NOW AND THEN

Antinuclear antibody determination in a routine laboratory

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Abstract
Pitfalls in the method for demonstrating antinuclear antibodies (ANA) by the indirect immunofluorescence technique are described and the use of international standard preparations outlined. Determination of the optimal border dilution dividing positive from negative results is discussed.

Each laboratory is a unique setting; it must define its own method, which should rarely be changed. One should not rely on copying methods from other laboratories or commercial firms, but the reproducibility of the nuclear substrate, the conjugate, and other variables should be controlled daily by the use of a control serum which has been related to the WHO standard preparation for ANA of the homogeneous type. Since many sera contain mixtures of different ANA, the results of routine tests are best expressed in titres or expressions of the intensity of fluorescence. The ANA test using the immunofluorescence technique should be used as a screening method for other tests allowing a more defined interpretation of the ANA.

Each laboratory should individually determine the border between positive and negative results. Therefore about 200 sera from local healthy controls equally distributed over sex and age, and 100 sera from local patients with definite SLE should be tested. Since the local clinicians should become acquainted with this border it should rarely be changed.

Finally each laboratory should participate regularly in national and international quality control rounds, where sera known to be difficult to interpret are tested. The judgement of the organisers of these rounds should stimulate improvements in the participating laboratories.

(Ann Rheum Dis 1996;55:723-727)

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In this article I shall try to describe problems which might arise when performing the immunofluorescence technique (IFT) for demonstrating antinuclear antibodies (ANA) in a normal routine laboratory for clinical immunology. It is not the purpose to describe "the ideal or optimal method" since most variations on the method originally described by Holborow et al [1] and Friou [2] are of minor importance. Those who are setting up a new laboratory and want to install the immunofluorescence technique for ANA determination in a modern way are referred to the recent article by Humbel. [3]

It is obvious that the results of the ANA test should be reproducible and in agreement with the results of other laboratories. Therefore I shall also discuss the use of standards and the need for quality control in this article.

Whatever method used, the purpose of ANA determination is generally to screen patients suspected from generalised autoimmune diseases, that is, systemic lupus erythematosus (SLE), rheumatoid arthritis, Sjögren's syndrome, scleroderma, polymyositis, or mixed connective tissue disease. Clinical and paraclinical studies are needed to reach a definitive diagnosis. In the ideal situation nearly all SLE patients are ANA-positive and nearly all healthy subjects ANA-negative. Therefore I shall also discuss the way to decide which ANA titre should be considered positive and which negative.

Pitfalls of the immunofluorescence technique for ANA determination
The variable factors in ANA determination with the immunofluorescence technique are:

(1) Nuclear antigen substrate.

(2) Specificity and avidity of the ANA.

(3) Specificity, avidity, fluorochrome labelling, and concentration of the conjugate.

(4) Incubation conditions, washing, and mounting.

(5) Microscopy and reading.

NUCLEAR SUBSTRATE
In principle all nuclear substrates can be used for ANA determination. In daily practice, however, it is evident that there are differences between the substrates. These differences hardly arise if only sera from SLE patients are tested. Such sera react with most substrates. In the same way, most sera from healthy control subjects are negative with all kinds of substrates. If, however, sera from patients with other autoimmune diseases, like rheumatoid arthritis, chronic active hepatitis, or myasthenia gravis, are tested, many differences are

Sera from 86 patients with myasthenia gravis tested for ANA in one laboratory on seven different substrates: human salivary gland (Hu sal), human thyroid (Hu thy), human adrenocortex (Hu adr), rat kidney (Rat kid), rat stomach (Rat sto), rat liver (Rat liv) and rat diaphragm (Rat dia). The results were compared to the LE-cell test and anti-dsDNA determinations with the IFT on Crithidia luciliae (aDNA Crit) and the Farr assay (aDNA Farr).

