromatin mass not stained. Possible antibodies are anti-Sm and/or anti-U1 RNP, while it is associated with MCTD, SLE, or SSc.

Multiple nuclear dots: It can be described as countable discrete nuclear speckles (6-20 nuclear dots/cell). It is indicative for the presence of anti-sp100 or anti-PML proteins antibodies observed in PBC, SARD or DM.

Few nuclear dots: It can be described as countable discrete speckles (1-6 nuclear dots/cell in most cells). These are known as Cajal bodies or coiled bodies and it is clinically associated with SjS, SLE, SSc, or PM.

Homogeneous nucleolar: It can be described as diffuse

fluorescence of the entire nucleolus, while the metaphase plate shows no staining. It is indicative for the presence of anti-PM-Scl or anti-Th/To. And it is clinically associated with SSc or SSc/PM overlap.

Clumpy nucleolar: It can be described as irregular staining of the nucleoli and Cajal bodies with a peri-chromosomal staining at the metaphase plates. Anti-fibrillarin can give this pattern which is clinically associated with SSc.

Punctate nucleolar or nucleolar speckled: It can be described as densely distributed but distinct grains seen in the nucleoli of interphase cells. In metaphase cells, up to 5 bright pairs of the nucleolar organizer regions (NOR) can be seen within the chromatin body. The cytoplasm of mitotic cells may be slightly positive. This pattern indicated the presence of anti-NOR-90, anti-RNA polymerase I and it is associated with SSc or SjS.

Smooth nuclear envelope or nuclear rim, or nuclear membrane, or membranous: It can be described as homogeneous staining of the nucleus with greater intensity at its outer rim and no staining at the metaphase and anaphase chromatin plates. There is a peculiar accentuation of the fluorescence at the points where adjacent cells touch each other. It indicates the presence of antibodies targeting the laminins and it is clinically related with SLE, SjS or seronegative arthritis.

Punctate nuclear envelope or nuclear membrane pores: The nuclear envelope reveals a punctate staining in interphase cells, with accentuation of fluorescence at the points where adjacent cells touch each other. No staining of the metaphase and anaphase chromatin plates. The antigenic target is the nuclear pore complex proteins (i.e. gp210) and it is clinically associated with PBC.

PCNA-like: It can be described as pleomorphic speckled nucleolar staining, with variability in size and brightness of the speckles. In interphase, some cells are negative (G1 phase), some are intensely stained (S-phase) and some present rare and scattered speckles with occasional nucleolar staining (late S and early G2 phases). Mitotic cells are not stained. The antigenic target is the PCNA and is associated with SLE or other conditions.

CENP-F-like: It can be described as nuclear speckled pattern with striking variability in intensity with the strongest staining in G2 phase and weakest/negative staining in G1. The centromeres are positive only in prometaphase and metaphase, revealing multiple aligned small and faint dots. Prometaphase cells frequently show a weak staining of the nuclear envelope. During anaphase and telophase, some sera demonstrate intense staining in the ring located at the midzone (i.e. mid-body, stem body) where the division of the daughter cells is taking place. The surrounding cytoplasm of the mitotic cells is diffusely stained. It is clinically associated with cancer or other conditions.

Cytoplasmic patterns

Cytoplasmic fibrillar linear or actin-like: This pattern is characterized by decorated cytoskeletal fibres, sometimes

with small, discontinuous granular deposits. Typical staining show striated actin cables spanning the long axis of the cells. It indicated the presence of anti-actin and/or anti-non-muscle myosin antibodies and it is associated with MCTD, Chronic Active Hepatitis (CAH), Liver Cirrhosis, Myasthenia Gravis, Crohn's disease, PBC or long term haemodialysis. This pattern is rarely found in SARD.

Cytoplasmic fibrillar filamentous: This pattern is characterised by staining of microtubules and intermediate filaments spreading from the nuclear rim. It indicates the presence of anti-cytokeratin and/or anti-vimentin antibodies. It clinically associated with numerous different infectious or inflammatory conditions.

Cytoplasmic fibrillar segmental: This pattern can be described as enhanced decoration of short segments, periodic dense bodies, along the stress fibres. It indicates the presence of anti-alpha actin, anti-vinculin and/or anti-tropomyosin antibodies. It is clinically associated with Myasthenia Gravis, Crohn's disease, or Ulcerative Colitis.

Cytoplasmic discrete dots/GW body-like: Staining of GW bodies in the pattern appears cloudy, almost homogeneous throughout the cytoplasm of interphase cells with high numbers in late S/G2 cells. It is clinically associated with different conditions.

