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Flow Cytometry Immunophenotyping (FCI) of Fine Needle Aspirates (FNAs) of Lymph Nodes

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A B S T R A C T

Flow cytometry immunophenotyping (FCI) has an important role in the clinic work-up of fine needle aspirates (FNAs) of lymph nodes. Its standardization has been defined by proposed analytical protocols and procedures used to assure proper analytical results also in those non-routine samples. In Institute of Clinical Chemistry, »Merkur« University Hospital, FCI is accredited method according to laboratory accreditation standard ISO 15189. According to this laboratory accreditation standard, participation in external quality assessment (EQA) programs is a prerequisite for assuring integrity and quality of the entire laboratory process. A critical analysis of our institutional experience in the feasibility of FCI of the material obtained by FNA of lymph nodes with suspected lymphoma represented the purpose of the study. During an eight-year period in Institute of Clinical Chemistry, »Merkur« University Hospital, a total of 1295 FNA analysis was done, 245 of them with a possible diagnosis of B-cell Non-Hodgkin lymphomas (B-NHL) formed the basis of the study. Lymphocytes were isolated on density gradient according to Boyum et al. The average feasibility of FNAs for FCI analysis was 86 % (ranged 78–93%). An acceptable total cell number in FNAs for FCI analysis (4257) was established. In total population of respondents statistical significances in expressions of cellular antigens CD3, CD5, CD22, CD23, CD19 and CD5 on B-cells (CD5+CD19+) between patient´s with final diagnosis of benign, reactive lymphoid proliferations and patient´s with diagnosis of B-NHL were found. EQA results analysis showed that all results were either inside target values ($\overline{X}$±1SD) or inside accepted values ($\overline{X}$±2SD). Compatibility of the restriction of imunoglobulins light chains determinated by FCI and cytomorphology diagnosis depends on the choice of criterion values of the light chains ratio which determine the monoclonality. According to the matrix of shares of all classified data of retained neural network, ranges of diagnostic sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and prevalency of 82%, 72%, 93%, 48%, and 72% were produced. As a conclusion, FCI is a reliable methodology for phenotyping FNAs of lymph nodes with suspected B-NHLs detecting their clonality easily.

Key words: *flow cytometry immunophenotyping (FCI), fine needle aspirates (FNAs)*

Introduction

Flow cytometry immunophenotyping (FCI) as a fast, objective and quantitative multiparametric method has become the preferred method for the lineage assignment, detection of clonality and aberrant antigen coexpression, as well as for quantitation of malignant cells. It is based on the determination of various cell differentiation (CD) markers that form a specific immunophenotypic antigen profile of cell lineage and its maturation stage of every single cell in specimen, but can Nalso detect monoclonal B-cell populations that by cytomorphology may be interpreted as reactive. High morphological similarity of benign lymphoproliferative lesions (reactive lymphoid proliferations) and Non-Hodgkin´s lymphomas (NHLs) requires FCI as essential ancillary technique to cytomorphology to their differential diagnosis. FCI is usually performed, but not limited, on routine specimens such as

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peripheral blood (PB) or bone marrow (BM) aspirates. In Institute of Clinical Chemistry, »Merkur« University Hospital, FCI of lymphoid cells is accredited method according to laboratory accreditation standard ISO 15189, even in non-routine samples which include liquor, ascites/pleural effusions or solid tissue (lymph nodes). A single cell suspension preparation is crucial. Paradoxically, FCI is rarely performed on cytological specimens which are cell suspensions, such as fine-needle aspirates (FNAs), and therefore ideal for FCI. Analytical protocols and procedures are proposed and used as a part of FCI standardization for ruling out the most common sources of variability[1-5]. The rare submitting of FNAs for FCI dictates that every attempt should be made to assure proper analytical result and an objective, useful result interpretation[6,7]. Today only a few reports describe FCI performed in FNAs as rare, »non-routine« specimen[8–17]. The purpose of the study was a critical analysis of our institutional experience in the feasibility of FCI of the material obtained by FNAs of lymph nodes with suspected B-NHL.

