

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

New procedures in the interpretation of the indirect immunofluorescence assays in
autoantibody testing: possibilities and limitations of computer-aided pattern
recognition

by Gábor Nagy

Supervisor: Péter Antal-Szalmás, PhD, DSc



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Head of the **Examination Committee:** Attila Bácsi, PhD, DSc

Members of the Examination Committee: Bertalan Fodor, PhD, DSc
Lajos Gergely, PhD

The Examination takes place in the discussion room (ÉTK 2.209) of the Department
of Immunology, Faculty of Medicine, University of Debrecen on 2 May 2024 at
11:00 am.

Head of the **Defense Committee:** Attila Bácsi, PhD, DSc

Reviewers: Ildikó Fanny Horváth, PhD
Eszter Nagy, PhD

Members of the Defense Committee: Bertalan Fodor, PhD, DSc
Lajos Gergely, PhD

The PhD Defense takes place in the lecture hall of Building A, Department of
Internal Medicine, Faculty of Medicine, University of Debrecen on 2 May 2024 at
1:00 pm.

Introduction

The indirect immunofluorescence technique has been present in autoimmune diagnostics for several decades. This method has led to the discovery of quite a few new antibodies by detecting unknown autoantibodies either on tissue sections or cell layers. In contrast to other methods, its main advantage is the ability to detect numerous, potentially unknown antibodies in a single step. However, its drawback is that conventional microscopic interpretation is tiresome and time-consuming. Nevertheless, it cannot be replaced by other methods, primarily because the antigen conformation is preserved in this assay and production expenditure is low.

The rising incidence and prevalence of autoimmune diseases have resulted in increased demand for autoantibody detection including indirect immunofluorescence assays and need for automation. In recent years, computer-assisted fluorescence microscopy systems have emerged, capable of capturing a digital image of the field of view, displaying it on a screen for the observer, even analyzing it using image processing algorithms. Of course, before the routine diagnostic application of such computer-aided procedures, it is crucial to verify the characteristics of these new evaluation means and determine whether they provide results comparable to conventional reading.

Our studies focused on examining the possibilities and limitations of such automated microscopic and image analysis systems in the field of anti-neutrophil cytoplasmic and antinuclear antibody diagnostics, comparing its performance with conventional interpretation.

Autoimmunity, Autoimmune Diseases

In autoimmunity, the immune system initiates an immune response against the body. Though it was previously considered pathological, we now know that limited form of autoimmunity occurs in individuals and has a physiological role. However, pathological autoimmunity can be triggered by environmental factors in genetically susceptible people, leading to the development of autoimmune diseases and damaging organs, tissues, and cells. Autoimmune diseases altogether affect 5-7% of the population, a recent epidemiological study showed that they may involve as many as one out of ten individuals. Both the prevalence and the number of new cases (incidence) have been demonstrated to increase in recent decades.

The Clinical Significance of Autoantibody Detection

Many autoimmune diseases have a chronic course, often beginning with a long preclinical phase in which the signs of the disease are either barely detectable or entirely absent. This phase may be followed by an undifferentiated stage, in which is not yet clear which autoimmune disease is likely to develop. Therefore, use of biomarkers that provide information about the underlying autoimmune process is crucial.

While most of the elements of autoimmunity can be examined by laboratory tests, the detection of autoantibodies is of utmost practical importance. In pathological autoimmunity, the appearance of these abnormal antibodies may serve as indicators of the disease, although they do not directly contribute to the initiation of the disease (pathognomonic). Other autoantibodies play a pathogenic role, actively participating in cell, tissue, and organ damage.

Methods for Detecting Autoantibodies

Fortunately, pathological antibodies can be distinguished from natural autoantibodies by several characteristics: high concentration, strong affinity, IgG isotype (occasionally IgM or IgA), specific target antigen. Based on these features, several methods are suitable for sensitive and specific detection of autoimmune

antibodies. In routine laboratory practice, the predominant methods include indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA), and immunoblotting. The fourth method that is spreading in the past decade is luminescence immunoassay.

All four common methods share the principle that, following the binding of the antibody to the autoantigen fixed to a solid phase and removal of nonspecific antibodies through washing, detection is accomplished using a second, tagged antibody (conjugate). The strength of the signal detected on the solid phase is in the main proportional to the concentration of autoantibody (immunometric), but occasionally competitive assays are also in use.

Indirect Immunofluorescence Assay (IFA)

In the IFA, the antigen is present in a tissue section or a cell layer (substrate) which is fixed onto a microscopic slide. Knowledge of the antigenic target of the antibody is not necessary for detection, which allows discovery of new, unknown autoantibodies. The antibody binds to the substrate according to the location of the autoantigen, and a corresponding fluorescent pattern is observed, from which we can infer the target of the autoantibody. The disadvantage is that antibodies against antigens with the same location cannot be differentiated; they yield identical patterns, and some proteins may not be expressed in sufficient quantities in the substrate to detect the corresponding antibody. In such cases, an antigen monospecific test (e.g., ELISA, immunoblot) must be used, where purified or recombinant forms of the antigen are employed to bind the autoantibody. The prerequisite for these tests is the prior identification and characterization of the autoantigen.

