Autoantibody Detection Using Indirect Immunofluorescence on HEP-2 Cells

Ulrich Sack, a Karsten Conrad, b Elena Csernok, c Ingrid Frank, d Falk Hiepe, e Thorsten Krieger, f Arno Kromminga, g Philipp von Landenberg, h Gerald Messer, i Torsten Witte, j and Rudolf Mierau k for the German EASI (European Autoimmunity Standardization Initiative)

a Institute of Clinical Immunology and Transfusion Medicine, Medical Faculty of the University, Leipzig, Germany
b Institute of Immunology, Medical Faculty of the Technical University, Dresden, Germany
c Department of Rheumatology, University Lübeck and Rheumaklinik Bad Bramstedt, Bad Bramstedt, Germany
d Laboratory Dr. Tiller, Munich, Germany
e Charité-Universitätsmedizin Berlin, Berlin, Germany
f Institute of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
g Laboratory Lademannbogen, Hamburg, Germany
h Institute of Clinical Chemistry and Laboratory Medicine, Johannes Gutenberg University, Mainz, Germany
i Department of Dermatology and Allergology, Ludwig-Maximilians University, Munich, Germany
j Department of Clinical Immunology, Medical School Hannover, Hannover, Germany
k Laboratory at Rheumaklinik Aachen, Germany

The detection of autoantibodies is an important element in the diagnosis and monitoring of disease progression in patients with autoimmune diseases. In laboratory diagnostic tests for connective tissue and autoimmune liver diseases, indirect immunofluorescence on HEP-2 cells plays a central role in a multistage diagnostic process. Despite the high quality of diagnostics, findings at different laboratories can differ considerably due to a lack of standardization, as well as subjective factors.

The present paper formulates recommendations for the standardized processing and interpretation of the HEP-2 cell test for the detection of non-organ-specific (especially antinuclear) antibodies. It provides requirements regarding the diagnostic tests used, instructions for laboratory procedure and evaluation, and recommendations for interpretation. For an optimal laboratory diagnostic process, it is useful to have an informative, tentative clinical diagnosis and an experienced laboratory diagnostician. In addition, the following key elements are recommended: initial screening using indirect immunofluorescence on carefully chosen HEP-2 cells beginning with a serum dilution of 1:80 and evaluation under a microscope with powerful illumination; results from a
titer of 1:160 upwards being considered positive; internal laboratory quality control; and standardized interpretation. The aim is to improve diagnostic tests and care of patients with autoimmune diseases as a central concern of the European Autoimmunity Standardization Initiative (EASI).

Key words: autoimmune diseases; autoantibodies; antinuclear antibodies; methods; laboratory diagnosis; standardization; HEp-2 cells; microscopy; immunofluorescence

Introduction

Non organ specific autoantibodies (AABs) are directed against highly conserved antigens of the body’s own cells. Diagnostically relevant target structures are predominantly located in the cell nucleus (antinuclear antibodies, ANA), but also in the cytoplasm. Screening for these AABs constitutes a major part of the diagnostic procedure for connective tissue and autoimmune liver diseases. Evidence is usually collected in a multistage diagnostic process in which initial screening is carried out using indirect immunofluorescence testing (IIF) on HEp-2 cells, an epithelial cell line derived from a human laryngeal carcinoma. HEp-2 cells have replaced the frozen sections of organs, which were originally used as a substrate. Used in primary screening, the main advantage of IIF on HEp-2 cells is that it provides a good overview of most of the diagnostically relevant non-organ-specific AABs and their concentrations.

Originally, immunological laboratories prepared their own HEp-2 cells, and diagnostic use of these cells was subject to great variability because of individual culture and fixation conditions. Nowadays preparations of acceptable quality are available from manufacturers of diagnostic equipment on standardized microscope slides as in vitro diagnostic material. The use of cells in IIF cannot be replaced by the use of lysed HEp-2 cells in enzyme immunoassays because only immunofluorescence can deliver information about all diagnostically relevant AABs.