No of sera	Hu sal	Hu thy	Hu adr	Rat kid	Rat sto	Rat liv	Rat dia	LE cell	aDNA Crit	aDNA Farr
1	+	+	+	+	+	+	+	+	−	−
1	+	+	+	+	+	+	+	−	+	−
2	+	+	+	+	+	+	+	−	−	−
1	+	+	+	+	+	+	−	−	+	−
1	+	−	+	+	+	+	+	+	−	−
2	+	+	+	+	+	−	+	−	−	−
1	−	+	+	+	+	−	+	+	−	−
1	+	+	+	+	−	−	+	−	−	−
1	+	+	+	−	−	−	+	−	−	−
1	+	−	+	−	+	−	+	−	−	−
2	−	+	−	+	+	+	−	−	−	−
1	−	+	+	+	+	−	−	−	−	−
1	−	−	+	+	+	+	−	−	−	−
2	+	+	+	−	−	−	−	−	−	−
1	−	+	−	+	−	−	+	−	−	−
1	−	−	−	+	+	+	−	−	−	−
1	+	+	−	−	−	−	−	−	−	−
3	+	−	+	−	−	−	−	−	−	−
1	+	−	−	+	−	−	−	−	−	−
3	+	−	−	−	−	+	−	−	−	−
1	+	−	−	−	−	−	+	−	−	−
1	−	+	−	+	−	−	−	−	−	−
1	−	+	−	−	+	−	−	−	−	−
1	−	+	−	−	−	+	−	−	−	−
2	−	−	−	−	−	+	+	−	−	−
2	+	−	−	−	−	−	−	−	−	−
3	−	+	−	−	−	−	−	−	−	−
1	−	−	+	−	−	−	−	−	−	−
1	−	−	−	+	−	−	−	−	−	−
1	−	−	−	−	+	−	−	−	−	−
44	−	−	−	−	−	−	−	−	−	−
86	24	23	20	19	17	16	15	3	2	0

observed. In sera from such patients the titre of the ANA is generally about 10-fold lower than in sera from SLE patients. When we tested sera from patients with myasthenia gravis on different nuclear substrates, a complete disorder resulted (table).[4]

The conclusion should be that no substrate is perfect. In the past human leucocytes in blood smears and rat liver tissue were mostly used. Autoantibodies to neutrophils, however, make ANA recognition difficult if leucocytes are used as the only substrate. Now most laboratories have switched to cultured cell lines. The latter nuclei are somewhat easier to read and the distinct fluorescence patterns somewhat more easily recognisable. The most important advice is: *choose one substrate and stick to it.* The technicians and, what is more important, the clinicians will learn to interpret the results and their shortcomings and will get used to them.

SPECIFICITY AND AVIDITY OF ANA

Many different ANA can be demonstrated with the immunofluorescence technique. A problem is that in sera from patients with generalised autoimmune diseases several of these antibodies may be present in high titres at the same time. Since the titres of these antibodies nevertheless show mutual differences, titration of the serum to study can reveal a certain type of antibodies showing a certain pattern of nuclear fluorescence.

Homogeneous patterns are frequently observed in patients with SLE and rheumatoid arthritis, speckled or granular patterns in

patients with Sjögren's syndrome or scleroderma, and nucleolar fluorescence in patients with progressive systemic sclerosis. However, these associations are of limited value for the clinician.

If the immunofluorescence technique for ANA determination is used, as it should be, as a screening method, other techniques like double immunodiffusion, counter immunoelectrophoresis, immunoblotting, enzyme linked immunosorbence (ELISA), or Farr assays can reveal antibodies of much greater clinical significance, such as anti-dsDNA, antiSm, anti-nRNP, anti-Ro(SS-A), anti-La(SS-B), anti-topoisomerase I (Scl 70), or anti-Jo1.

Only the discrete speckled pattern of nuclear fluorescence is informative, since the observation should lead to the performance of the immunofluorescence technique on mitotic cells, which enables antibodies to centromeres to be demonstrated. These antibodies show a strong association with limited cutaneous scleroderma, which was earlier called CREST syndrome (calcinosis, Raynaud's phenomenon, oesophageal dysfunction, sclerodactyly, telangiectasia).

SPECIFICITY, AVIDITY, AND CONCENTRATION OF THE CONJUGATE

Nobody likes to miss ANA of a certain isotype. Therefore many laboratories prefer a conjugate which is directed against all classes of immunoglobulins. Since all important ANA are present in the IgG class, an antihuman IgG conjugate is normally sufficient. Furthermore Humbel warns that IgM ANA and IgA ANA frequently occur in sera from healthy subjects.[3] This is not, however, our experience.

The specificity and avidity of each new batch and each new substrate should be compared with a national standard antihuman Ig conjugate. Such a national conjugate should have been compared with the WHO FITC conjugated sheep antihuman Ig international standard (480010).[5] Such a comparison should be obtained by performing a block or chessboard titration of twofold dilutions of both the national and the WHO conjugate against twofold dilutions of a local ANA-positive control serum. These block titrations are described and discussed in detail by Beutner *et al*,[6] Feltkamp,[7] and Johnson *et al*.[8]

The optimal concentration of the conjugate *in your laboratory and for your substrate only* is usually a concentration which is two steps of a twofold dilution series higher than the so called plateau endpoint. Never rely on well meant advice such as: "We have a good experience with this conjugate at a dilution of 1:50". Never think that if, for instance, in your hands a conjugate is doing well at a dilution of 1:80 for the demonstration of autoantibodies against human thyroid tissue, it can be used in the same dilution on HEp-2 cells for the demonstration of ANA.