Principle of the IFA

On a microscopic slide, up to twenty distinct areas separated by hydrophobic coatings are created and then used to fix tissue sections or cell substrates to. Beside fixation, permeabilization of cell membranes is carried out, rendering antigens, even those located within the cell nucleus, accessible to antibodies. Optimal sample

dilution is determined during assay development. Antibodies from the sample, typically serum, bind during the first incubation period if the corresponding antigen is present in the substrate. Non-specific antibodies are removed during the first washing step. In the second incubation period, a secondary antibody against human immunoglobulin labeled with fluorescein isothiocyanate binds to the autoantibody. The isotype of detected antibody depends on the conjugate. Unbound conjugate is removed during the second washing step. As a final step, the slide is covered by mounting medium and coverslip glass.

Biochip IFA Slide

At Euroimmun, during the production of IFA tests, the substrate is affixed not to a regular microscope slide but to a thin glass slide, which is then cut into 2x2 mm squares. These squares, each covered with a different substrate, are used as 'biochips.' Up to six different types of these biochips can be mounted directly next to each other on a special plastic slide, forming up to ten complex reaction fields on a single slide.

Evaluation of the Indirect Immunofluorescence Assay

Processed IFA slides are interpreted under a fluorescence microscope. The fluorescent signal appearing on a dark background ensures the high analytical sensitivity of IFA. The traditional method of IFA slide evaluation is visual inspection under microscope.

The autoantibody labelled with the secondary antibody provides a signal corresponding to the location of the antigen in the substrate, creating a characteristic fluorescent pattern. Quantity of the antibody can be determined by titration (endpoint titer). The standard procedure involves preparing a series of dilutions starting from the level of the screening dilution and determining the highest dilution at which the specific pattern is still positive.

Computer-Aided Evaluation

The microscope slides are moved by a computer-controlled, motorized microscope. The slide is taken out from the magazine, the reaction fields are positioned one by one under the objective, and digital image of the field of view is taken. The fluorescence intensity can be derived from the brightness of pixels of the digital image. Since the intensity is proportional to the antibody level, it is possible to estimate the endpoint titer of the sample based on the fluorescence intensity observed in the screening dilution.

Analysing the captured image, computer algorithms can qualify the sample as negative or positive. Advanced versions can even recognize patterns and classify positive samples. Another computer assisted method is the evaluation of patterns by a human observer on the computer screen. Using images taken of serial dilutions of a sample, the endpoint titer value can be read on the screen, too.

Detection of Anti-Neutrophil Cytoplasmic Antibodies (ANCA)

ANCA Detection in Conditions with Vasculitis and Other Diseases

The discovery of anti-neutrophil cytoplasmic antibodies (ANCA) was made possible by the indirect immunofluorescence (IFA) technique in the 1980s. Later antigens were identified as proteins in the granules of neutrophil granulocytes. The detection of ANCA serves as a valuable diagnostic marker for numerous inflammatory and autoimmune diseases, especially vasculitides.

Methods for ANCA Detection

IFA using ethanol-fixed neutrophil granulocytes was the first tool for ANCA detection. Subsequently, after the identification of antigens, the two most important antibodies (anti-myeloperoxidase (MPO) and anti-proteinase-3 (PR3)), could be measured using the ELISA method. In recent years, luminescent immunoassays became available.

Indirect Immunofluorescence ANCA Test

ANCA patterns are described by international recommendations and their supplements. The classic diffuse cytoplasmic 'C-ANCA' pattern has a strong association with anti-PR3 antibodies, while anti-MPO antibodies are responsible mostly for classic perinuclear pattern ('P-ANCA'). Furthermore, 'atypical C-ANCA' and 'atypical ANCA' patterns are also distinguished. Some laboratories use formaldehyde-fixed neutrophil granulocytes in addition to ethanol-fixed cells, which helps recognize antinuclear and ANCA patterns. Formalin sensitive atypical P-ANCA and atypical C-ANCA patterns can also be observed using this technique, beside formalin resistant P-ANCA and C-ANCA patterns.

The Significance of Antinuclear Antibody (ANA) Detection

Detection of antinuclear antibodies plays pivotal role in diagnosing systemic autoimmune diseases. All patients with mixed connective tissue disease, nearly 100% in systemic lupus erythematosus (SLE), the majority of patients with systemic sclerosis, 50-70% of Sjögren's syndrome patients, nearly half of rheumatoid arthritis patients and a significant portion of those with autoimmune myositis are ANA positive. The clinical importance of ANA stands out in autoimmune liver diseases, especially in autoimmune hepatitis and primary biliary cholangitis. ANA is frequently encountered in some other inflammatory conditions (juvenile idiopathic arthritis (JIA), inflammatory bowel diseases).

The HEp-2 Indirect Immunofluorescence ANA Test

The use of the HEp-2 cell-based IFA is attributed to structure of antigens in the test, mainly because of their human origin. Over a hundred autoantibodies can be detected on this IFA substrate. Numerous nuclear and cell cycle-dependent antigens are expressed, which enables the detection of a wide range of autoantibodies. Harmonization of pattern names began in the 2000s and led to the development of the International Consensus on HEp-2 ANA Patterns (ICAP) in 2014, which was subsequently published on the <https://anapatterns.org> website. The website was

translated into several languages including Hungarian and provides descriptions and images of patterns, along with antigen and disease associations.