Cytoplasmic dense fine speckled: The pattern appears cloudy, almost homogeneous throughout the cytoplasm. It indicates the presence of antibodies against P ribosomal proteins and it is clinically associated with "anti-synthetase syndrome", PM/DM, SLE, juvenile SLE or neuropsychiatric SLE.

Cytoplasmic fine speckled: It characterised by scattered small speckles in the cytoplasm mostly with homogeneous or dense fine speckled background. This pattern is indicative for the presence of anti-Jo-1 antibodies and it clinically associated with anti-synthetase syndrome, PM/DM, limited SSc.

Cytoplasmic reticular/mitochondria-like: It can be described as coarse granular filamentous staining extending throughout the cytoplasm due to the presence of anti-mitochondrial antibodies. It is common in PBC and SSc but it is rare in other SARD.

Polar/Golgi-like: It can be described as discontinuous speckled or granular perinuclear ribbon-like staining with polar distribution in the cytoplasm.

Rods and rings: The staining looks like distinct rod and ring structures in the cytoplasm of interphase cells.. It is clinically associated with HCV, post-IFN/ribavirin therapy.

Mitotic patterns

Centrosome: It can be described as distinct centrioles (1-2/cell) in cytoplasm and at the poles of mitotic spindle.

Spindle fibres: The spindle fibres between the poles are stained in mitotic cells, associated with cone-shaped decoration of the mitotic poles. Spindle fibres cover both NuMA-like and non-NuMA patterns.

NuMA-like: It can be described as nuclear speckled

staining with spindle fibres. It is clinically associated with SjS and/or SLE.

Intercellular bridge or stem body or midbody: Staining of the intercellular bridge that connects daughter cells by the end of cell division, but before cell separation.

Mitotic chromosomal coat: Punctate coloration of chromosomes in pro- and metaphase with no staining of interphase cells.

International Consensus on ANA Patterns- Towards harmonisation

During the second ICAP workshop²⁴, two alternative recommendations for reporting of ANA test results were discussed. These alternatives only differed with respect to cytoplasmic and mitotic patterns being considered ANA positive or negative. In these proposals, the report should consist of 3 items: type of assay used, test result, and, if appropriate, an advice on the clinical correlation.

The item “type of assay” should specify the method used, i.e., IIF on HEp-2 cells or alternative HEp-2 substrates, addressable-laser bead immuno-assay (ALBIA), enzyme linked immunosorbent assay (ELISA). The second item of the report should contain information on the ANA test being positive or negative, the IIF pattern, and the autoantibody titre. The results should be reported in the sequence: positive/negative, pattern and titre. The nomenclature for ANA patterns should be according the consensus reached in the first ICAP workshop. The autoantibody level can be expressed as titre, fluorescence intensity or other arbitrary units. In case of mixed patterns, all nuclear patterns are reported first and next cytoplasmic, and then mitotic patterns. In the third item of the report, an advice on reflex testing in the clinical context should be given. Since the clinical associations with ANA patterns are partially defined and at best, the patterns hint at the antigens recognized and merely the cognate autoantibodies are associated with certain diseases or manifestations of diseases, it was strongly advised against providing clinical associations.

Conclusions

ANA by indirect immunofluorescence has been used for more than 50 years as an identified assay in autoimmunity and is still the “gold standard” for the screening of ANA. International Autoantibody Standardization (IAS) and International Consensus on ANA Patterns (ICAP) are involved in the standardization and harmonization of ANA testing and nomenclature. Their recommendations suggest that the results of ANA should be reported as positive or negative, type of fluorescence pattern and titre. However, further studies and discussions are needed to achieve the best outcome both in evaluation of the ANA testing as much as in their optimal assessment in context of the diagnostic approach of SARDs.

