IMMUNOPHENOTYPING - A TOOL FOR THE DIFFERENTIAL DIAGNOSTICS OF LYMPHADENOMEGALY

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Background: Flow cytometry is a method that enables the automated quantification of a set of parameters for a large number of cells or cell-like particles. It is also possible to analyze solid tissues after reduction to a single cell suspension. One of the applications of flow cytometry is immunophenotyping.

Aim: The authors try to introduce the basic physical and biological principles of flow-cytometry to the broader public and to prove the benefits of method in lymph nodes assessment.

Method: A common method of immunophenotyping with the help of labeled monoclonal antibodies applied to lymph node cells was used.

Results: Flow cytometry revealed the infiltration of the extirpated lymph node with leukemic cells.

Conclusions: The authors demonstrate flow cytometry as a relatively less common but efficient way of lymph node assessment in a patient with an anamnesis of acute lymphoblastic leukemia. They also discuss the contribution of immunophenotyping to the process of the differential diagnostics of lymph nodes enlargement.

INTRODUCTION

Flow cytometry is a method that enables the automated quantification of a set of parameters for a large number of cells or cell-like particles during each analysis session. It is also possible to analyze solid tissues but they have to be reduced to single cell suspension.

Cells or particles flow in a sheath fluid through a detection device. It is possible to collect 2 morphological parameters plus 1 or more fluorescence signals per single cell.

To allow the correct detection and measurement of morphological differences and staining patterns it is important to choose the right source of light and to adjust the instrument setting according the cell type to be analyzed (cell cultures, leucocytes, platelets, bacteria, spermatozoa, yeast) and the analysis to be performed (phenotyping, cell cycle, apoptosis, membrane properties, etc.)¹.

The single cell is exposed to the light source and is carried into flow cell where it meets the laser beam. The sheath fluid is drawn into a very thin stream in which the particles are distributed linearly (hydrodynamic focusing). The laser beam is focused in an elliptical shape and this is very important.

The beam coming out of the laser is radially symmetric, but the intensity varies with distance from the axis of the beam. If intensity versus distance from the axis is plotted, a Gaussian/normal distribution will be seen. In order to measure scatter and fluorescence signals from cells with a precision of a few percent, it is necessary that illumination be uniform within that same few percent over

the entire width of the sample or core stream. As long as the sample is flowing, we know that the cells will get through the plane, defined by the intersection of the axes of the illuminating beam and the collection lens, in which the observation point lies. However, while, under ideal conditions, we would like to have cells strung out along the axis of flow like beads on a string, there is probably some variation in position of cells in the core stream. If the beam is focused to a very small spot, the variation in intensity of illumination reaching cells at different positions will be too high to permit precise measurements.

Measurements show that if the diameter of the focused beam is about 100 μm , there will be only about 2 % variation in intensity over the width of a 20 μm sample stream. But if cells travel through the instrument at velocities in the range of 2-5 m/sec, it will take 20-50 μs for a cell to traverse a 100 μm beam. During this time most of the beam will be illuminating objects other than the cell, and any scatter or fluorescence signals from these objects will increase background noise level. Therefore current conventional devices focus the laser light into elliptical focal spot 5-20 μm high and about 100 μm wide to give optimal results².

When a particle passes across the light beam, it scatters some of the light in all directions. The amount scattered mainly forward (forward scatter = FS) and the amount scattered at 90° to the direction of the light beam (side scatter = SS) are measured. Forward scatter correlates with the cell volume, while side scatter is influenced by parameters such as the shape of the nucleus, cytoplasmic granules and the membrane projections. As a function of

Fig. 1. Emission spectra of 4 common fluorochromes (FITC = fluorescein isothiocyanate, PE = phycoerythrin, PerCP = peridinin chlorophyll protein, APC = allophycocyanin)⁵.

the morphological characteristics of cells, scatter signals will always be generated during flow analysis. They are called intrinsic parameters.

Fluorescent dyes, when added to the probe, give off their own light (fluorescence). These days there are a large number and wide range of different types of fluorescent probes on the market. The choice depends on the excitation source, the detection system and the specific aim of the analysis.

The instrument's light detectors collect the scattered and fluorescent light and convert it into electronic signals proportional to the amount of light collected.

Voltage pulses need to be amplified for optimal visualisation. The amplification process accentuates the differ-

ences between cell signals and this consequently increases the resolution among cell populations with different characteristics (for example, the differentiation of viable from non-viable cells, or non-specific fluorescence from antigen-specific fluorescence after staining with a fluorescent monoclonal antibody).