Composite, Multiple, and Mixed ANA Patterns

Pattern recognition is complicated due to the simultaneous presence of autoantibodies which can be detected by the HEp-2 IFA. If more than one pattern co-occurs, multiple and mixed patterns should be differentiated. In the case of multiple patterns, two or more clearly distinguishable patterns can be observed in a single serum sample. Differentiating mixed patterns poses an even greater challenge since at least two autoantibodies causing different patterns may be present in the patient's sample, and there is overlap in the location of the fluorescence signal, which makes it impossible to clearly identify the individual components. The observed fluorescent signal depends on the concentration and affinity of each autoantibody, the dominant pattern may partially or completely obscure the others. It is also important to make a distinction between multiple/mixed patterns and composite patterns (e.g., Scl-70-like or PCNA-like), where various cell components exhibit different fluorescence staining in different phases of the cell cycle, but all staining is caused by a single autoantibody.

Aims

I. During comparison of conventional ANCA IFA interpretation with computerized image analysis and on-screen evaluation we wanted to answer these:

1. What is the reproducibility of pattern qualification and intensity? Does the repeatability differ among the three types of evaluation?
2. Is the estimated titer based on single well fluorescence intensity linear with the endpoint titer within the measurement range? Can it be used instead of traditional titration based on serial dilution?
3. How much agreement exists between the results of qualitative rating and classification of samples using the image analysis algorithms or on-screen reading and conventional microscopic evaluation?
4. Does the presence of interfering antinuclear or anti-cytoplasmic antibodies in the sample affect ANCA evaluation?

II. Questions to be answered by investigation of interference between ANA patterns and comparison of computer-assisted evaluation means:

1. What is the proportion of samples with more than one co-occurring ANA pattern among those sent for routine HEp-2 IFA testing? Which patterns are most frequently combined?
2. In the case of mixed patterns, is there interference between patterns? What is the extent of interference? At what titer does it occur? Can one pattern completely mask the other?
3. Is there a difference in the recognition of the examined patterns in the presence of an interfering pattern between automated image analysis, on-screen and conventional microscopic evaluation?

Methods

Frequency of single and mixed ANA patterns in the routine diagnostic practice

We collected all of the ANA HEp-2 results from the period of 01/03/2019 to 31/12/2019 into an Excel file. The positivity/negativity, pattern(s) and titer(s) were loaded in separate columns; maximum 3 nuclear, 1 cytoplasmic and 1 mitotic pattern was recorded in one sample. The frequency of the different individual and mixed patterns were calculated.

Patients and samples

Samples used in the ANCA comparison study

Serum samples consecutively sent to the Department of Laboratory Medicine, University of Debrecen for routine ANCA testing over a period of three months were collected and analysed. Tests were ordered for patients referred to an outpatient or inpatient clinic in the Northern Great Plain region of Hungary covering the disciplines of immunology, rheumatology, nephrology, gastroenterology and hepatology. The 575 samples were drawn from 570 individuals, 5 patients provided two samples. Top reasons for referral were suspicion of or follow-up for acute and chronic renal failure, ulcerative colitis, Crohn's disease, systemic autoimmune diseases, vasculitis and autoimmune liver disorders.

Samples used in ANA pattern interference study

Serum samples sent to the Department of Laboratory Medicine, University of Debrecen for routine ANA testing were selected based on their IFA staining pattern on HEp-2 cells (EUROIMMUN AG, Lübeck, Germany). Sera positive in the 1:80 screening dilution were further diluted 1:320, 1:1,280, 1:5,210, and these dilutions were tested by HEp-2 IFA assay and evaluated on a EuroStar II Plus fluorescence microscope (EUROIMMUN AG) to determine the end-point titer. Samples which

showed only either a nuclear homogeneous (AC-1) or a nuclear fine speckled (AC-4) pattern at a titer of 1:5,120 were selected.

ANCA IFA test

Indirect immunofluorescence testing was performed using a reagent kit designed to be used with EUROPattern (EPa), EUROIMMUN's computer-aided immunofluorescence microscope (Granulocyte Mosaic 13; catalogue number: FC 1201-1010-13; EUROIMMUN AG, Lübeck, Germany). One reaction area on the microscope slide contains three biochips (2×2 mm, substrate coated cover slips), covered by ethanol or formaldehyde-fixed human neutrophil granulocytes or granulocytes scattered on a HEP-2 cell layer, respectively.

Automated image analysis categorizes the samples as 'not evaluable', 'negative', 'C-ANCA', 'P-ANCA' or 'atypical ANCA'. In order to use the same categories which were available in automated analysis 'C-ANCA atypical' and 'atypical ANCA' results were merged into a single 'atypical ANCA' entity during on-screen validation and traditional visual interpretation. Furthermore, based on the evaluation of fluorescence patterns on the HEP-2 cells and the ethanol fixed granulocyte cell layer, samples with interfering antibodies to common, not neutrophil granulocyte specific nuclear or cytoplasmic cell components (antinuclear antibodies - 'ANA' or anti-cytoplasmic antibodies - 'ACyA') were included in a separate 'ANA/ACyA interference' group since these antibodies make the evaluation of ANCA pattern uncertain or impossible. Based on these during comparisons samples were classified into 5 categories: 'negative', 'P-ANCA', 'C-ANCA', 'Atypical ANCA' and 'ANA/ACyA interference'.

