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Assessment of lymphocyte proliferation for diagnostic purpose: Comparison of CFSE staining, Ki-67 expression and ^{3}H-thymidine incorporation

Jan Laštovička *, Michal Rataj, Jiřina Bartůňková

Department of Immunology, 2nd Medical School and University Hospital Motol, Prague, Czech Republic

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ABSTRACT

The capability of lymphocytes to respond to antigenic or mitogenic stimulation is an important feature in the diagnosis of various immunodeficiencies and immune disorders. We used large cohorts of both immune compromised patients and healthy controls to measure lymphocyte proliferations by means of three methods: CFSE staining, Ki-67 expression and ^{3}H-thymidine incorporation. The advantages and disadvantages of each method was then evaluated for use in routine clinical diagnostic. The statistical analysis was performed between the outcomes and the correlation between all three methods was computed. CFSE and Ki-67 assay correlated well with the r = 0.767, correlation between Ki-67 expression and ^{3}H-thymidine incorporation was 0.546 and correlation between CFSE staining and ^{3}H-thymidine incorporation was 0.337. The differences between these three methods concerning complexity, sensitivity and reliability as well as the financial aspects are discussed hereafter. CFSE and its analogues provide the cheapest and reasonable choice for measuring lymphocyte proliferation, while Ki-67 represents a more expensive, but more sensitive and robust method. The original ^{3}H-thymidine assay does not bring any advantages and cannot compare to the competition presented by modern flow cytometric methods available today.
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1. Introduction

The capability of lymphocytes to respond to antigenic or mitogenic stimulation is an important feature in the diagnosis of various immunodeficiencies and immune disorders. Alternative methods for assessment of lymphocyte proliferation are available, but not all of them are suitable for routine clinical use. Robustness, ease-of-use and cost are the most important factors when considering the practical application for clinical diagnostics. Within this paper we compare three different methods, which are contemporarily used for assessment of proliferation, and evaluate their capability to meet the requirements of a routine immunological laboratory.

Historically, the thymidine uptake method was the first approach for the laboratory evaluation of lymphocyte proliferation. This method remains present in the repertoire of many immunological centers worldwide, for it yields relatively reliable outcomes with sufficient sensitivity at reasonable cost, despite pitfalls which include the use of radioactive labels. However, modern non-radioactive methods based on flow cytometry evaluation slowly displaced this approach and replaced the ^{3}H-thymidine at first by other thymidine analogues like 5-bromo-2-deoxyuridine [1]. The disadvantage of BrdU assay lies in the necessity of DNA denaturation, since the antibodies against BrdU cannot reach it in native DNA due to steric reasons. This problem was later solved through other analogues like 5-ethynyl-2-deoxyuridine, and with a detection method that is not antibody-based, so it does not require DNA denaturation. Unfortunately this method is rather expensive and therefore remains restricted to research purposes. As one could expect, the correlation between all these incorporation assays is very strong [2]. Moreover these new methods overcome the main drawback of a non-cytometric approach, which is to quantify only overall proliferation without the ability to discern

Abbreviations: PHA, phytohemagglutinin; PBMC, peripheral blood mononuclear cells; CFSE, carboxy fluorescein succinimidyl ester; BrdU, 5-bromo-2-deoxyuridine; CPM, counts per minute; SI, stimulation index; FASCIA, Flow-cytometric assay for specific cell-mediated immune-response in activated whole blood; 7-AAD, 7 amino-actinomycin D.

* Corresponding author.
E-mail address: jan.lastovicka@lfmotol.cuni.cz (J. Laštovička).

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the individual lymphocyte subpopulations. Supplementary labelling of cells with fluorochrome-labelled antibodies facilitates the identification and monitoring of various cell phenotypes.

Parallel to the aforementioned thymidine analogues the entire collection of flow cytometric methods for measuring lymphocyte proliferation was developed. They are based on four basic principles.

FASCIA [3,4], simple flow cytometric method, is based upon quantification of the total amount of lymphoblasts in cultures of lymphocytes stimulated with mitogen. The test is performed from the whole blood sample and the correlation to ^{3}H-thymidine assay was reported as good (r = 0.78).

Another group of flow cytometric tests uses DNA staining with various DNA dyes like propidium iodide [5]. The limitation of this analysis is that these DNA content-based dyes cannot differentiate between quiescent cells in G_0 and cells in G_1 phases and cannot discern between DNA and RNA, necessitating treatment with nucleases to distinguish between RNA and DNA staining [6].

Recent possibilities for determination of lymphocyte proliferation were discovered in the tracking of some intracellular molecules, which are closely related to cell division. These molecules like Ki-67, PCNA or nucleophosmin (NPM, B23) [7] appear predominantly when the cell leaves G_0 phase [8,9]. The most suitable molecule for detection of proliferation, apart from that mentioned above, seems to be the Ki-67.

