



Original Contribution

COMPARATIVE ANALYSIS OF IMMUNOPEROXIDASE AND IMMUNOFLUORESCENT ASSAYS ON DIFFERENT SUBSTRATES FOR SCREENING ANTINUCLEAR ANTIBODIES

Victoria Sarafian^{1*}, Marianna Murdjeva², Marian Draganov³, Pepa Ganchevska¹

¹Department of Medical Biology, Medical University, Plovdiv, Bulgaria

²Department of Microbiology and Immunology, Medical University, Plovdiv, Bulgaria

³Department of Developmental Biology, University of Plovdiv, Bulgaria

ABSTRACT

The aim of this study was to compare the information obtained from the immunofluorescent and immunoperoxidase assays (IFA and IPA, respectively) on the standard HEp-2 cell line and the new serum-free cell line McCoy-Plovdiv as screening substrates for screening antinuclear antibodies (ANA). 126 sera from patients with connective tissue diseases were examined simultaneously on HEp-2 and McCoy-Plovdiv cells with both IFA and IPA. Commercial fluorescent and peroxidase labeled conjugates and kits were used. An optimized protocol for IPA on McCoy-Plovdiv cells was then developed. Best results were obtained when sera and conjugates were dissolved in 0,2% Tween-X100. Both techniques were compared on the commercial and the new cell line and showed identical specificity and type of reactivity (homogenous, nucleolar, speckled, rim). The new serum-free cell line McCoy-Plovdiv is a biologically suitable and financially cost-effective substrate for detection of ANA. We suggest the application of IPA as an informative and reliable technique that could be used as an alternative to IFA for ANA testing in small immunological laboratory settings.

Key words: Antinuclear antibodies, Immunofluorescent assay, Immunoperoxidase assay, McCoy-Plovdiv, HEp-2, Cell cultures

INTRODUCTION

Antinuclear antibodies (ANA) play a significant role in the pathogenesis of systemic autoimmune diseases. Their detection is a major diagnostic parameter that confirms correct clinical diagnosis. ANA are also important prognostic markers for clinical and therapeutic monitoring of autoimmune diseases (1)

In spite of the vast array of immunological techniques for ANA detection, IFA is still considered the "golden standard" for their identification (2). The IPA is usually applied in laboratories lacking fluorescent microscopy. In both methods the HEp-2 cell line is used as a substrate.

McCoy-Plovdiv is a novel serum-free cell line derived from the original synovial cell line McCoy (3). It has been shown to be suitable and cost-effective substrate for

cultivation of different pathogens (4, 5) and for cytotoxic studies (6).

In view of this, the aim of the present study is to compare the specificity and informative value of IFA and IPA on the standard HEp-2 cell line and the new serum-free cell line McCoy-Plovdiv in ANA screening.

MATERIALS AND METHOD

126 sera from patients with connective tissue disorders were examined simultaneously on HEp-2 and McCoy-Plovdiv cells with both IFA and IPA.

IFA: McCoy-Plovdiv cells grown on slides or commercially available HEp-2 cells (*The Binding Site*, UK) were incubated for 30 min at room temperature with sera diluted 1:40. After washing in PBS for 3 min an anti-IgG - FITC conjugate (National Center for Infectious and Parasitic Diseases, Sofia or *The Binding Site*, UK) was applied and left in the dark for 30 min. After another washing, the slides were cover-slipped. The reaction was read on a Nikon fluorescent microscope at magnification 400x. The intensity of

* Correspondence to: Dr Victoria Sarafian;
Department of Medical Biology, Medical
University – Plovdiv; 15a V. Aprilov Blvd;
Plovdiv-4000, Bulgaria; Tel. +359 32/602 531;
E-mail: sarafian@abv.bg

fluorescence was estimated as 1+ (weak reactivity), 2+ (medium), 3+ (medium strong), and 4+ (strong fluorescence).