ANA IFA test

The ANA HEP-2 IFA test was evaluated by the EUROPattern microscope (EUROIMMUN AG) and digital images were taken using the 20x objective by the built-in camera and stored in png and jpeg formats. Positive-negative discrimination, ANA pattern recognition and titer estimation was first performed by automated image analysis using the built-in classifier algorithms (EPa) (version:

3.7.289.14128). Next, the digital images were presented to users on a calibrated computer display, who checked and evaluated the patterns on-screen. Finally, the slides were evaluated by means of conventional visual reading under an epifluorescence microscope.

Laboratory performance testing of ANCA IFA

For reproducibility testing, three low or medium positive samples with C-ANCA, P-ANCA and atypical ANCA patterns were tested five times in three runs on separate days. For testing linearity, we used two samples with C-ANCA, two samples with P-ANCA and one sample with atypical ANCA pattern, and diluted them two-fold serially. Estimated titer was determined in each dilution by EPa and plotted against the expected titer calculated from the end-point titer by conventional evaluation and the dilution factor. End-point ANCA titers of 32 samples (11 C-ANCA, 15 P-ANCA, 6 atypical ANCA) were determined by means of conventional method (serial two-fold dilution between 1:10 and 1:1,280). Titers estimated by EPa based on the image taken from the 1:10 screening dilution were plotted against end-point titers, and Spearman rank correlation was calculated. Total evaluation time necessary to read an ANCA testing batch was recorded for all three ways of evaluation.

Data analysis, visualization and statistical tests

Descriptive statistics, visualization and statistical analyses were performed using Microsoft Excel and GraphPad Prism (9.1.2.) software packages. A probability value of < 0.05 was considered statistically significant.

Results

Results of the ANCA comparison study

Reproducibility of pattern identification and end-point titer estimation

Based on the automated image analysis all 15 tests gave the same pattern for the P-ANCA positive sample, the C-ANCA positive sample tested as expected 14 times and the sample with atypical pattern 13 times. Estimated end-point titers showed good replicability, all results were in the range of median $\pm$ one dilution step for all three patterns. Reproducibility of patterns were perfect during on-screen validation and conventional evaluation, too: same pattern for all three samples during repetitions. Reproducibility of fluorescence intensity was also acceptable.

Linearity of end-point titer estimation

Dilution curves showed a closely linear response in the range of 1:10 to 1:80 but plateaued when titer estimation reached its maximum at 1:320. Interestingly, both C-ANCA positive samples showed high dose hook effect at high antibody concentrations.

Correlation of titer estimation with the end-point titer

When all patterns were included Spearman r coefficient was 0.70 ($p < 0.001$). Stronger correlation could be observed on titers of P-ANCA ($r = 0.74$, $p < 0.001$) positive samples. Correlation on titers of atypical and diffuse cytoplasmic ANCA positive sera was good but not significant ($r = 0.66$, $p = 0.33$ and $r = 0.58$, $p = 0.06$, respectively).

Discrimination between negative and non-negative samples

Altogether 568 samples were analysed. Of the 401 samples which were negative by conventional visual reading 328 (81.8%) were classified as negative by automated image analysis, too. The number of false positive samples was 73, from which 6, 19 and 48 were classified as P-ANCA, C-ANCA and atypical ANCA, respectively. All these samples showed weak/borderline positivity by the EPa system. The

concordance of negative results increased to 95.3% (382/401) when the images were validated on screen.

167 samples were not negative as read under the conventional microscope: 23 (4.0%) P-ANCA, 17 (3.0%) C-ANCA, 47 (8.3%) atypical ANCA and 80 (14.1%) not evaluable due to interfering antibody (ANA/ACyA interference). Presence of ANA accounted for the majority of these not evaluable results (74/80, 93%). Among not negative samples, agreement between conventional and automated reading was excellent (161/167=96.4%) and became perfect (100.0%) after on-screen validation. Only 5 atypical ANCA results and 1 sample with interfering ANA/ACyA were negative by EPa, all of them were correctly classified during user validation.

Altogether, counting the results of negative and not negative categories, 86.1% (489/568) of automated image analysis results were congruent with standard, conventional interpretation, while total agreement reached 96.7% (549/568) after images had been reviewed on screen.

Sample classification

EUROPattern automated reading correctly classified 392 samples (69.0%; 328 negative, 20 P-ANCA, 14 C-ANCA, 30 atypical ANCA) if ANCA categorization was also taken into consideration. Percentage of correctly identified samples rose to 95.1% when recognition was done by the user. Rate of correctly identified samples was highest in P-ANCA positive (87.0%) and lowest in atypical ANCA positive sera (63.8%) when the software algorithm was used on its own. It should be emphasized that no P-ANCA or C-ANCA positive sample was categorized as negative by the image analysis algorithm. Rates of correctly categorized samples ranged from 91.3% to 95.7% if pattern was identified on computer screen by a specialist.

Influence of antinuclear and anti-cytoplasmic antibody on sample classification

Agreement between automated sample classification and standard reading was significantly weaker if ANA could be detected on HEp-2 cells (Cohen's kappa:

0.534 versus 0.335), but significant impact of ACyA could not be seen. There was no difference in the agreement between results of on-screen validated and conventional reading independently of presence of ANA or ACyA.

