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The assessment of intracellular cytokine expression with three-colour flow cytometry technique

Zastosowanie techniki cytometrii przepływowej w ocenie wewnątrzkomórkowej ekspresji cytokin

Cytokines are protein or glikoprotein molecules that play the crucial role in regulation of cell proliferation and differentiation. They can be secreted by T and B lymphocytes, monocytes, macrophages, mast cells, fibroblasts, endothelial cells, platelets. They act in auto- or paracrine way after binding with specific membrane receptors [8]. Following such binding signaling cascade is generated which influence the proliferation, maturation and apoptosis of cells. Cytokines connecting with many cells are mediators of immunological and inflammation responses and influence hematopoesis. [4]. The accurate determination of cytokine levels can be difficult. In general, released cytokines interact with target cells which are often adjacent to the producer cells, resulting in short lifetimes of the circulating protein. In addition producer cells may secrete very small amounts of cytokine [1]. Commonly used ELISA method can detect total quantities of cytokine. There are also some methods such as Northern blotting, RNase protection assays, RT-PCR that measured cytokine messenger RNA, but these methods are time consuming and often need prior separation of cells.

The new technique that has made possible to identify cytokine producing cells at the level of the individual cell is flow cytometry [4]. The generation of monoclonal antibodies (MAb) against intracellular cytokines conjugated to different fluorochromes allows simultaneous flow cytometric analysis of the individual cell phenotype and its cytokines expression. It is possible to detect intracellular cytokines in virtually every cell subset of interest identified according to the expression of typical surface molecules [2].The flow cytometry analysis of cytokines allows to determine cytokines both in *peripheral blood mononuclear cells* (PBMCs) isolated by gradient density centrifugation and in whole blood sample, without cells separation.

This study was set up to introduce basis of the method of intracellular cytokine detection in activated PBMCs with use of three-colour flow cytometry technique on the example of detection IL–2, IL–4, IFN gamma in normal individual CD3+, CD4+ and CD8+ lymphocytes.

INTRODUCTION OF THE METHOD

A total of 20 ml of peripheral blood was taken into heparinised tube (sodium heparin). PBMCs were isolated by density gradient centrifugation, then washed with PBS without Ca^{2+} and Mg^{2+}, finally resuspended at a concentration of 2 x 10^2 cells/ml in culture medium. This medium was consisted of RPMI 1640 with 1% 2 mM L–glutamine, 1% antibiotic, 10% heat inactivated calf serum. Because of the small amount of cytokine spontaneously produced by cells, detection of intracellular cytokines recquires prior cell activation.

There are some different T cell stimulation procedures such as PMA and ionomycin, anti–CD3 and anti–CD28, superantigen, PHA, but they yield a similar cytokine profile [3]. We used PMA and ionomycin activation assay in our procedure. PMA (Phorbol 12–myristae 13–acetate) was used at a final concentration 25 ng/ml of cell culture and ionomycin at a final concentration of 1ug/1ml of cell culture. The very important step during cell stimulation in culture is the addition of protein secretion inhibitor, that inhibits cytokine secretion and increase the signal of stained cells detectable [4]. These inhibitors reversibly block the cell Golgi complex that prevent secretion of synthetised cytokines. Brefeldin A and monensin are the best known Golgi complex inhibitors and their inhibition effect are comparable [2]. We used Brefeldin A at a concentration of 10 ug/ml of cell suspension.

PBMCs culture with PMA, ionomycin and Brefeldin A were incubated for 4 hours at 37 C in 5% CO_2 atmosphere. After this stimulation period cells were washed with PBS without Ca^{2+} and Mg^{2+}, divided into tubes at a concentration about 5 x 10^5 cells per tube and submitted to membrane and cytoplasmic staining procedure. The first step was membrane staining by monoclonal antibodies — PerCP conjugated anti–CD3 (Becton Dicinson) and FITC conjugated anti–CD4 and anti–CD8 (Dako). 10 μl of each MAb was added to appropriate tubes and incubated 15 min at a room temperature, then washed. Following membrane staining the fixation of cells with paraformaldehyd was performed. This fixation step in cytokine detection procedure allows preservation of cell morphology and intracellular antigenicity prior to next step-permeabilisation. Cells are permeabilised with a detergent such as saponin, whitch allows anti-cytokine antibody to penetrate cell membrane and connect with cytokine inside the cell. To fix and permeabilise cells we used ready-to-use IntraPrep kit (Immunotech). Because of reversible manner of saponin action, anti-cytokine antibodies should be added in the presence of saponin. We used monoclonal antibodies PE-conjugated anti IL–2, IL–4, IFN gamma and followed up the IntraPrep procedure. These stained cells were measured in flow cytometer directly after procedure. Intracellular staining procedure recquire following controls assays, also used in our procedure:

- unstimulated control — used to assess the level of cytokine synthesis without in vitro activation. It is prepared by incubating PBMCs in the presence of Brefeldin A without PMA and ionomycin;
- isotype control — PE and FITC conjugated control monoclonal antibodies were used to detect non-specific binding on CD3+ PerCP cells;