IPA: An optimized protocol of IPA for screening ANA on the serum-free cell line McCoy-Plovdiv was developed. Briefly, cells were grown on sterile slides till 80% confluent state, then washed in PBS and fixed in acetone. Patients' sera were diluted 1:40 in PBS, as well as in PBS/TweenX-100 with different concentrations of the detergent - 0,05%, 0,1% and 0,2%. McCoy-Plovdiv cells treated with diluted sera, together with the appropriate Controls (PBS instead of patient serum), were incubated for 30 min in

a humid chamber at room temperature. After washing in PBS, a purified goat anti-mouse IgG-peroxidase conjugate (*The Binding Site, UK*) was applied for another 30 min in a humid chamber in a dark environment. The conjugate was diluted 1:200, 1:300, 1:400 in PBS, as well as 1:200 in 0,25TweenX-100/PBS. The reaction was visualized using the substrate aminoethylcarbazole. A nuclear red-brownish stain confirmed positive reactivity. When the HEp-2 cell line was used as a substrate for ANA screening, the IPA was performed according to the manufacturer's instructions.

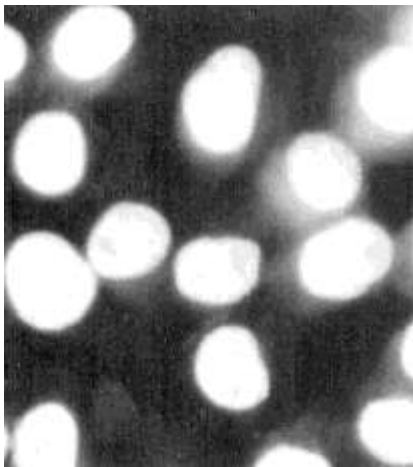


Figure 1a. Homogenous pattern of reactivity.

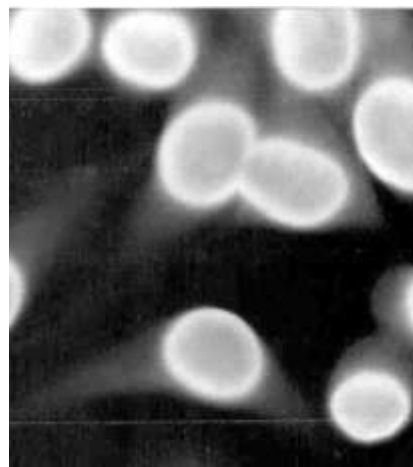


Figure 1b. Homogenous/rim pattern of reactivity.

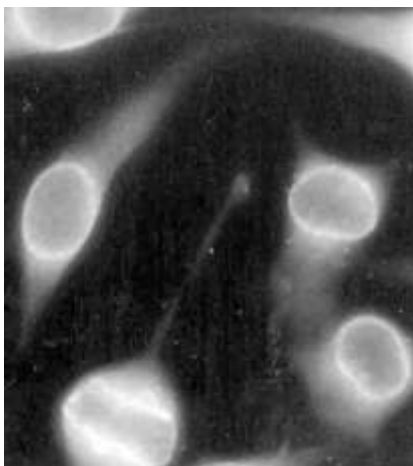


Figure 1c. Rim pattern of reactivity.

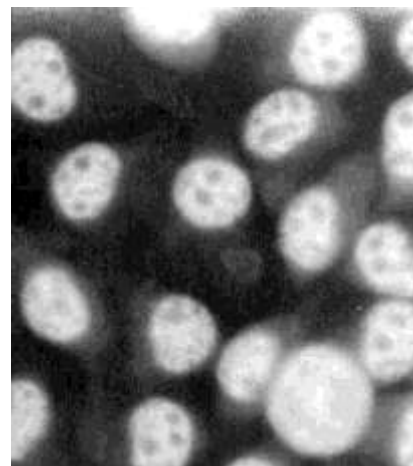


Figure 1d. Speckled pattern of reactivity

Figure 1. IFA for screening ANA in McCoy-Plovdiv cell line.