Impact on evaluation time and workflow

Autofocusing, image capture and automated evaluation took 63.8 ± 1.5 seconds per reaction area, which is in accordance with the 20 seconds/biochip reading time stated in the specification sheet. Conventional visual interpretation took 79.5 ± 9.0 seconds/reaction area, while much less time was needed to evaluate one sample on screen (46.8 ± 2.4 seconds, $p < 0.001$).

Results of the ANA pattern interference study

Frequency of single and mixed ANA patterns in the routine diagnostic practice

Altogether 10,587 ANA HEp-2 IFA tests were performed in our laboratory between 01/03/2019 and 31/12/2019. From the 4,291 nuclear positive samples 2,786 (64.9%) possessed only 1, 1,396 (32.5%) carried 2, while 109 (2.5%) showed 3 different nuclear patterns. In the case of cytoplasmic and mitotic positivity 15 (1.4%) and 4 (2.5%) samples had two different cytoplasmic or mitotic patterns, respectively. Combination of nuclear homogeneous and nuclear speckled pattern was by far the most prevalent.

Qualitative agreement of conventional, on-screen and automatic readings with expected pattern in the presence of interfering pattern: Effect of nuclear fine speckled pattern on recognition of nuclear homogeneous pattern

Samples with only a homogeneous pattern could be perfectly identified by conventional (100%) and on-screen evaluation (100%) while the EPa analysis was significantly inferior (60.7%, $p < 0.0002$). In the presence of the disturbing speckled

pattern, qualitative agreement was 100% using conventional and almost perfect (97%) using on-screen evaluation when the titer of homogeneous pattern was higher than that of speckled pattern. Match percentage of EPa image analysis (83%) was slightly but significantly lower ($p=0.0002$ and $p=0.0028$). Agreement decreased if titer of interfering pattern was the same (conventional: 90.2%, on-screen: 86.9%, EPa: 78.7%) or higher (conventional: 55.2%, on-screen: 50.0%, EPa: 54.2%) than the titer of homogeneous pattern.

Effect of nuclear homogeneous pattern on recognition of nuclear fine speckled pattern

The identification rate of a single speckled pattern was 100% for conventional and on-screen assessments, while showed only 63.6% ($p<0.0001$) in the case of EPa. Recognition percentage of speckled pattern in the presence of the homogenous pattern was 87% and 90% by conventional and on-screen readings, respectively, if the level of fine speckled pattern was higher than the interfering homogeneous pattern. Performance of EPa automatic analysis was good (69.8%), but significantly weaker ($p=0.0052$ and $p=0.0007$). When the titers of the two patterns were the same, match% was 14.8% using EPa, while nuclear speckled pattern was correctly identified at a significantly higher level using conventional (45.9%, $p=0.0002$) and on-screen (54.1%, $p<0.0001$) evaluation means. Match percentage was below 20% when level of interfering pattern was higher than the titer of speckled pattern (conventional: 2.7%, on-screen: 10.7%, EPa: 1.3%).

Correlation between the observed and expected fluorescence intensity/titer of the examined pattern in the presence of the interfering pattern:

Examined pattern: nuclear homogeneous, interfering pattern: nuclear fine speckled

Fluorescence intensity of homogeneous pattern by conventional and on-screen evaluation means showed high level of correlation with expected titer in the absence and presence of nuclear fine speckled pattern (Spearman's rank correlation coefficient r : 0.79-0.99). On the other hand, estimated titer values of EPa automatic image analysis were much less accurate. In the presence of the interfering speckled pattern lower but significant correlation could be observed between the expected and observed homogeneous titers (r : 0.44-0.72, all p values < 0.01). The titer of the interfering speckled pattern did not significantly alter the correlation between the expected and observed titer/FI of the examined homogeneous pattern.

Examined pattern: nuclear fine speckled, interfering pattern: nuclear homogeneous

Rank correlation was high (r : 0.80-1.00) between the observed and expected FI/titer of speckled pattern by conventional or on-screen readings but only if the interfering homogeneous pattern was absent or its titer was less than 1:1,280. Automatic image analysis showed high to low level correlation in this range (r : 0.83-0.33). We found low correlation ($r < 0.5$) in all evaluation types if the homogeneous pattern was present in titer 1:1,280 or 1:5,120. Altogether, correlation decreased remarkably as titer of interfering pattern increased.

Detailed interference analysis of fine speckled (AC-4) on homogeneous (AC-1) ANA pattern identification evaluated by on-screen image analysis, traditional microscopic reading and EUROPattern

Fluorescence intensity of 1:80 (1+) homogeneous pattern by conventional and on-screen evaluation was significantly ($p < 0.001$) decreased if titer of interfering nuclear speckled pattern was 1:1,280 or higher. Unfortunately, the same could not be judged using EPa because all the homogeneous samples in the absence of the interfering pattern were classified as “undetermined” and no estimated titer was reported. However, similarly to the other two types of evaluation, in most of the sera, in which the expected titer of homogeneous pattern was 1:80 and the titer of speckled pattern was $\geq 1:1,280$, homogeneous pattern was not recognized. The same underestimation of nuclear homogeneous pattern can be seen if homogeneous pattern is 1:320 or 2+, but the differences are not significant. Significant negative interference cannot be seen if titer of homogeneous pattern is 1:1,280 or 1:5,120 irrespective of how high the titer of speckled pattern is.