The final approach on how to detect proliferation is to stain the cytoplasm of the cell. There are tens of various dyes available which bind to certain cytoplasmic structures and remain in the cell for extended periods of time, often for the whole life span. These fluorescent dyes are easily detectable by flow cytometry and allow tracking every cell division.

We leveraged a large cohort of both immune compromised patients and healthy controls to measure lymphocyte proliferations by means of three methods: CFSE staining, Ki-67 expression and ^{3}H-thymidine incorporation. We then evaluated the advantages and disadvantages of each method for use in routine clinical diagnostics. The following aim was to illustrate how these methods correlate together in the groups of evaluated patients.

2. Materials and methods

2.1. Patients

Blood samples were taken from 190 patients of the Department of Immunology, University hospital Motol in Prague, in various stages of immune recovery after bone marrow transplantation. These samples were examined in this study together with the control group (48 healthy individuals). The signed consent from all patients and donors was obtained and the experiments were performed with the agreement of the local ethical committee.

2.2. PBMC preparation

Human peripheral blood mononuclear cells were isolated using the Ficoll-Paque density gradient centrifugation as described earlier [10].

2.3. ^{3}H-thymidine assay

For ^{3}H-thymidine assay cells were cultivated for 72 h in 96 well microtitre plates (Nunc) in X-VIVO medium (Lonza) supplemented with 10% FCS in concentration $0.5x10^{6}$/ml with or without PHA 5 μg/ml (Sigma Aldrich) in triplicates, 2×10^{5} cells per well. For the last 18 h of cultivation ^{3}H-thymidine (TH-6[6/3H] 1 mCi, Perkin Elmer) was added for a final concentration of 0.2 MBq/ml. Cells were then harvested and ^{3}H activity was measured by means of liquid scintillation counting (Tricarb, Perkin Elmer). From the cpm (counts per minute) values the stimulation index (SI) was calculated (PHA stimulated/unstimulated cpm).

2.4. CFSE staining

For CFSE staining, $2 - 5 \times 10^{6}$cells were resuspended in 1 ml PBS and 1 μl of 0.25 mM CFSE (Cell-Trace CFSE Proliferation Kit, Molecular Probes, Invitrogen) was added (final concentration 2.5 μM), mixed well and incubated for 15 min in darkness, stirring occasionally. Then the reaction was stopped by adding 2 ml of PBS with 5% FCS, followed by centrifugation. This wash step was repeated 3 times, and the final instance included X-VIVO medium (Lonza) supplemented with 10% FCS. Cells were then counted and resuspended in X-VIVO medium with 10% FCS. Stained cells were then cultivated in 1 ml tissue culture plates (Nunc) for 72 h with or without PHA 5 μg/ml.

2.5. Ki-67 expression

Isolated PBMC were cultivated for 72 h as described above with or without PHA 5 μg/ml, then harvested and stained with *anti*-CD3 PB (BioLegend, clone HIT3a) for 20 min, permeabilized for 30 min at 4 °C with Fixation/Permeabilization solution (eBioscience) according to manufacturer's instructions, washed, and stained with *anti*–Ki-67 PE (BioLegend, clone Ki-67) at 4 °C for 30 min.

2.6. Flow cytometry

Flow cytometry samples were analyzed on BD FACS CANTO II (BD Biosciences). 7-AAD (Life Technologies) was used to exclude dead cells in CFSE stained cells. The average amount of dead cells in CFSE samples was 5% (data not shown). The samples for Ki-67 measurement were stained randomly for viability before intracellular staining was performed and the viability proved to be >99% (data not shown). At least 50.000 cells were analyzed in each sample. Data were analyzed using FlowJo software (FlowJo LLC).

2.7. Statistics

Pearson's test was used to determine bivariate correlations. Statistical analyses were performed using GraphPad Prism 4 (GraphPad).

3. Results

3.1. Influence of CFSE staining on lymphocyte proliferation

The negative influence of CFSE on lymphocytes viability, previously described by our group [10] was confirmed in our recent experiments. CFSE in 5 μM concentration, which is routinely used and recommended for lymphocyte proliferation studies [11], influenced markedly the proliferation as measured either by ^{3}H thymidine assay (Fig. 1A) or by Ki-67 expression (Fig. 1B). In the Ki-67 assay 5 μM concentration of CFSE suppressed the proliferation of PBMC by 10%. In the majority of outcomes the CFSE histograms of both controls and patients look like Fig 3a, with detectable peaks of every cell division. However, in some cases, especially in patients with severe immune deficiency the cell division is not visible at all and the histograms look like Fig. 2b, c, d, e.

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