In spite of the now-acceptable quality of the available HEp-2 preparations for immunofluorescence, the results from different laborato-

Reagents and Test Preparation

HEp-2 Cells

HEp-2 cells are available from numerous suppliers as a CE-certified diagnostic aid. They can also be obtained from cell banks (such as American Tissue and Cell Collection CCL-23; http://www.atcc.org) as a cell line for scientific investigations. The cells have been found to be heterogeneous in their morphology, antigen expression, and cell division behavior. Modified HEp-2 cells also exist in which the expression of particular antigens, such as Ro60 has been increased by transfection. Certain criteria are important for the judgment of the cells’ quality and can be heavily influenced in the production process by cell culture conditions, cell preparation, microscope slide preparation, fixation, and the processing instructions given. These criteria are:

1. cell density and distribution on the microscope slide
2. number of mitoses (at least 3 to 5 mitoses per visual field at 200× magnification)
3. expression of target antigens for relevant autoantibodies
4. maintenance of morphology
5. background fluorescence

Before it is used in the laboratory, the quality of each batch should be tested using defined monitoring procedures. There is so far no standardization of the preparation of HEp-2 cells or the composition of the test kits among manufacturers, although this would make it possible to ensure that differences in titer levels and fluorescence patterns detected on evaluation do not arise as a result of preparation procedures. Furthermore, only standardized procedures make possible longitudinal measurements and comparison of results from different laboratories. Batch monitoring requires the use of one negative serum and at least three positive serum samples with different fluorescence patterns resulting from defined antibody reactivity (e.g., centromeres, dsDNA, Ro/SS-A). These must be used in alternation each time the test is run.

Workplace

The preparation of IIF tests and subsequent evaluation by fluorescence microscopy are typically carried out in separate areas. Immunofluorescence laboratories it is especially important to ensure low levels of dust. The microscopy room must be large enough to allow two people to work simultaneously and must be adequately ventilated. Considerable amounts of heat can be produced, especially when microscopes with high-pressure mercury lamps are used. A network connection for laboratory computers simplifies working procedures and documentation.

Assay Procedure

All samples to be processed must be identifiable at all times with the help of the order specification and sample labeling.

Processing of Samples

Pre-analytic procedures are not critical for detection of autoantibodies. Hemolytic, lipemic, and icteric sera should be recorded in the protocol, as they may influence the test system.

As a rule the reagents used have a CE certificate, so that analysis must be carried out according to the manufacturer’s instructions. Deviations from incubation times, dilutions, or buffering systems can influence the test results when the microscope slides are evaluated and must therefore be validated in the laboratory. Samples are processed at room temperature (20 to 24°C).

When microscope slides from different manufacturers are used, it is necessary to ensure that the corresponding test components are used in each case. Buffer systems and conjugate concentrations, in particular, are usually adjusted for the relevant substrates on the microscope slides.

It is also necessary to be aware of the expiry date and the correct storage method for each of the reagents used. With conjugate, in particular, a decrease in intensity is otherwise to be expected, which can lead to a lower titer of the autoantibody of interest.

The volumes of serum and conjugate to be pipetted or dripped onto the appropriate application sites are often not clearly defined. Microscope slides frequently have application sites of different sizes. It is necessary to ensure that the whole application site is covered with serum/conjugate, but an application site should not be allowed to overflow because this immediately puts the next application site at risk of contamination. It is recommended that individual laboratories determine the optimal volumes for each microscope slide and include these in the corresponding internal instructions (Standard Operating Procedures, SOPs).

The individual incubation stages should be carried out in a humidity chamber to prevent the sample from desiccation, which would significantly reduce the sample volume per
application site. Easily cleanable flat plastic boxes are used for this purpose. The floor of the box is covered with a porous carrier material, such as cellulose, which is easy to moisten and should be renewed regularly. The microscope slides should not be placed directly on top of this material.

The actual test starts when the serum, which has been diluted with the buffers supplied within the test kit (usually phosphate-buffered isotonic saline, PBS), is added to the HEp-2 cell monolayer. Under no circumstances should serum spill over onto adjacent fields. The incubation time is usually 30 min, normally followed by three 10-min washes in PBS. When cuvettes are used, the washing time is shortened to $3 \times 2$ to 5 min. The use of automatic washing equipment should be considered only after rigorous evaluation since it often gives unsatisfactory results because of increased background staining or cell detachments.