At the same time, significant ($p = 0.0147$) overestimation of FI of homogeneous pattern by on-screen evaluation occurred in samples with 1:1,280 homogeneous pattern if confounding speckled pattern was present at a titer of 1:320. EPa estimated titer is the less accurate to determine fluorescence intensity of homogeneous pattern, as shown by the considerable overestimation of titer in the absence of speckled pattern. It is also clearly visible, though it was not analysed using statistical tests, that concurrent speckled pattern increases variability of titer determination.

Detailed interference analysis of homogeneous (AC-1) on fine speckled (AC-4) ANA pattern identification evaluated by on-screen image analysis, traditional microscopic reading and EUOPattern

Analysis of FI of samples with 1:80 speckled pattern by conventional/on-screen evaluation revealed a significant decrease (p values: <0.0001 to 0.0378) if titer of interfering homogeneous pattern is $\geq 1:320$. This negative interference was not proved on the EPa platform, probably due to the high rate of false negative results of these borderline positive sera. On samples with titer of speckled pattern $\geq 1:320$ homogeneous pattern at a titer of $\geq 1:1,280$ caused significant underestimation (p values: <0.0001 to 0.0158) in all combinations but two. However, data show that there is a clear negative interference in these two cases as well, but underestimation is not significant if compared with FI of samples without interfering pattern or significant (p=0.0051) only at titer of 1:5,120. Both may be the consequence of low statistical power of data. Minor overestimation (p=ns) of FI of speckled pattern by on-screen evaluation can be discovered on samples in which the titer of interfering homogeneous pattern is 1:80. Imprecision of EPa estimated titer is striking on samples with borderline positive speckled pattern and no homogeneous pattern. Similar as with interference by speckled pattern, uncertainty of fluorescence intensity quantitation of speckled pattern is higher in the presence of a second homogeneous pattern.

Discussion

Results of the ANCA comparison study

In the field of ANCA testing, our aim was to test the Euroimmun EUROPattern (EPa) automated IFA microscopy system and compare the computer-assisted assessment methods with conventional reading on a large number of routine samples from specialists in immunology, rheumatology, nephrology, gastroenterology and hepatology, covering all indications for ANCA testing.

We first tested the analytical reliability of the IFA system. The repeatability test showed good reproducibility for the EPa automatic assessment. Low or borderline positive samples showing PANCA, C-ANCA and atypical ANCA patterns were classified as expected by the algorithm in all runs, with only one test giving negative result.

The linearity of the titer calculated from the fluorescence intensity of the image was acceptable in the range 1:10-1:100, but above this the curve plateaued. This is probably due to the large difference between the fluorescence intensity and the range of light intensities that can be digitised by the 8-bit camera.

Importantly, some strongly positive samples showed a high dose hook effect. This phenomenon is also known to occur in IFA: fluorescence quenching or steric inhibition of conjugate binding may reduce the fluorescence intensity if the concentration of autoantibody is extremely high.

In our study, 70.6% of sera were negative and 81.8% of these were correctly classified by automated image analysis. More importantly, 328 of the 334 samples negative by automated image analysis were also classified as negative by conventional evaluation. Only 6 samples were non-negative, but the evaluator correctly classified all of them on screen.

The image analysis algorithm groups images into four different categories: negative, C-ANCA, P-ANCA or atypical ANCA. The software of the instrument installed in our laboratory was not able to distinguish antinuclear or anti-cytoplasmic

interference from ANCA, which formed the fifth category ('ANA/ACyA interference') in conventional and on-screen evaluation.

In our study, 69.0% of the samples were categorized by EUROPattern image analysis according to the results of conventional microscopic reading, whereas this rate was 95.1% for on-screen evaluation. Out of 401 negative samples, only 19 were incorrectly categorised as positive, while out of 167 non-negative samples, 9 were categorised as negative by the on-screen observer. These results also show that computer assessment alone is not sufficiently accurate.

The misclassification of the EPa was mainly due to two reasons. i) It classified negative samples as positive. Looking at these misclassifications separately, we saw that most of them were samples with fluorescence intensities very close to the cut-off value, which were still classified as negative by conventional assessment, but were already considered positive by EPa. ii) Another common cause of incorrect EPa categorisation was ANA and/or ACyA interference. These samples were mostly classified by EPa as atypical ANCAs, as both antinuclear and anti-cytoplasmic antibodies are positive on ethanol-fixed granulocytes, while formalin-fixed neutrophil cells are negative, similar to the atypical pattern. The majority of these cases were classified correctly on-screen.

There was only one previous study on the EUROPattern microscope/software evaluation for ANCA, but that was also performed on different patients and using different reagents. In this study, Csernok et al. evaluated patients with different types of vasculitis, healthy individuals and patients with autoimmune and inflammatory disorders, but excluded inflammatory bowel and autoimmune liver diseases. The sensitivity and specificity of the automated IFA for vasculitis were 89.8% and 89.2%, respectively, and the ROC AUC was 0.904.

The use of automated IFA microscopes is expected to result in shorter overall analysis times. In our study, it reduced the evaluation time by 41%.

Results of the ANA pattern interference study

Computer algorithms that analyse digital images can provide reliable data on ANA positivity/negativity but identifying ANA patterns is still challenging. When comparing pattern classification by algorithms with conventional microscopic evaluation, the agreement ranges from 43.0% to 84.7% for Aklides, 69.0% to 87.1% for EPa, 53.8% to 70.8% for NovaView, 26.0% to 63.0% for Zenit G-sight, and 70.8% for Helios.

