GUIDELINES FOR ANTINUCLEAR ANTIBODY TESTING

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12.1 Introduction

Testing for autoantibodies has become an important part of clinical diagnostics, estimation of prognosis and, thus, planning of follow-up and possible therapeutic approach. Finding a strongly expressed autoantibody in early disease where the full spectrum of clinical manifestations is yet not present can guide further exploration to reveal subclinical tissue or organ involvement and thus give a more precise overview of the incipient disease.

Some autoantibodies represent more characteristic predictors of a certain disease than a particular clinical manifestation or histopathological finding. This fact particularly pertains to the disease-specific autoantibodies. Nevertheless all positive autoantibody finding need to be set into a clinically meaningful context to be useful for clinical diagnostics. With the advent of many new and very sensitive technical platforms and assay formats for detecting autoantibodies clinicians and laboratory scientists need to collaborate closely to reveal the clinical usefulness before results coming from a new technology can be as certain and informative as the results derived by use of classical technologies (e.g. double immunodiffusion, counter-immuno-electrophoresis, passive haemagglutination etc.). Such thorough work on clinical utility must precede any introduction of new technologies and assays for diagnostics in a laboratory.

12.2 The use of antinuclear antibodies (ANA) in rheumatology

The most indispensable parts of clinical diagnostics relate to the clinical history, family history, and manifestations found clinically. Diagnostic aids such diagnostic imaging, histopathology/ immunopathology, simple laboratory tests to detect signs of inflammation, autoantibody testing and specialist evaluations are secondary to the clinical setting found at presentation. The use of one or a few screening tests - rationally ordered after setting a tentative diagnosis - can lead to low cost but high quality diagnostics. Simple screening for ANA using indirect immunofluorescence technique (IF) and a sensitive cellular substrate is an appropriate strategy in unfolding clinical and laboratory diagnostics. A positive result can lead to exploration for antibodies known to be important for that particular diagnosis and for the IF result found. Though the term ANA relates to autoantibodies directed to nuclear antigens only, the term is very commonly used in a broader sense to describe any antibody giving rise to a positive staining pattern on a cellular substrate (i.e. including those that target cytoplasmic structures). In this presentation this broader definition of ANA will be used. The most

popular cellular substrate used for such ANA screening today is the human epithelial cell line HEp-2 cells derived from a laryngeal carcinoma, and the preferred conjugate used for visualization of antibody binding is specific for human IgG.

12.3 ANA screening using HEp-2 cells.

ANA can roughly be divided into those that recognize antigens in five different regions of the cell: the nuclear envelope, the nucleoplasm with its organelles, the nucleoli, the mitotic spindle apparatus and the cytoplasm with its organelles. In the following I will thus use the term ANA for all of the antibodies that can be seen by IF testing using HEp-2 cells. Although the cell contains thousands of different proteins only very few of these have been found to have autoantigenic properties. The reason why cellular proteins are turned into autoantigens are partly unknown, but events taking place during inflammation and cell death seem to cooperate with a number of genes in causing this antigen transformation.

The precise recognition of a particular well-defined HEp-2 cell staining pattern on one hand can lead the laboratory scientist to determine the most likely autoantigens recognized and on the other hand indicate known relationships to a limited number of likely diagnostic entities. In this way a particular positive ANA screening result can guide further specific ANA testing but also be useful for unravelling a precise clinical diagnosis/ prognosis.

Some laboratory scientists have stated that the precise categorization of an IF staining pattern cannot be reached at by most laboratory technicians, but this is clearly wrong. With the use of reference images and unique terms for each pattern, precisely defined by a team of experts, can result in the development of very accurate recognition skills in most laboratory workers as proven by international multi-centre studies. Among the multitude of clearly defined IF patterns seen in a clinical immunology laboratory, the majority can be used directly by clinicians to promote diagnostic work-up if the laboratory has the ability to explain the most likely clinical associations seen with a positive screening ANA result. The majority of these patterns can not be detailed further by specific ANA testing using available routine enzyme-immuno-assay (EIA) technology since either the autoantigen is not clearly known or it is not available in a form that can be used in presently used kit formats. Among the many ANA patterns known only around 10-12 specific ANA targets can be detected in an EIA, immuno-blotting or line-immuno-assay format.