Materials and Methods

During an eight-year period (2000–2008) in Institute of Clinical Chemistry, »Merkur« University Hospital, a total of 1295 FNA analysis was done. In the final cohort 245 of them (128 men and 117 women) with a possible diagnosis of B-NHL formed the basis of the study. At each FNA 5–10 passes in the lymph node were completed. Each FNA was obtained using a new 23 gauge disposable needle and 10 ml syringe as heterogeneous cell suspensions. Lymphocytes were isolated on density gradient according to Boyum et al. The absolute number of leukocytes was established on a Sysmex-XE 2100 hematologic analyzer. All patients samples and clinical details were obtained upon receiving patients consent and the ethical-committee approval. The reference diagnosis was obtained by standard cytological and histological methods[17]. In internal quality control validity of flow cytometer optical alignment and fluidics system (FlowCheck Fluorospheres; Coulter PN 6605359), quality of reagents, erythrocytes lysis procedure and cell staining (Immuno-Trol Cells Kit, Coulter, PN 6607077), quality of monoclonal antibodies (Cyto-Trol™ Control Cells Kit, Coulter, PN 6604248) and specific analytical conditions were determined. The FCI analysis of cellular antigens was done using directly fluorochrome-conjugated monoclonal antibodies in simultaneous dual- or three- color immunofluorescence: CD45 (DAKO F0861), CD14 (DAKO R0864;), CD3 (DAKO C7067), CD19 (DAKO C7066), CD5 (DAKO F0795), CD23 (DAKO R7108), CD10 (DAKO R0848), CD20 (DAKO F0799), CD22 (DAKO R7061), CD24 (DAKO F7134), CD38 (DAKO F7101), CD138 (DAKO R7229), FMC 7 (DAKO F7110), KAPPA (DAKO F0434), LAMBDA (DAKO R0437). Fixation and whole blood lysis of FNAs was done (Coulter Q-PREP, Q-PREP/ImmunoPrep Reagent System PN 7546946) before quadrant analysis on instrument (Coulter EPICS- XL, System II™ Software, Coulter Corporation, Miami, Florida). Lymphocyte gate was set by forward light scatter (FS) and side scatter (SS) and validated by CD45 and CD14 monoclonal antibodies. For greater precision in identification of lymphocyte subsets quadrant analysis based on coexpression of antigens defined by dual- or three- color combinations was used[17]. Standardized analytical protocols and procedures assured validity of FCI process. In daily internal quality control validity of flow cytometer optical alignment parameters (CV FS, CV FL1, CV FL2, CV FL3 and CV FL4) all parameters were inside target value (≤2.00)(CV FS 1.81; CV FL1 0.91; CV FL2 0.99; CV FL3 1,13; CV FL4 1.20) and all were acceptable with 5.4–10.0% accuracy. According to laboratory accreditation standard ISO 15189 participation in external quality assessment (EQA) programs is a prerequisite for assuring integrity and quality of the entire laboratory process. Therefore *a*nalytical results were confirmed by participation in EQA program CEQUAL organized by Becton Dickinson (Germany). CEQUAL analysis of cellular lymphocyte antigens was done by own directly fluorochrome-conjugated monoclonal antibodies according to own laboratory procedure and according to the method and procedure of Becton Dickinson (BD). EQA results analysis showed that all results were either inside target values ($\overline{X}$±1SD for CD19, CD3, CD16+CD56+, CD3+CD8+, CD3+HLAD/DR+) or inside accepted values ($\overline{X}$ ± 2SD for CD3 and CD3+CD4+).

In statistics data analysis multivariate statistical method (neural network) was used because of complex system with a large number of measured parameters.

Results

From a total of 1295 FNAs the average feasibility for FCI analysis was 86% (ranged 78 – 93%) (Figure 1). The quality of lymphocytes obtained by FNA as a material for FCI was very satisfactory. In total population of respondents (128 men, 117 woman) there was no statistical significance in age (p=0.05*)*, but a statistical significance in

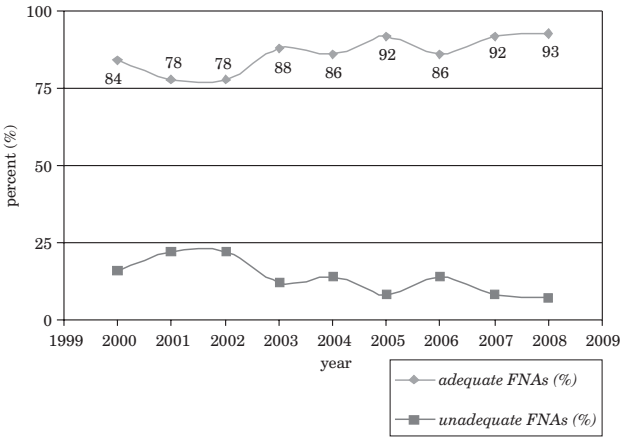

Fig. 1. The FNAs average feasibility for FCI analysis during an eight-year period (2000–2008).

age between patient's with final diagnosis of benign, reactive lymphoid proliferations and patient´s with diagnosis of B-NHL (p=0.00) was found.