Table I. Cytoplasmic IL–2, IL–4, IFNg expression on CD3+, CD4+, CD8+ cells in a normal individual. The data is presented as a percentage of CD3+ gated cells

CYTOKINE		CD3+	CD3+/CD4+	CD3+/CD8+
IL–2	Unst.	0,31	0,11	0,19
	St.	18,1	13,87	2,01
IL–4	Unst.	0,03	0,03	0.0
	St.	2,49	2,38	0,11
IFNγ	Unst.	0,68	0,12	0,0
	St.	10,94	5,56	4,32

Unst. — unstimulated cells; St. — stimulated cells

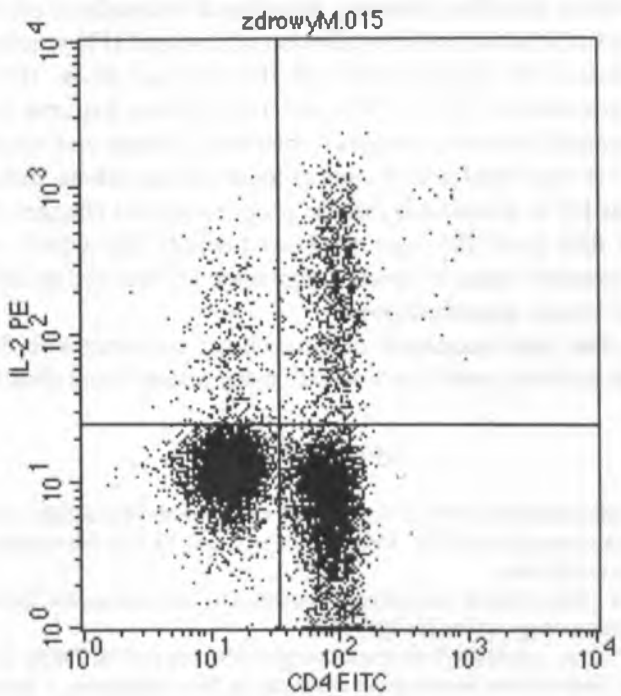

Fig. 1. Expression of IL-2 on CD3+/CD4+ cells in a normal individual

- activation and permeabilisation control — assessment of cytoplasmic expression CD69 (early activation marker) was used in permeabilised cells the nuber of activation cells and to checque the permeabilisation step. In our procedure we had 98.8% of CD69+/CD3+ cells after activation step.

Our samples were analysed by three-colour flow cytometry technique using BD FACSCalibur instrument and CellQuest software. We estimated the percentage of IL–2, IL–4, INF gamma positive CD3, CD4, CD8 cells gated on CD3 cells. Results are shown in table I and Figure 1.

APPLICATION OF THE METHOD

The analysis of cytokine production by flow cytometry technique gives a possibility to study immune response that is mediated by different cytokines. It becomes a very useful tool in immunological research easier then commonly used methods [1]. It allows to detect cytokine producing cells at the level of the individual cell [4] that makes this procedure most useful among others. The pattern of cytokine production can be used to analyse both the number and phenotype of cells that respond to various immune stimulation. These patterns provide significant information about responses to pathogens, vaccines, pahrmacological substances and other immune stimulation. Profile of cytokine stimulation can also allow for the distinction between normal and abnormal T–cell function under various conditions [7].

It makes evidence that flow cytometry detection of intracellular cytokines has also clinical application. Simultanous determination of IL–4 and IFN gamma producing T cells helps to identify Th1 and Th2 cells with IFN gamma+/IL–4– IFN gamma–/IL–4+ phenotype respectively [2]. The Th1 and Th2 cytokine patterns have now been implicated in several immune responses – infections, allergy and autoimmunity [6]. This technique has been used in such clinical conditions as asthma, multiple sclerosis, atopic dermatitis [2] in monitoring disease progression and changes after drug administration. A shift from Th1–type cytokines towards Th2–type is concidered as evidence and a possible cause of cancer progression [5] thus testing of Th1/Th2 balance can help in cancer immunotherapy.

It becomes clear that assessment of intracellular cytokines with flow cytometry technique is easy and very useful method with many research and clinical application.

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STRESZCZENIE

Cytokiny są cząsteczkami odgrywającymi kluczową rolę w regulacji procesów wzrostu i różnicowania komórek. Produkowane są przez komórki układu immunologicznego, a swoje działanie wykazują po połączeniu ze specyficznymi dla poszczególnych cytokin receptorami błonnowymi. Ocena cytokin wewnątrzkomórkowych przy użyciu techniki cytometrii przepływowej jest nową metodą badawczą pozwalającą na określenie ekspresji cytokin na poziomie pojedynczych komórek w niemal wszystkich odpowiednio wyznakowanych fluorochromami komórkach. Jest to łatwa i bardzo przydatna technika immunologiczna, mająca wiele zastosowań nie tylko badawczych, ale i klinicznych. Praca przedstawia podstawy metody trójkolorowego cytometrycznego oznaczania wewnatrzkomórkowej ekspresji cytokin na przykładzie oceny wybranych cytokin w limfocytach T osoby zdrowej.