RESULTS

The application of both immunological techniques on both cellular substrates McCoy-Plovdiv and HEp-2 showed identical ANA-reactivity and intensity profiles. The

following reactivity patterns were detected: homogenous, speckled, rim, nucleolar, speckled/rim, homogenous/rim, homogenous/scattered. They were observed

in both the IFA and IPA (**Figure 1** and **Figure 2**, respectively).

The reactivity patterns in both immunological techniques were identical.

The diagnostic sensitivity with McCoy-Plovdiv cells was 89,6%, while it was 92,7% with HEp-2 cells.

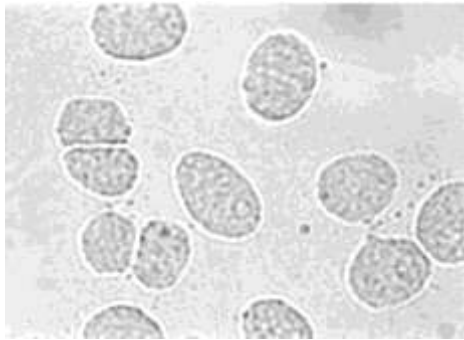


Figure 2a. Homogenous pattern of reactivity. HEp-2 cells.

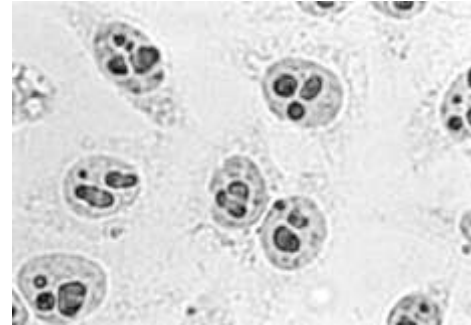


Figure 2b. Nucleolar pattern of reactivity. HEp-2 cells.

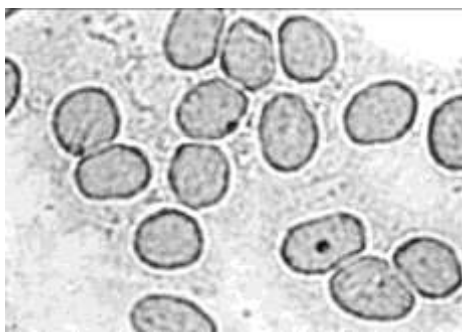


Figure 2c. Homogenous/rim pattern of reactivity. HEp-2 cells.

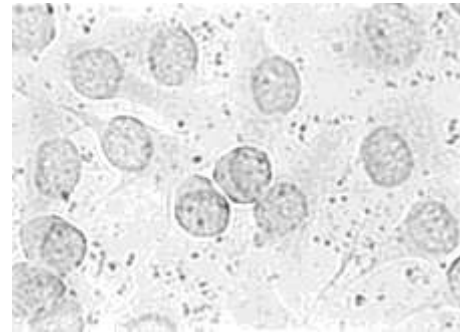


Figure 2d. Homogenous/rim pattern of reactivity. McCoy-Plovdiv cells.

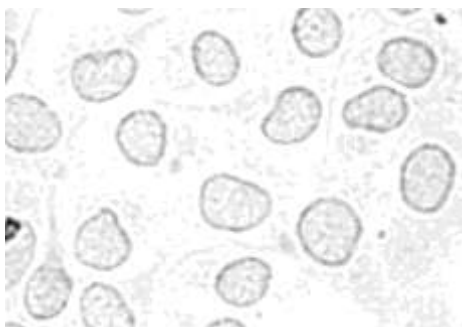


Figure 2e. Rim/speckled pattern of reactivity. McCoy-Plovdiv cells.