For individual patterns, the percentages of correct identification vary over an even wider range. Homogeneous pattern ranged from 46.7% to 95.0%, speckled pattern from 11.1% to 76.9%, nucleolar pattern from 33.3% to 100%, and centromere pattern from 25.0% to 92.7% when identified by the different automated systems. The high variability is partly due to differences in instrument capabilities and partly due to differences in study design.

In computer-aided systems, the proportion of correctly recognised patterns is higher when the captured digital image has been evaluated on-screen by an expert in ANA pattern reading rather than by the image analysis algorithm. The match rates between on-screen and conventional microscope evaluation were similarly high for EPa (94.6%), NovaView (97.0%) and Zenit G-sight (90.0%).

The most difficult task in ANA IFA evaluation is the classification of samples with more than one pattern at a time. To the best of our knowledge, currently only EUROPattern's image analysis algorithm is able to categorize such multiple or mixed patterns and identify their individual components among automated computer-aided systems. The percentage of cases where EUROPattern's image analysis software identified the dominant pattern according to the classical microscopic evaluation was relatively high: 87.5% and 95.2%, respectively. In contrast, all components of the multiple/mixed pattern were correctly identified by the automated system in only 33.3% to 68.4%. Based on these data, the accurate classification of ANA patterns is still a major challenge for automated, computer-

assisted systems, which is even more pronounced in the case of multiple co-occurring ANA patterns.

This is very important in routine diagnostics, as the proportion of samples with multiple ANA patterns is relatively high. Over the 10-month period we studied, more than a third (36.3%) of positive samples had multiple/mixed patterns, and similar rates have been reported in previous studies.

A major problem in the assessment of computer-based ANA pattern recognition systems is the lack of adequate reference samples with multiple/mixed patterns, i.e. samples with all patterns and their titres accurately known. Serum samples from routine diagnostics are not adequate for this purpose, as even experts experienced in conventional microscopic ANA reading cannot provide accurate results in the case of mixed patterns. To solve this problem, a new approach was used. We prepared and mixed serial dilutions of high-titer single positive samples positive for only one out of two patterns, to produce well-defined mixed samples with known patterns and titers. HEp-2 IFA assays were performed on these mixtures and the presence and strength of the two different patterns were evaluated in all mixtures. The interaction of the two ANA patterns was first investigated by examining the most common pattern combination, the homogeneous nuclear pattern and the fine speckled nuclear pattern.

Our results show that the homogeneous nuclear pattern interferes more with the identification of the fine speckled nuclear pattern than vice versa, but both patterns interfere with the identification of the other. In a significant proportion of mixed-pattern samples, the homogeneous pattern may even completely mask the speckled pattern in the same sample, and, although much less frequently, the low-titer homogeneous pattern may remain completely hidden next to a high-titer speckled pattern. In some cases, the masked component may become visible by titration, i.e. serial dilution of the sample.

Knowledge of the interference between ANA patterns is important for the development of well-functioning laboratory screening algorithms. Recently, an

ANA testing protocol has been published that proposes a sequence of second-line autoantibody tests based on the pattern detected on HEp-2 cells. Based on the type and titer of the pattern, detailed information on how one pattern may interfere with the identification of another can be conveyed by annotating the specific finding.

In addition to masking, interestingly, in some cases the presence of an interfering antibody increased the intensity of the pattern being tested. The enhancement was statistically significant for on-screen readings of a homogeneous pattern with a titer of 1:1280 when the interfering speckled pattern was 1:320. The overestimation of the strength of the homogeneous pattern may have been due to the fact that the fluorescence of the speckled pattern was partially combined with the signal of the homogeneous pattern during the reading.

When evaluating different mixed homogeneous-speckled patterns, conventional microscopic and on-screen reading showed very similar results. However, the classification based on computer image analysis alone showed poorer performance and the titer estimation was even less accurate. These results show that current computer-based pattern recognition algorithms identify patterns relatively well even for mixed pattern samples, but worse than conventional and screen-based human assessment, and that titer estimation based on fluorescence intensity is not yet very reliable. The latter draws attention to the fact that determining the intensity of a fluorescent pattern is not a simple luminance measurement, at which a digital camera would obviously be better than the human eye.

Our approach to study interference between ANA HEp-2 patterns is relatively novel. In the literature, we found only two studies dealing with interference between ANA patterns, but in these studies, strongly positive samples were used for different patterns (without determining the titer) and then mixed in different proportions. Moreover, these studies were not designed to analyse interference. In contrast, the aim of our study was to prepare mixtures of samples in which two different ANA patterns are present at different but known titres and to compare the expected and observed pattern intensities. This allowed not only the detection of interference

between the patterns, but also its detailed analysis and characterisation. In addition, we were able to compare computer image analysis and on-monitor evaluation of the mixed patterns with conventional reading. To the best of our knowledge, this experimental setup has not yet been used to investigate interference between ANA patterns.