When washing the individual microscope slides, it is important first to remove all the serum or conjugate from each application site by briefly rinsing with washing buffer before placing the slides into the cuvette with washing buffer. This procedure is intended to prevent high-titred and high-avidity antibodies on other application sites from causing cross-contamination and false-positive results, even when incubation times are short.

After addition of the fluorescent-labeled secondary antibody (conjugate) and an incubation period which normally lasts 30 min, the slide is washed again. The use of fluorescein isothiocyanate (FITC)-labeled secondary antibodies to human IgG is very common and, in our opinion, ideal. The value of additional use of secondary antibodies to other immunoglobulin classes needs to be investigated further. The FITC-labeled secondary antibodies are often treated with 0.01 g/IL Evans Blue to make the fluorescence signal easier to distinguish.

To complete the test, the microscope slides are covered with a mounting medium containing glycerine (about 80% glycerine in PBS). This usually contains an antibleaching substance. The ideal quantity of mounting medium per microscope slide should be fixed for each laboratory individually. A large excess of mounting medium can lead to fogging and poor focus when slides are evaluated microscopically. Manufacturer-specific differences in mounting medium should be taken into consideration here.

The practice of cleaning or drying around the application site with a paper towel or swab, which has become established at some laboratories, is unnecessary and causes errors by wiping substances into the cells and introducing dust and fibers. Gentle tapping of the microscope slide and drying on an absorbent surface are sufficient.

After evaluation, the prepared slides can be kept in a refrigerator and analyzed for up to 24 h. However, after longer periods, reanalysis is difficult due to diffusion of the antibodies and bleaching-out of the fluorescent dyes, especially when the reactivity is weak. A prepared slide should therefore be hermetically shrink-wrapped and stored at $-20^\circ C$ in case another analysis needs to be carried out later for monitoring or comparison purposes.

**Serum Dilution**

A serum dilution of 1:80 is used in screening for antibodies. The majority of test kits are designed so that 95% of sera from healthy control subjects show no staining, while sera with diagnostically relevant autoantibodies are detected. Reagents put together by individual laboratories (laboratory-made tests) must be adjusted during validation so that, at this dilution, negative sera from healthy blood donors are not recorded. The adjustments carried out by the manufacturer during batch monitoring must always be verified at the laboratory. It may be necessary to check whether changes of the testing procedure need to be made. However, these must also be validated. The
TABLE 1. Typical Nuclear and Cytoplasmic Fluorescence Patterns of Anto-Antibodies

<table>
<thead>
<tr>
<th>Patterns</th>
<th>HEp-2 Fluorescence</th>
<th>Common Antigens</th>
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<tbody>
<tr>
<td><strong>nuclear patterns</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homogeneous</td>
<td>homogeneous or fine granular nuclear fluorescence,</td>
<td>dsDNA, nucleosomes, histones</td>
</tr>
<tr>
<td></td>
<td>chromatin in metaphase positive</td>
<td></td>
</tr>
<tr>
<td>fine granular</td>
<td>fine/intermediate granular nuclear fluorescence,</td>
<td>Ro/SS-A, La/SS-B, Ku, Mi-2</td>
</tr>
<tr>
<td></td>
<td>negative metaphase chromatin</td>
<td></td>
</tr>
<tr>
<td>coarse granular</td>
<td>coarse granular nuclear fluorescence with numerous</td>
<td>U1-RNP, Sm</td>
</tr>
<tr>
<td></td>
<td>condensations, excluding nucleoli</td>
<td></td>
</tr>
<tr>
<td>nucleolar</td>
<td>nucleolar fluorescence</td>
<td>Scl-70 (chromosomal association); PM-Scl, Fibrillarin, Th/To</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>centromere</td>
<td>dot count according to interphase chromosome</td>
<td>CENP-B</td>
</tr>
<tr>
<td></td>
<td>number AND mitosis chromatin</td>
<td></td>
</tr>
<tr>
<td>nuclear dots</td>
<td>multiple nuclear dots (commonly 13 to 25 per nucleus)</td>
<td>Sp100</td>
</tr>
<tr>
<td>pleomorphic</td>
<td>heterogeneous staining of interphase nuclei</td>
<td>PCNA</td>
</tr>
<tr>
<td><strong>cytoplasmatic patterns</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>homogeneous</td>
<td>cytoplasm homogeneous to fine granular</td>
<td>Rib-P (with positive nucleoli)</td>
</tr>
<tr>
<td>granular</td>
<td>fine to intermediate granular cytoplasm (or granular dots)</td>
<td>Jo-1</td>
</tr>
<tr>
<td>mitochondrial</td>
<td>Fine-stitched cytoplasmatic fluorescence</td>
<td>AMA-M2</td>
</tr>
<tr>
<td>cytoskeletal</td>
<td>cytoskeleton associated fluorescence</td>
<td>Actin</td>
</tr>
</tbody>
</table>