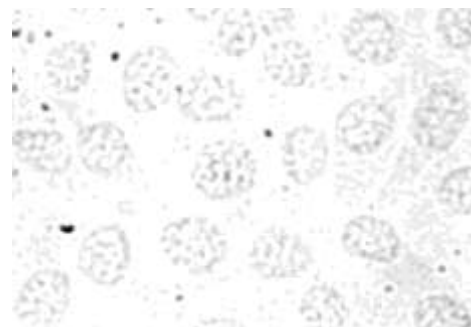


Figure 2f. Speckled pattern of reactivity. McCoy-Plovdiv cells.

Figure 2. IPA for screening ANA in HEp-2 cells and in McCoy-Plovdiv cell line.

DISCUSSION

This paper presents a comparative study of IFA and IPA for ANA screening using a substrate made in our laboratory. The results obtained with this new substrate, the serum-free McCoy-Plovdiv cell line, were judged against those from a standard cell line HEp-2 known as the classical substrate for ANA

testing (7). The diagnostic and prognostic importance of ANA-detection in clinical practice is growing in the sphere of systemic connective tissue disorders (8, 2, 9). It therefore necessitates the relentless search for new, rapid, cost-effective and elucidative laboratory techniques.

Our investigation puts forward an optimized protocol for ANA screening using

the IPA on McCoy-Plovdiv cells. Unlike HEp-2 cells, the McCoy-Plovdiv cell line shows a better reactivity in IPA when both sera and IgG-peroxidase conjugate are diluted in 0.2% Tween-X100. Unlike the IFA, where no permeabilization is needed, such a step is required in IPA probably because of the higher molecular weight of the peroxidase enzyme compared to the FITC-molecule. In order to reach and stain cell nuclei larger pores on both plasma and nuclear membranes are needed. The fixation step with ice-cold acetone and the subsequent incubations with sera and conjugates diluted in a mild detergent like Tween-X100 guarantee the reagents' entry into the nucleus. In the manufacturer's guide neither details for fixing HEp-2 cells nor the permeabilization step was given. So we assumed that HEp-2 cells had not been previously treated with any detergent. A possible explanation for the increased entry of the peroxidase conjugate in HEp-2 nuclei could be due to the larger nuclei of these cells compared to those of the McCoy-Plovdiv cells. Our comparative investigation has revealed identical reactivity patterns in the two immunological techniques on both cellular substrates. The diagnostic sensitivity of IPA on McCoy-Plovdiv cells was 89,6%, which was quite comparable with that on HEp-2 cells that was 92,7%. Bearing in mind that serum-free cell lines in culture medium exclude the interaction between serum components and the structures or molecules of the cell lines, this new cell line could be a reliable substrate candidate for immunological assays. In addition, the cost-effectiveness of such a substrate should not be neglected. It has been shown that the McCoy-Plovdiv cell line could be about 20 times cheaper than its parent cell line – the

serum-supplemented cell line McCoy (3). A similar conclusion could have been drawn if the HEp-2 cell line were considered.

On the basis of our comparative analysis of both cellular substrates we hereby submit that the McCoy-Plovdiv serum-free cell line would be a biologically suitable and inexpensive substrate for ANA screening. The application of the IPA as an informative and reliable technique for ANA testing is being recommended. It could be utilized as an appropriate alternative of IFA in small immunology laboratory settings lacking complicated fluorescent equipment. Our study offers not only an additional approach to routine diagnosis but also optimizes ANA diagnostic assays by being both informative and cost-effective.

On the basis of our comparative analysis of both cellular substrates we hereby submit that the McCoy-Plovdiv serum-free cell line would be a biologically suitable and inexpensive substrate for ANA screening. The application of the IPA as an informative and reliable technique for ANA testing is being recommended. It could be utilized as an appropriate alternative of IFA in small immunology laboratory settings lacking complicated fluorescent equipment. Our study offers not only an additional approach to routine diagnosis but also optimizes ANA diagnostic assays by being both informative and cost-effective.

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