Our studies have some limitations. First, we only investigated the interference of the two most common ANA patterns, nuclear homogeneous and nuclear fine speckled. It is very likely that the interference effects of other pairs of ANA patterns on each other may differ. Second, the mixing of two ANA-positive patterns alters the idiotype/antiidiotype network, which may affect the reactivity of autoantibodies, altering the observed pattern. Thirdly, IgA and IgM class antibodies from a sample with an interfering pattern, which cannot be detected with the anti-IgG-FITC conjugate, may bind to HEp-2 cells and alter the binding of IgG class autoantibodies that cause the observed pattern. This potential problem could be eliminated by isolating and using antibodies of the IgG isotype. Fourth, we used patient samples that could be replaced with standard homogeneous and fine speckled pattern positive preparations. An even cleaner approach could be to mix monoclonal antibodies (e.g. anti-dsDNA and anti-Ro60), but this would be to investigate interference between non-human antibodies. Fifth, mixed pattern samples were tested with only type of HEp-2 ANA reagents.

Summary

Background: The indirect immunofluorescence test is one of the oldest methods used to detect autoantibodies. It has many benefits, but the interpretation of fluorescence images is a complex, time-consuming task, which is still considered markedly subjective, despite the steps towards harmonisation. Automation, computerised procedures can increase throughput, reduce variability of results and workload of expert. However, the introduction of computer-aided techniques into routine diagnostics requires that the possibilities and limitations of the new procedures be understood and compared with the conventional evaluation means.

Methods: In these studies, we used the EUROPattern fluorescence microscope for computer-assisted evaluation of indirect immunofluorescence assays in antinuclear (ANA) and anti-neutrophil cytoplasmic antibody (ANCA) testing. In the field of ANCA diagnostics, we compared the results of image analysis and pattern recognition algorithm (EPa) and on-screen evaluation with conventional microscopic evaluation on a series of routine diagnostic or follow-up samples of patients with small-vessel inflammation, autoimmune disease and other inflammatory disease states sent to our laboratory. In the ANA study, we investigated the interference between HEp-2 ANA patterns and compared the evaluation modalities.

Results: We found reproducibility of ANCA pattern recognition and intensity estimation suitable. At the same time, relationship of single well titer estimation and end-point titer was not linear above 1:80, even significant underestimation occurred in case of strong C-ANCA positivity. Total agreement of image analysis algorithm with the conventional evaluation was 86.1% for all (568) samples, 81.8% for negative samples and 96.4% for non-negative samples. Overall concordance was 96.7% for on-screen readings, while agreement rates for negative and non-negative cases were 95.3% and 100%, respectively. Rate of correct classification of ANCA patterns was 69.0% by EPa and 95.1% during on-screen evaluation.

Significant interference between ANA patterns was demonstrated if homogeneous and fine speckled nuclear patterns were present in the sample at the same time. Fine speckled pattern interferes with the recognition of homogeneous pattern, and vice versa. Due to the influence of the homogeneous pattern, the speckled pattern is not even visible in the vast majority of cases if its titer is lower than that of the homogeneous pattern. Quality of on-screen recognition and intensity estimation of patterns is similar to conventional evaluation, but computer-assisted image analysis by itself is inferior.

Conclusion: The EUROPattern automated microscope system is suitable for interpretation of ANA and ANCA indirect immunofluorescence slides if performed by an observer on the computer screen. Without validation of the user, the image analysis algorithm – at least the version used in our laboratory - cannot be used to report results, as it appeared to be inferior both for positive-negative classification and pattern recognition on sera with mixed ANA patterns and samples sent for routine ANCA testing, as compared to conventional reading. Titer estimation based on fluorescence intensity of a single well cannot substitute traditional titration. The use of automated fluorescence microscopes makes darkrooms and paper worklists unnecessary, while providing faster evaluation and storage of fluorescence images.

New scientific achievements

I. Comparison of the conventional, EUROPattern computer image analysis and on-screen ANCA IFA assessment:

1. The reproducibility of pattern classification and strength was adequate, with no significant differences between the three types of evaluation.
2. The endpoint titer estimation was not linear within the measurement range, in addition, for C-ANCA patterns with strongly positive samples, a high dose hook effect was observed.
3. There was a significant discrepancy between the automated image analysis and conventional assessment in both sample classification and rating, and therefore the results of the computer algorithm cannot be reported without user validation. The reason for incorrect qualitative assessment was most often the rating of negative samples as borderline positive atypical ANCA. In most cases, incorrect pattern recognition was caused by incorrect classification of ANA/ACyA positive samples.
4. The on-screen evaluation showed a very good agreement with the reference conventional microscopic reading.

II. Examination of the interference between ANA patterns, comparison of computer aided evaluation methods:

1. Among samples sent to the Department of Laboratory Medicine for HEp-2 IFA, it is common to see more than one ANA pattern at a time, with homogeneous and speckled nuclear patterns occurring together most frequently.
2. In samples with mixed patterns, the homogeneous pattern interferes with the speckled pattern and vice versa. The disturbing effect of the homogeneous pattern is greater.
3. In the presence of a disturbing second pattern, the detection and assessment of strength of the examined pattern is similar on-screen and worse using computer image analysis as compared to conventional reading.

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List of publications related to the dissertation

1. Nagy, G., Földesi, R., Csípő, I., Tarr, T., Szűcs, G., Szántó, A., Bubán, T., Szekanecz, Z., Papp, M., Kappelmayer, J., Antal-Szalmás, P.: A novel way to evaluate autoantibody interference in samples with mixed antinuclear antibody patterns in the HEP-2 cell based indirect immunofluorescence assay and comparison of conventional microscopic and computer-aided pattern recognition.
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