The amplification can be either linear or logarithmic. Linear amplification enhances the differences among strong pulses (e.g. cell scatters), while logarithmic amplification is for weak pulses or analysis processes, which generate both weak and strong pulses (e.g. cell antigens).

Each fluorescent dye has its own excitation spectrum and its emission spectrum. During multicolor staining the fluorochromes emissions spectra will overlap as each fluorescence detector will measure its own specific fluorescent light and a variable amount of light emitted by other fluorescent probes. The result will be a signal over-evaluation and poor separation of cell populations (Fig. 1.). Each combination of fluorochromes demands a fluorescence compensation – i.e. selective subtraction of the interfering signals from each fluorescence signal after detector sensing. This is done with the help of calibration beads and instrument system software. There can be variation in emission spectra of labels (e.g. fluorescein emission intensity increases as pH rises from 7,0 toward 8,0). The more colors are measured, the more complicated the situation becomes³.

The outcome of the acquisition are histograms which show the signal intensities versus the cell counts or 3 parameter histograms which result from the combination of 2 signal intensities and the number of cells⁴. When analysing acquired data the flow cytometer software can generate other types of graphs (e.g. contour plots, density plots, tomograms and overlays).

Fig. 2. Flow cytometric plot of nodular suspension shows clearly 2 different populations of cells.

Fig. 3. Dot-plot shows the expression of CD 10 on some cells from the node.

Within the sample analyzed various cell subpopulations can be found. Some of them are unwanted (debris or aggregates) and irrelevant to the actual acquisition (some markers we study on populations of lymphocytes only). Using region gate we can restrict the analysis to population(s) of the given morphological characteristics (using their morphological signals FS and SS). A region gate is drawn around the selected population and then the fluorescence plots are gated into the selected region. Or the cell population of interest is identified on the basis of the expression intensity of an antigen or a dye and a gate region is drawn around it.

Afterwards the fluorescence plots are gated into the selected region. The analysis software allows the creation of multiple gate regions.

One of the applications of flow cytometry is immunophenotyping. Flow cytometry tests for surface markers of white blood cells (mainly) to determine the stage of cell maturation. Proteins on the surface of cells must be mapped properly to classify acute and chronic leukemias and non-Hodgkin lymphomas.

CASE REPORT

22-year-old male was followed up for acute B-lymphoblastic leukemia (B-ALL) in a second complete remission for 3 years, when he was examined for a 10 days lasting lymphadenomegaly in the outpatient clinic of the Department of Hemato-oncology. Spiramycine treatment has been started by a general practitioner several days before. The patient denied any other complaints.

Physical examination revealed enlarged painless neck lymph nodes. Ultrasonography showed multiple lymph nodes in neck and right supraclavicular area, in size 13x10 and 17x11 mm. Blood count was normal and nothing indicated the possibility of a leukemia relaps.

Nevertheless, diagnostic lymph node extirpation with histologic examination and immunophenotyping was recommended given the anamnesis of B-ALL and unsatisfactory antibiotherapy outcome.

Immunophenotyping of lymph node revealed infiltration with cells slightly bigger than normal lymphocytes, that were CD19, CD10, CD24 and CD34 positive (Fig. 2, 3). The same phenotype (CD19, CD10, CD24, CD34, HLA-DR, CD79a, TdT and cyclambda positive) was found when immunophenotyping of leukemic clone from bone marrow was carried out 4 years previously. Hence the test confirmed relapse of acute B-lymphoblastic leukemia. The result was available within 3 hours from extirpation. The flow cytometry enabled timely detection

of the relapse and the start of appropriate treatment. Subsequent analysis of the bone marrow and the results of histopathology confirmed the diagnosis.

DISCUSSION

The issue of the differential diagnosis of enlarged lymph nodes is quite extensive. It is not necessary in the majority of patients to extirpate the node. Being invasive, node extirpation is the last step in examination when the results (anamnesis, physical examination, radiological imaging, serological assessments, tumor markers, etc.) create the suspicion of malignity and no other causation of lymphadenopathy has been found.

It is appropriate to mention here that examination of the entire node or the relevant part of it in the case of bulk nodes appears to be ideal. Aspiration cytology once often performed does not seem to produce important information for differential diagnostics and was therefore dropped as inconclusive by hemato-oncological departments.

According to our experience it is relevant to complement the histopathology with other specialized methods such as immunophenotyping. Of course it is essential to consider the sample size – histopathology continues to be the most important method even today. The other laboratory tests can be performed only if the sample size is adequate. The above-described history of our patient demonstrates that flow cytometry is a rapid and useful method in the differential diagnostics of lymphadenopathy.

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