12.4 Use of ANA for diagnosis and estimation of prognosis

It is well known that some ANA are used as diagnostic criteria as part of a systemic rheumatic disease diagnosis e.g. systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD), Sjögren's syndrome (SjS), but other ANA serve as an important diagnostic support for diagnosis e.g. scleroderma (SSc), poly-/dermatomyositis (PM/DM), secondary SjS, secondary anti-phospholipid antibody syndrome (APAS), and juvenile chronic arthritis (JCA) (Table 1).

Table 1. *ANA as diagnostic criteria or support for diagnosis in rheumatic disease.*

Disease	Criteria	Support
SLE	ANA, anti-dsDNA, anti-SM	
MCTD	Anti-U1RNP (high titer)	
SjS	Anti-SSA(Ro)/-SSB(La)	
SSc		Anti-centromere, -topo I etc *.
PM/DM		Anti-tRNA synthetases etc. *
2^0 APAS		ANA, anti-dsDNA etc. *
JCA (oligoarticular)		ANA *

*See text about the various ANA.

It is assumed that the ANA found in a patient with any of these diseases somehow reflect genetic predisposition and lesional pathology in a particular individual. Since involvement and severity of different organs is directly related to disease prognosis the revelation of a particular ANA in a patient can guide the clinician in the follow-up and surveillance of incipient organ manifestations so that rational therapy can be instituted early.

A specially illustrating example of such relationships is SSc, where anti-centromere antibodies mostly are associated with a slowly developing form of limited SSc which has a good long-term prognosis, while anti-topoisomerase I (anti-Scl-70) antibodies relate to a more rapidly progressing form of diffuse SSc commonly complicated by fibrosing alveolitis and a more cumbersome prognosis. Anti-RNA polymerase I antibodies have been found to be associated with a particularly severe form of rapidly progressing diffuse SSc, that commonly involves the kidneys and manifests with malignant hypertension, cardiovascular disease and cerebral infarctions. In SSc patients that harbour anti-U1RNP antibodies the disease is practically always overlapping with another immuno-inflammatory rheumatic disease e.g. SLE, PM/DM or MCTD, and the prognosis may be very different from case to case. Similar clinical subsyndromes have been found in SLE, primary SjS, PM/DM, JCA. Each subsyndrome is thus associated with presence of a particular specific ANA, and the nature of that ANA should be revealed if at all possible.

Some ANA can be difficult to reveal with certainty, probably due to different properties or different epitope specificities seen in relationship to a njumber of diseases. Nevertheless, credible results are absolutely necessary in order to allow a meaningful use of the serologic information. A typical example is that of anti-double stranded DNA (-dsDNA), where independent studies have reached the same conclusion, i.e. anti-dsDNA that are characteristic for SLE can only be disclosed by using at least two different methods for their demonstration.

This may be explained by the fact that production of some types of anti-dsDNA can be a

normal response to certain infections or to any type of long-standing tissue injury. In our laboratory we have reached at a strategy where we start screening for anti-dsDNA using an EIA that is known to be broadly reacting and thus has a low specificity for SLE, but then a positive result is followed up by use of a Crithidia luciliae IF test which is highly specific for SLE if found positive. Only a if a positive IF test is found we report anti-dsDNA to be present. A positive result of the EIA only is not reported as positive. With that approach we have shown that the sensitivity in SLE patients is around 45-50 % and the diagnostic specificity around 97 %.

12.5 How can we judge the clinical utility of results from solid phase assays?

It is clear from many reports that a positive test for ANA using EIA or some other solid phase based technology does not correspond well to what is found by the classical double immuno-diffusion or counter-immuno-electrophoresis techniques which rely on presence of precipitating antibodies. Before one can use results from such solid phase assays in clinical work-up it is necessary to explore the diagnostic potential by studying sera from local populations of immuno-inflammatory diseases. Healthy donor controls cannot be used for a clinically meaningful cut-off setting. Results derived from the study of a prototype disease must be compared with those of inflammatory disease controls that manifest features somewhat similar to the prototype disease ("critical controls"). By constructing receiver-operation curves and choosing a preferred high level of specificity cut-off can be set. After that the sensitivity for the prototype disease can be seen from the curve. By setting a similar high level of specificity for diagnosis different assays for the same antibody can be rationally compared. Tests that are used to establish diagnosis need to have a high diagnostic specificity whereas the sensitivity is less important. Even rarely found ANA relate to a particular subsyndrome and prognosis.