Since most laboratories now use commercial conjugates, the important variable of the fluorescein/protein ratio of conjugates will not be discussed here. It is sufficient to state that

F/P ratios between 2 and 3 generally give a satisfactory positivity and a low background staining.[9] If a laboratory is satisfied with a certain batch of a conjugate, it should try to obtain a sufficient amount of it for the coming years.

INCUBATION, WASHING, MOUNTING

These procedures have been recently described by Humbel.[3] It suffices here to underline in particular that the performance of a large number of tests at the same time might prolong the period during which diluted serum samples sit on the slide. This leads to evaporation of serum or phosphate buffered saline (PBS) before the actual incubation period is started. Such drying increases the salt concentration, leading to elution of antigens or antibodies. Also the pH of the PBS should be controlled regularly. Evaporation of CO_2 might increase the pH of sera and buffers, thereby promoting the elution of anti-DNA antibodies. Most of us are so used to acid elution that we forget that anti-DNA dissociates from DNA mainly at high pH.[10]

MICROSCOPY AND READING

This is not the place to discuss the optimum fluorescence microscope. Nevertheless some general remarks will be made. A microscope with epi-illumination and interference filters is preferable. Alignment of light source and diaphragm and lenses is of utmost importance. The objectives should have the highest possible numerical aperture, even if this lowers the image quality at low magnifications. The magnification of the eyepieces should be as low as possible. Of course the interference filters should be optimal for the fluorescent dye of the conjugate.

Semiquantitative results can be obtained by the performance of a twofold dilution series, expressing the results as a titre or in international units (IU) per ml. The performance of the IFT on dilution series is time consuming and expensive. Many laboratories prefer, therefore, to obtain semiquantitative results by testing only one or two serum dilutions—for example 1:20 and 1:160—and further quantifying the antibody content of the serum by indicating the intensity of the fluorescence, for example, negative -, dubious (+), weak positive +, positive ++, or strong positive +++. The dubious results should be repeated until a decision between (weakly) positive and negative can be made.

The use of standards in the ANA determination

To control the reproducibility of the ANA determination, the same positive and negative control serum must be included every day. The positive control serum has to be related to a national standard for ANA of the homogeneous type, in the way recently described.[11] This national standard should have been referred to the WHO standard preparation for ANA of the homogeneous type (66/233). How this should be done and where this standard and other standard preparations can be obtained was recently described.[11][12]

It is evident that the daily use of a standard preparation leads to an increase in reproducibility in a particular laboratory: all the results of one day should be corrected if the standard serum shows up to a twofold aberration in the regular titre of this serum, and all results of the day should be abandoned and the tests repeated if the aberration is more than twofold. It is open to discussion whether the results of all ANA determinations in a routine laboratory should be expressed in titres or in IU ml⁻¹. If all sera to be tested only had ANA of the homogeneous type the answer would be simple since expression in IU ml⁻¹ would decrease the variability between the results of different laboratories. The figure, taken from a study by the Dutch working group on standardisation of rheumatoid serology, shows an example of the decrease of interlaboratory variations if the results of an ANA determination of the homogeneous type were expressed in IU ml⁻¹ instead of titres.[13]

Unfortunately in daily practise not all ANA are of the homogeneous type. It would be confusing for a clinician if one result of an ANA determination was expressed in IU/ml and another in a titre. In conclusion, therefore, it is recommended to use the ANA standard for the homogeneous type to standardize the positive control serum in a laboratory, but to express the routine results in a titre or as the intensity of the fluorescence at a certain dilution.

Besides the quantitative international standard for ANA of the homogeneous type, prepared and distributed under the auspices of the WHO, qualitative reference sera for some other types of ANA also exist. These reference sera were made available by the American Arthritis Foundation, together with the Centers for Disease Control (CDC). The types of nuclear fluorescence which they distinguish are: ANA speckled (AF/CDC 3), ANA nucleo-
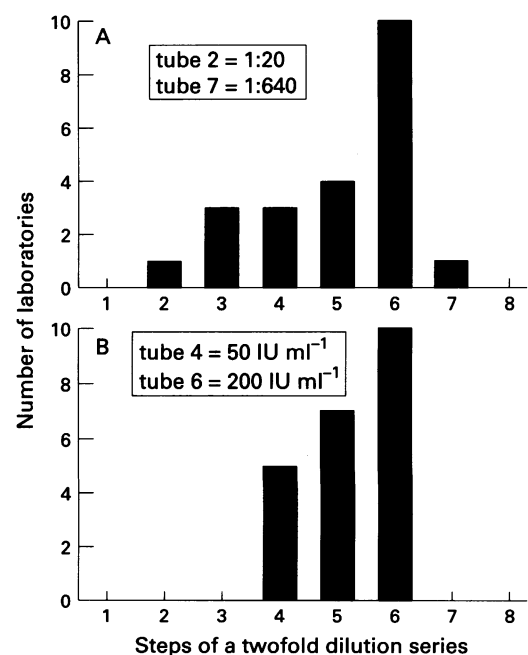
Results found by 11 laboratories testing serum for antinuclear antibodies (homogeneous) on HEp2 cells on two occasions: top, expressed as titres; bottom, expressed as IU ml⁻¹.