manufacturers should offer an adjustment here to accord with the consensus protocol.

In the case of a positive result, the test is repeated using a geometric series of dilutions according to the strength of the primary fluorescence (1:160 to 1:5120). Titers of at least 1:160 are taken to be diagnostically relevant provided the test has been carried out and evaluated correctly. A titer of 1:80 can be seen as borderline because, in the majority of cases with this titer, no diagnostically relevant ANA specificities are found (Table 1).

Microscopy

Precise pattern evaluation is carried out at 400× magnification (combination of 10× eyepiece and 40× objective). First the intensity of the fluorescence is judged. The evaluation must take into account several mitoses so that a clear decision on the presence or absence of fluorescence as well as a statement with regard to the basic pattern can be made (Table 1).

The light intensity should not be less than that provided by a 50-watt mercury vapor lamp. Today, light emitting diodes (LEDs) are increasingly being used as a light source. Their constant brightness and long life (over 10,000 h of use) make them a real alternative to mercury vapor lamps (100 to 300 h of use depending on type; adjustment is necessary when bulbs are changed).
Efforts to standardize fluorescence intensity were not yet ultimately successful. Approaches such as the use of defined gray filters, standardized fluorescent beads,\textsuperscript{6} or computer-assisted image analysis programs\textsuperscript{7–10} are currently being evaluated.

**Evaluation and Interpretation**

HEp-2 cells contain a broad spectrum of detectable autoantigens. Primarily attention is directed toward nuclear patterns, but cytoplasmic patterns should also be considered. Mixed
patterns are common. The individual patterns must be specified in the report, with details of different titers if appropriate.

Certain patterns in IIF indicate disease-relevant AAB specificities and are an indication for using specific immunoassays. In an effective multistage diagnostic process it is therefore necessary to identify the autoantibodies that are typically responsible for a particular pattern. In the case of fluorescence patterns that cannot be characterized more closely, and whose clinical relevance is unknown, a note should at least be included to inform the clinician about the pattern so that future investigators can recognize it again.\(^{11}\)

Antinuclear antibodies are subdivided into a few basic patterns. These patterns are often also present as mixed patterns. These can be differentiated by means of observation at different dilutions because, for example, a strong homogeneous fluorescence can mask other patterns at low serum dilutions and thus mask them. Depending on the suspected diagnosis it is useful to pay specific attention to the fluorescence patterns named in Figure 1. The evaluation must be carried out by experienced workers who regularly undergo further training in autoimmune diagnostic procedures. Increasingly, attempts are being made to use computer-assisted interpretation aids; initial experiences in this area have recently been published.\(^{6,8}\)

Depending on the diagnostic questions posed and the fine characterization required for a differential diagnosis, screening should be followed by further diagnostic procedures based on specific immunoassays (radio, enzyme, or dot/line immunoassays) using highly purified (natural or recombinant) autoantigens.

The diagnostic report must include the possible target antigens as well as a description of the fluorescence patterns found and their titers. Above all, the report needs to contain an assessment of the findings in the light of the diagnostic questions posed. The latter applies particularly for negative results. Because, in older patients, low-titred autoantibodies are often not an indication of illness, differential tests can be omitted if there is no clinical indication. It is not sufficient to report findings with the comment “positive ANA” without giving details of the pattern.

**Conflicts of Interest**

The authors declare no conflicts of interest.

**References**