It is very important to prove the value of a test for early diagnostics. In early disease a positive ANA result has a relatively higher impact on clinical decision-making than later in follow-up. The most informative ANA results are those that are unique to one diagnostic entity (disease-specific).

12.6 What should be done to establish serologic positivity in borderline cases?

Since the early start of the European consensus studies the recommendation has been to confirm or refute doubtful borderline results ("grey area" results) by e.g. performing two different techniques. Alternatively one can agree with clinicians to report such results with a written "caveat notice" that the result cannot be used with confidence for differential diagnostics. Another possibility is to agree on calling all such results "negative". This has to be discussed between laboratory personnel and clinicians, so the policy is always the same.

12.7 Use of algorithms

Collaboration between clinicians and laboratory scientists may also lead to agreement on the use of practical algorithms for test ordering, for rational stepwise exploration of

a preliminary result at screening, and for interpretation of a positive final result. As an alternative to an algorithm for test ordering it may be practical to set up the order form in such a way that the doctor can tick either a tentative diagnosis or one or more single tests (Fig 1). Thereby the flexibility of test ordering is maximal and people who may be uncertain about which tests will be rational to do can learn from the form.
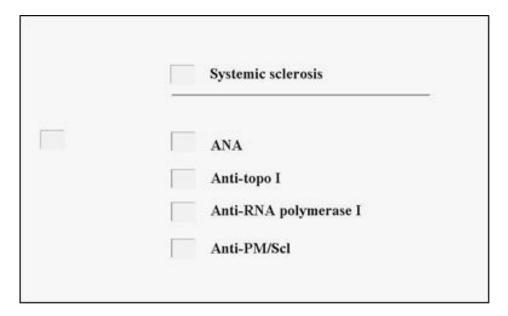

Figure 1. Choice of test packages or single specified orders on test order form.

12.8 Use of international serum standards in the laboratory

The IUIS/WHO/CDC/AF International Committee on Standardization of Autoantibodies in Rheumatic and Related Diseases have established a repository of well-characterized freeze-dried sera ampoules into glass vials as standards or alignment tools for producing national and local serum standards. These standards can be ordered free of charge from Center for Disease Control in Atlanta, GA. Until now 14 different standards are available and in the coming year more standards will be made available.

12.9 Efforts to harmonize clinical/laboratory collaborative work

For five years annual meetings have been organized in the Nordic countries to make clinicians aware of the importance of collaborating with clinical immunology laboratories to optimize diagnostics and make the diagnostic process more appropriate for differential diagnostic use.

Clinicians and laboratory scientists have discussed a number of items that are handled very differently in different centres with the aim to harmonize such activities to the benefit of the end user, the patient. This led to the formation of a European steering group of leading scientists in rheumatology, called EASI (European Autoimmunity Standardization Initiative). Senior people from rheumatology and clinical immunology are now being recruited from hopefully every European country with the task to plan national discussions of the items laid forward as suggestions from the steering committee, hoping that controversies between different country policies can be bridged

and the suggested measures amended in such a way that all nations get a unified concept of how to interact across each country. The final plan is to have European open meetings where these plans and strategies are presented by all national delegates for fruitful discussions. The 5th International Autoimmunity Congress in Sorrento next year will set up such a general session for interested parties.

12.10 Modern technical platforms and new assays.

Many new assay platforms and new technologies to detect and quantify specific ANA have been introduced by the industry, now is the time to find out what should be their role in future autoimmune serodiagnostics using the strategy outlined above. There is no doubt that precision and speed of testing can be made much better with automation, but that is just a small part of rationalizing laboratory work and may not contribute to better diagnostics. We need to know the clinical implications of getting positive results that are not substantiated by IF methodology or precipitation techniques. We need to have many more autoantigens ideally expressed on solid phases (addressable laser bead assays, multiplex assays etc.) so that true pathological ANA are binding but polyreactive low affinity (diagnostically unimportant) ANA are not. We also need to look at the possible value of quantitating various ANA as part of disease surveillance, an area of research that has been much neglected until now. Hopefully we can also start to look at pathobiological effects of certain ANA (e.g. the complement-fixing properties of lupus-related ANA) as compared to the same ANA specificity in other diseases. We know very little about the ANA found in inflamed tissues and fluids compared to the corresponding serum ANA. Until now there are no indications that high quality detection of ANA using a solid phase principle can take the place of HEp-2 cell ANA demonstration by IF, and there are multiple reasons for that.

