Immunofluorescence assay for autoantibodies to nuclear antigens (ANA) on HEp-2 cells

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Anti-nuclear antibodies (ANA)

Anti-nuclear antibody (ANA) testing by indirect immunofluorescence (IFT) is a fundamental laboratory method for the detection of autoantibodies in systemic autoimmune rheumatic diseases (SARD) from which clinical entities such as lupus erythematosus, mixed connective tissue disease, Sjögren's syndrome, systemic sclerosis, polymyositis, dermatomyositis and overlap syndromes are typical examples (TAN EM [1982], HOCHBERG MC [1997]).

The common name ANA (anti-nuclear antibodies) dates back to the early days of its description to be associated with clinical syndromes such as the lupus erythematosus (HOLBOROW EJ et al. [1957], FRIOU GJ [1957], HOLMAN HR and KUNKEL HG [1957], FRIOU GJ [1958], FRIOU GJ et al. [1958]). For a long time, tissue sections from rodent liver served as antigen substrate. At present, HEp-2 cells (monolayer culture of eukaryote cells derived from a human carcinoma) are in use for reasons of better reproducibility of cell consistency, staining process and staining patterns in daily routine.

The historical label "ANA" is imprecise because not only "nuclear antibodies" but also antibodies directed at various other cellular compartments are equally relevant in autoimmune rheumatic diseases. All these autoantibodies detectable on HEp-2 cells are collectively termed antibodies of the "ANA family".

ANA immunofluorescence testing can reveal a great number of antigenic entities that are useful for diagnostic purpose. ANA test strategies may vary among laboratories, but ANA testing with the immunofluorescence technique (IFT, indirect immunofluorescence test) is still the most important platform for screening of anti-cellular antibodies in suspected autoimmune rheumatic diseases (KAVANAUGH A et al. [2000], DELLAVANCE A et al. [2005], AGMON-LEVIN N et al. [2014], MAHLER M et al. [2016], HEROLD M et al. [2018]). Finally, it is essential that nomenclature and interpretation of ANA patterns follow the internationally revised recommendations (AGMON-LEVIN N et al. [2014], CHAN EKL et al. [2015].

Even if ANA testing is a milestone in the diagnosis of systemic autoimmune rheumatic diseases (SARD), ANA is not disease specific. More than about 95% of people with lupus erythematosus (LE) have a positive ANA test, and a negative test can help to exclude this diagnosis. However, one has to realize that only about 10% of people with a positive ANA result have LE, and up to 15% of healthy persons have a positive ANA test. Thus, ANA tests alone do not confirm diagnosis of LE or any other systemic autoimmune disease (TAN EM et al. [1997]).

ANA fluorescence patterns are very informative, but the pattern is not as valid as a defined disease marker. Positive ANA results and staining patterns need to be assessed in context with

the clinical issue and followed by verification of the antibody specificity by an antigen specific immunoassay, for example with ELISA or immunoblot methods (KAVANAUGH A et al. [2000], SOLOMON DH et al. [2002]. Negative test results do not need controls with the exception that a connective tissue disease is supposed to be in development or the clinical diagnosis needs a revision. Antinuclear antibodies against SS-A proteins are sometimes not detectable with ANA IFT. In diagnostic unclear situations (e.g. Sjögren's syndrome and some forms of LE) it is necessary to search for SS-A antibodies by an appropriate immunoassay.

Detection of ANA with HEp-2 cells

ANA serology with HEp-2 cells stands in the beginning of SARD diagnostics. The method of indirect immunofluorescence for ANA testing is quite laborious when compared to ELISA immunoassays, line immunoassays, bead immunoassays and others, but ANA IFT on HEp-2 cells is the standard technique as endorsed by experts (ACR American College of Rheumatology, Position statement [2015], MERONI PL und SCHUR PH [2010]). The advice gives reasons for the existence of multiple antigens within a single test assay to detect the bulk of relevant antinuclear antibodies. Thus, a number of antibody entities is detectable such as antibodies directed against components of cytoplasm, membranes and mitotic proteins being directive for the diagnosis of certain diseases.