lar (AF/CDC 6), and ANA centromere (AF/CDC 8). With these standards laboratories can check their local standards for these types of nuclear fluorescence. The IUIS/WHO/ILAR subcommittee for ANA standardisation considers these reference preparations to be of great importance. Further details and indications how they can be obtained were recently mentioned.[11] [12] [14]

Determination of the border between positive and negative

All normal healthy individuals have ANA. This becomes evident when you observe the results of your own serum in a 1:10 dilution in the western blotting technique with nuclear antigens. Since not all healthy individuals are as normal as you are, it is generally accepted that a certain test should give negative results in at least 95% of healthy normal controls. This can easily be achieved by increasing the dilution to be considered as the border between positive and negative.

On the other hand, we want nearly all sera from SLE patients to give positive results. If the diagnosis of SLE was based on the presence of ANA, like that of the mixed connective tissue disease on the presence of anti-nRNP, this would never be a problem. Fortunately this is not the case. Now the border dilution between positive and negative has to be chosen at the dilution at which at least 95% of the patients with definite SLE (ANA thus not forming a part of the criteria) is ANA positive.

It is evident that the two above paragraphs in fact discussed the terms diagnostic "specificity" (per cent negative controls) and "sensitivity" (per cent positive patients). Both these figures should be as high as possible.

A complication is the well known fact that ANA are far more frequent in healthy females than in healthy males and in elderly females than in young females. The border dilution should therefore certainly not accept positive healthy young boys and be less stringent on healthy old women.

Finally, it should be kept in mind that the border dilution settled in one laboratory referring to a certain population cannot be copied for another laboratory which not only serves another population but also performs the ANA test and readings in a somewhat different way. Fritzler *et al*,[15] indicating that in their hands a 1:40 dilution of the patients serum is optimal for screening for ANA on HEp-2 cells, underlined the fact that due to interlaboratory variations in the test conditions this border has no general validity.

In practice the following method for the determination of the border between positive and negative ANA test results is advocated:

> Ask your local blood bank for sera from normal donors. These sera should represent 10 samples of males between 20 and 24 years of age, 10 between 25 and 29, etc, up to 60-65 years. To these 90 sera should then be added 90 sera from female donors of the same age groups. The border dilution preferably allows negative results in over 99% of the males and over 95% of the females under 45 years of age. Assemble the names of the clinicians who referred 200 sera to your laboratory for ANA determination and where the result was in your opinion positive. Ask these clinicians to complete a questionnaire according to the revised criteria for SLE.[16] The sera from the patients fulfilling three of the first nine non-serological criteria can now be used to settle your border dilution for the ANA test. If the number of sera fulfilling the SLE criteria is less than 100, the study should be extended. Finally the choice of the border dilution should result in an ANA positivity of at least 95% of the patients with definite SLE.

When performing ANA determinations in a routine setting, it is of the utmost importance to realise that the test is mainly meant as a screening method before other more defined serological tests are performed. Patients with definite SLE who are ANA-negative exist, but if you find them in over 0.5% it is recommended that you restudy the method even though, as stated above, statistically 5% is acceptable. On the other hand you should inform your clinicians that a positive ANA test in a woman over 60 is not alarming, in contrast to positive result in a boy.

Each laboratory is a unique setting. This means that each laboratory, possibly together with the referring clinicians, should have its own policy on the performance of the test and the choice of the border between positive and negative results.

Participation in rounds for quality control

It is evident that the laboratory should be well equipped and that the rules for good laboratory practice (GLP) are respected. A protocol should indicate that each major change in the performance of the method, that is, a new cell line as substrate, another conjugate, another microscope, but also a new technician or a move to another building, should lead to intensification of quality control activities along the lines given above.

Of the greatest importance is regular participation (at a minimum once in the six months) in quality control rounds organised by external organisations. Such organisations will ask your laboratory to examine some known positive sera (sometimes representing hidden dilution series) and negative controls. Sera known to be difficult to interpret should also be included.

If for practical reasons your laboratory cannot participate in such externally organised quality control rounds, it is good to ask a colleague from another department in your organisation or hospital to act as a "fake doctor", sending by external post from time to time "blinded" samples of sera that you provided yourself for this purpose. If this fake doctor also asks difficult questions over the phone or in writing you will learn lessons on how to improve the organisation and quality of your laboratory for routine ANA determination.

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