References

- Hargraves MM, Richmond H, Morton R: Presentation of two bone marrow elements: the ‘tart’ cell and the “LE” cell. *Proceedings of the Mayo Clinic* 1948;23:25-28.
- Sundberg RD and Lick NB. L. E. cells in the blood in acute disseminated lupus erythematosus. *J Invest Dermatol* 1949; 12(2):83.
- Haserick JR. and Bortz DW. A new diagnostic test for acute disseminated lupus erythematosus. *Cleve Clin Q* 1949; 16(3):158-61.
- Lee SL, Michael SR, Vural IL. The L.E. (lupus erythematosus) cell; clinical and chemical studies. *Am J Med* 1951; 10(4):446-51.
- Holborow EJ, Weir DM, Gonson GD. A serum factor in lupus erythematosus with affinity for tissue nuclei. *BR Med J* 1957; 2(5047):732-4.
- Holman HR, Kunkel HG. Science. Affinity between the lupus erythematosus serum factor and cell nuclei and nucleoprotein 1957; 126(3265):162-3.
- Friou GJ, Finch SC, Detre KD. Interaction of nuclei and globulin from lupus erythematosus serum demonstrated with fluorescent antibody. *J Immunol* 1958;80(4):324-9.
- Friou GJ. Clinical application of a test for lupus globulin-nucleohistone interaction using fluorescent antibody. *Yale J Biol Med* 1958; 31(1):40-7.
- Beck JS. Variations in the morphological patterns of “autoimmune” nuclear fluorescence. *Lancet* 1961; 1(7188):1203-5.
- Moore AE, Sabachewsky L, Toolan HW. Culture characteristics of four permanent lines of human cancer cells. *Cancer Res* 1955; 15(9):598-602.
- Keach CL, McCluskey J, Gordon TP. Transfection and overexpression of the human 60-kDa Ro/SS-A autoantigen in HEp-2 cells. *Clin Immunol Immunopathol* 1994;73(1):146-51.
- Fritzler MJ, Miller BJ. Detection of autoantibodies to SS-A/Ro by indirect immunofluorescence using a transfected and overexpressed human 60 kD Ro autoantigen in HEp-2 cells. *J Clin Lab Anal* 1995; 9(3):218-24.
- Meroni PL, Schur PH. ANA screening: an old test with new recommendations. *Ann Rheum Dis* 2010;69(8):1420-2.
- Mahler M, Meroni PL, Bossuyt X, Fritzler MJ. Current concepts and future directions for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *J Immunol Res* 2014; 2014:315179.
- Chan EK, Damoiseaux J, Carballo OG, Conrad K, de Melo Cruvinel W, Francescantonio PL, Fritzler MJ, Garcia-De La Torre I, Herold M, Mimori T, Satoh M, von Mühlen CA, Andrade LE. Report of the First International Consensus on Standardized Nomenclature of Antinuclear Antibody HEp-2 Cell Patterns 2014-2015. *Front Immunol* 2015;6:412.
- Tan EM, Feltkamp TE, Smolen JS, Butcher B, Dawkins R, Fritzler MJ, Gordon T, Hardin JA, Kalder JR, Lahita RG, Maini RN, McDougal JS, Rothfield NF, Smeenk RJ, Takasaki Y, Wiik A, Wilson MR, Koziol JA. Range of antinuclear antibodies in “healthy” individuals. *Arthritis Rheum* 1997;40(9):1601-11.
- OpDe Beeck K, Vermeersch P, Verschueren P, Westhovens R, Mariën G, Blockmans D, Bossuyt X. Detection of antinuclear antibodies by indirect immunofluorescence and by solid phase assay. *Autoimmun Rev*. 2011 Oct; 10(12):801-8
- Conrad K, Röber N, Andrade LE, Mahler M. The Clinical Relevance of Anti-DFS70 Autoantibodies. *Clin Rev Allergy Immunol* 2017; 52(2):202-216.
- Mahler M, Fritzler MJ. The clinical significance of the dense fine speckled immunofluorescence pattern on HEp-2 cells for the

- diagnosis of systemic autoimmune diseases. *Clin Dev Immunol* 2012;2012:494356.
20. Damoiseaux J, von Mühlen CA, Garcia-De La Torre I, Carballo OG, de Melo Cruvinel W, Francescantonio PL, Fritzler MJ, Herold M, Mimori T, Satoh M, Andrade LE, Chan EK, Conrad K. International consensus on ANA patterns (ICAP): the bumpy road towards a consensus on reporting ANA results. *Auto Immun Highlights* 2016;7(1): 1.
 21. Damoiseaux J, Andrade LE, Fritzler MJ, Shoenfeld Y. Autoantibodies 2015: From diagnostic biomarkers toward prediction, prognosis and prevention. *Autoimmun Rev* 2015;14(6):555-63.
 22. Agmon-Levin N, Damoiseaux J, Kallenberg C, et al. International recommendations for the assessment of autoantibodies to cellular antigens referred to as anti-nuclear antibodies. *Ann Rheum Dis* 2014;73(1):17-23.
 23. <https://www.anapatterns.org/>
 24. Chan EK, Damoiseaux J, de Melo Cruvinel W, Carballo OG, Conrad K, Francescantonio PL, Fritzler MJ, Garcia-De La Torre I, Herold M, Mimori T, Satoh M, von Mühlen CA, Andrade LE. Report on the second International Consensus on ANA Pattern (ICAP) workshop in Dresden 2015. *Lupus* 2016;25(8):797-804.