12.11 Important issues in health cost estimation

Many scientists have wondered how to handle the increasing complexity and demand for autoimmune serodiagnostics. Many have switched from manpower-dependent to automated technical platforms trying to keep short-term costs low. One needs to realize that health costs are very low in the early phase of chronic diseases, total laboratory costs mounting to 2-3% of the patient-related costs in Sweden, whereas the heavy costs arrive during the later phases of such diseases. These long-term costs are dependent on many factors some of which are number of visits to clinics, length of stay and cost of stay in hospital, readmission rate, working days lost for the patient and family, productive years gained, economic compensation for inability to work etc. The best way to cut these long-term costs is to set an early diagnosis through the use of optimal clinical/serological diagnostics, making decisions about interventions as rational as possible and thus effect ultimate outcome.

12.12 Conclusions

ANA most likely reflect tissue lesion mechanisms, genetic predisposition and perhaps etiology, are associated to diagnosis, subsyndrome categorization, and prognosis, may help planning of clinical follow-up and therapeutic strategies, are of particular value in

early diseases cases, can best be revealed by IF using HEp-2 cells, and can be credibly interpreted by non-medical personnel. Modern testing platforms are perhaps easier in use but not better. To arrive at optimal clinical diagnostics patients need to donate blood for testing purposes, and clinicians and laboratory scientists need to collaborate closely.

Literature

1. Wiik A. Anti-nuclear autoantibodies: clinical utility for diagnosis, prognosis, monitoring, and planning of treatment strategy in systemic immunoinflammatory diseases. Scand J Rheumatol 2005; 34:260-8.

2. Bizzaro N, Wiik A. Appropriateness in anti-nuclear antibody testing: from clinical practice to strategic laboratory practice. Clin Exp Rheumatol 2004; 22:349-55.

3. Wiik AS, Gordon TP, Kavanaugh AF, Lahita RG, Reeves W, vanVenrooij WJ, Wilson MR, Fritzler M, and the IUIS/WHO/AF/CDC Committee for the Standardization of Autoantibodies in Rheumatic and Related diseases. Cutting edge diagnostics in rheumatology: the role of patients, clinicians, and laboratory scientists in optimizing the use of autoimmune serology. Arthritis Rheum (Arthritis Care & Research) 2004; 51:291-8.

4. Fritzler MJ, Wiik A, Fritzler ML, Barr SG. The use and abuse of commercial kits used to detect autoantibodies. Arthritis Res Ther. 2003; 5:192-201.

5. Fritzler MJ, Wiik A. Autoantibody assays, testing and standardization. In: Roe NR, Mackay IR (eds): The autoimmune diseases, 4th edition, Elsevier, London. (in press).

6. Tan EM, Feltkamp TEW, Smolen JS, Butcher B, Dawkins R, Fritzler MJ et al. Range of antinuclear antibodies in "healthy" individuals. Arthritis Rheum 1997; 40:1601-11.

7. Fritzler MJ, Wiik A, Tan EM, Smolen JS, McDougal JS, Chan EKL et al. A critical evaluation of enzyme immunoassay kits for detection of antinuclear antibodies of defined specificities. III. Comparative performance characteristics of academic and manufacturer's laboratories. J Rheumatol 2003; 30:2374-81.

8. Fenger M, Wiik A, Høier.Madsen M, Lykkegaard JJ, Rozenfeld T, Hansen MS, Danneskjold-Samsoe B, Jacobsen S. Detection of antinuclear antibodies by solid phase immunoassays and immunofluorescence analysis. Clin Chem 2001;50:2141-47.

9. Pottel H, Wiik A, Locht H, Gordon T, Roberts-Thomson P, Abraham D et al. Clinical optimization and multicenter validation of antigen-specific cut-off values on the INNO-LIATM ANA Update for detection of of autoantibodies in connective tissue disorders. Clin Exp Rheumatol 2004; 22:579-88.

10. Wiik A. Testing for ANA and ANCA - diagnostic value and pitfalls. In Hochberg MC, Silman AJ, Smolen JS, Weinblatt ME, Weisman MH (eds): Rheumatology, 3rd edition, Mosby, Edinburgh, pp 215-226.

11. Wiik A, Lam K. Report to the European Commission: On the usability of extended DOORS software for education and training, quality assurance and consensus formation. Deliverable D 09, version 2.1, 2001. The EU CANTOR project HC4003(HC)2000.