Principle of ANA assay (indirect immunofluorescence)

The indirect IFT method meets the originally developed principle of immunohistology by AH COONS and coworkers (COONS AH et al. [1942], COONS AH and KAPLAN MH [1950]). HEp-2 cells are the preferred antigenic substrate. These cells are growing as monolayer cultured cells. They originated from a human carcinoma (HeLa contaminant, see TOOLAN HW [1954], MOORE AE et al. [1955], CHEN TR [1988]). FITC labeled goat anti-human IgG (H-chain specific) conjugates serve to detect the bound human autoantibodies. The microscopic assessment of ANA immunofluorescence is described in international recommendations (AGMON-LEVIN N et al. [2014], CHAN EKL et al. [2015], HEROLD M et al. [2018], MERONI PL et al. [2018]).

Different types of ANAs give rise to typical staining patterns in the microscope. IFT patterns enable one to classify antibodies in a patient sample. Yet, dedicated IFT patterns (with rare exceptions) are not evidence for a defined disease marker. Positve ANA stainings require assurance by selective antigen specific immunoassays (AGMON-LEVIN N et al. [2014]).

Specimen type

Human serum or plasma,

1. Minimum quantity: 10µL
2. Stability: 8 days at 2 – 8 ° C. For longer storage, samples are maintained deep-frozen at – 20 ° C. Repeated thaw-freeze cycles must be avoided
3. Disturbing factors: haemolytic, lipemic, icteric samples, visible impurities, contamination by bacterial growth

Reference values

1. Negative: No reaction at serum dilution 1:80, ANA not present
2. Positive 1:80 Borderline titer of ANA, and further sample dilution needed
3. Positive > 1:80 Serial dilution of sample, and final titration value reported

Reagents, materials, equipment

1. Reagents
   - Antigen target: microscopic slides loaded with HEp-2 cells and frozen cut liver sections (BioCHIPS [Euroimmun], IVD certified)
   - FITC labeled goat anti-human IgG antibodies (IVD certified); dilution factor is marked on each batch. Otherwise, conjugates need to be titrated for optimal dilution
   - Positive control sera: human sera containing autoantibodies against cell nuclei with defined staining pattern (IVD certified). From each new batch (lot-number), the antibody titer is controled on arrival according to the package leaflet and followed by monthly controls. Aliquots are customized in small vials and stored frozen at – 20 º C until use. The day of testing, one aliquot is thawed and a fresh dilution is prepared with PBS-Tween buffer
   - Negative control serum: human serum, antibody free (IVD certified)
   - PBS-Tween: buffer salts for preparing phosphate buffered saline pH 7.4 (PBS) and Tween 20 (products IVD certified)
   - Ready-to-use embedding medium (IVD certified)
2. Materials
   - Eppendorf vials
   - Object tray
   - Washing tray, e.g. Coplin jar
   - Pipette-tips, combi-tips
   - Cover glass
3. Microscopy
   - Fluorescence microscope (epifluorescence), objective lens magnification 20 x and objective lens magnification 40 x
   - Special filter sets for FITC (fluorochrome)
   - Illumination unit for FITC fluorescence
   - Correct alignment of light path
   - Microcopy in a dimmed room (dark room)

Quality aspects, controls

Reproducible results depend on standardized procedures with correct reagents, dilutions and appropriate equipment. Diagnostic test results must fulfill the criteria of positive and negative reactivities by use of defined control samples as function controls (MOLDEN DP et al. [1984], SMOLEN JS et al. [1997], SOLOMON DH et al. [2002], AGMON-LEVIN N et al. [2014]).

1. Serological controls
   - Microscopical slides are reacted with positive and negative control sera for internal controls. The participation in external control programs is obligatory

- Positive control: a panel of sera with various defined staining patterns and known titer values are used (established on HEp-2cells)
- Negative control: an antibody free and ready-to-use serum serves as negative control

2. HEp-2 cells
   - From each lot, samples are allocated for examination of cell density, mitosis per field of view and fluorescent staining pattern by testing with defined control sets
3. Microscopy of IFT preparations
   - Slides are first viewed at 200 x magnification to assess the reactivities, then 400 x magnification is used to judge the IFT patterns
4. Personal qualification
   - Basic knowledge in microscopy/fluorescence microscopy is required
   - Basic knowledge is reinforced by training in a reference laboratory
   - Staff: constant checks of competence by yearly executed tests with defined preparations. Reference slides with distinct immunofluorescence patterns are obtained from a reference laboratory

Immunofluorescence, staining procedure

Prior to testing, all reagents and slides have to reach room temperature (18 – 22 ° C). Incubation procedures follow a standardized schedule, during all steps the EUROIMMUN Titerplane™-Technik is used.

1. Screening of patient sample
   - Patient sera are pretested at a dilution of 1:80 in PBS-Tween buffer
   - All positive screening tests are further processed by serum titration
2. Dilution of patient sample for titration
   - The needed number of test tubes is calculated from the fluorescent strength in the screening assay (f.e. n = 5); the vials are numbered consecutively
   - Each test tube contains a defined buffer volume (100 µL). Then, an equal volume (100 µL) from the screening test tube is transferred into the first test tube of this dilution panel; all test tubes are carefully mixed
   - From the first tube of this dilution panel, a defined volume (100 µL each) is transferred to the second tube. After thorough mixing further dilutions are prepared in the same manner
3. Incubation and washing schedule for ANA staining
   - Incubation of slides with diluted specimen sample (apply 25 µL to each field) for 30 min at room temperature (18 – 25 ° C) in a humidified incubator (wet box)
   - Rinse (quick dipping in PBS/Tween) and wash in PBS/Tween buffer for 2 x 5 min
   - Incubation with diluted FITC labeled goat anti-human IgG antibodies (apply 25 µL to each field) for 30 min at room temperature (18 – 25 ° C) in a humidified incubator (wet box)
   - Rinse (quick dipping in PBS/Tween) and wash in PBS/Tween buffer for 2 x 5 min
   - Slides are mounted under coverglass with ready-to-use embedding medium
   - Optional step: counterstain slides for 5 min with Evans blue 0.1% (w/v) in PBS buffer, rinse in PBS, drain and mount under coverslip
   - Protect slides from light, store in the dark (black box)
   - Slides are inspected with a fluorescence microscope

Microscopic records, medical reports

Microscopic results are logged in the worksheet. Any doubtful findings are counterschecked by a second person of experience.

Stainings are quoted using to the ICAP nomenclature (AGMON-LEVIN N et al. [2014], CHAN EKL et al. [2015]); relevant and defined HEp-2-cell staining patterns possess an alphanumeric code from anti-cell **AC-1** to anti-cell **AC-28**. The classification algorithm denominates three main groups (www.ANApatterns.org):

1. Nuclear pattern (AC-1 to AC-14)
2. Cytoplasmic pattern (AC-15 to AC-23)
3. Mitotic pattern (AC-24 to AC-28)

Recently, a further distinct ANA pattern (ANA pattern associated with antibodies to DNA topoisomerase I) was defined as **AC-29** and added to the above algorithm (ANDRADE LEC et al. [2018]).

Microscopic results from the worksheet protocol are entered into a computer file. The mask contains several entry fields and the possibility to select from predefined result options. A second person controls the inputs.

Result examples:

1. ANA negative: no defined staining pattern is observed, no further result options
2. ANA positive: by entering “positive”, the screen mask opens a submenu for options such as
   - Nuclear pattern: f.e. *nuclear homogenous (AC-1)*
   - Antibody titer: f.e. *1:1280*
   - Second nuclear pattern: f.e. *nuclear fine speckled (AC-4)*
   - Second antibody titer: f.e. *1:2560*
   - Cytoplasmic pattern: f.e. *cytoplasmic speckled (AC-20)*
   - Mitotic figures: f.e. *spindle fibers (AC-25)*

Patient cases are finally validated (medical validation). Text modules can be changed, completed or newly formulated if necessary. The significance of microscopic findings is evaluated.

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