Analytical evaluation

Based on the parameters of cell size (FS) and granularity (SS), a homogeneous specific cell population (population size of lymphocytes) was isolated from a heterogeneous cell suspension. A minimum acceptable total cell number in FNAs for FCI analysis was established through linear regression of cell number on a homogeneous specific cell population (population size of lymphocytes) on the measuring instrument (Coulter EPICS-XL) and an absolute lymphocyte number (x10^9/L) on referral instrument (Sysmex XE-2100) (Figure 2). Minimal absolute lymphocyte number on referral instrument (0.24x10^9/L) positively corresponded (r=0.93) with total number of cells on a homogeneous specific cell population (population size of lymphocytes) and therefore lower linearity level was equal to the minimum acceptable total cell number in FNAs needed for FCI analysis (4257 cells). Daily control of measuring instrument performance was consistent with the measurement criteria established at instrument setup (Figure 3). Measuring instrument precision calculated as variation coefficients (CV) of all parameters ($FS_{mean\ channel}$, AUX (FL3), $FL1_{\ mean\ channel}$, $FL2_{\ mean\ channel}$, $FL3_{\ mean\ channel}$, $FL4_{\ mean\ channel}$) with half peak variation coefficients of all parameters ($HPCV_{FS}$, $HPCV_{FL1}$, $HPCV_{FL2}$, $HPCV_{FL3}$, $HPCV_{FL4}$) were within

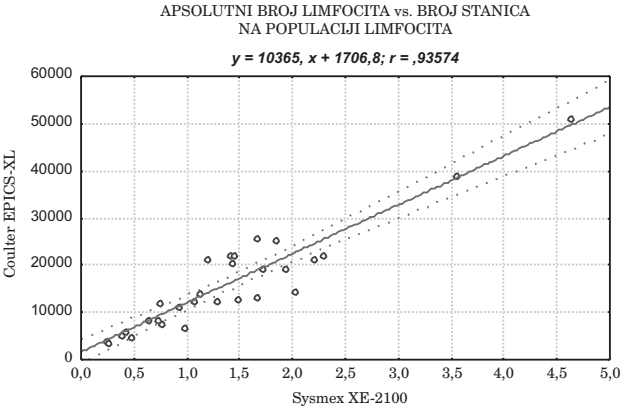

Fig. 2. Corellation of parameters of the refferal instrument (Sysmex XE-2100) and the measuring instrument (Coulter EPICS-XL); Sysmex XE-2100 – absolute lymphocyte number (x10^9/L) on the referral instrument (Sysmex XE-2100); Coulter EPICS-XL – cell number (N) on a homogeneous specific cell population (population size of lymphocytes) on the measuring instrument (Coulter EPICS-XL)

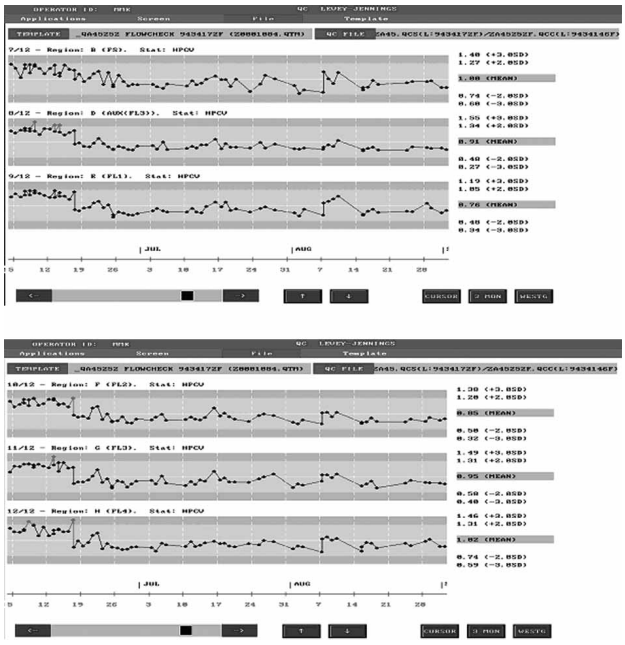

Fig. 3. Daily control of optical alignment and fluidics system on measuring instrument (Coulter EPICS-XL). Levy-Jennings graphic of half peak variation coefficients (HPCV) of all parameters.

TABLE 1
STATISTICAL SIGNIFICANCES IN EXPRESSIONS OF CELLULAR ANTIGENS BETWEEN PATIENT´S WITH FINAL DIAGNOSIS OF BENIGN, REACTIVE LYMPHOID PROLIFERATIONS AND PATIENT'S WITH DIAGNOSIS OF B-NHL (KRUSKALL-WALLIS TEST; STATISTICAL SIGNIFICANCE p<0.05)

Cellular antigen (CD)	Cellular expression (Median %)		Statistical significance (p) (p<0.05)
	Benign, reactive lymphoid proliferations	B-NHL	
CD3	55.3	31.4	0.000
CD5	45.9	22.7	0.000
CD20	29.7	63.7	0.001
CD22	50.8	44.6	0.532 (*NS*)
CD23	15.8	42.1	0.000
CD19	31.5	61.9	0.000
CD38	31.0	60.0	0.000
CD5+CD19+	1.6	53.4	0.000

NS – not statistically significant

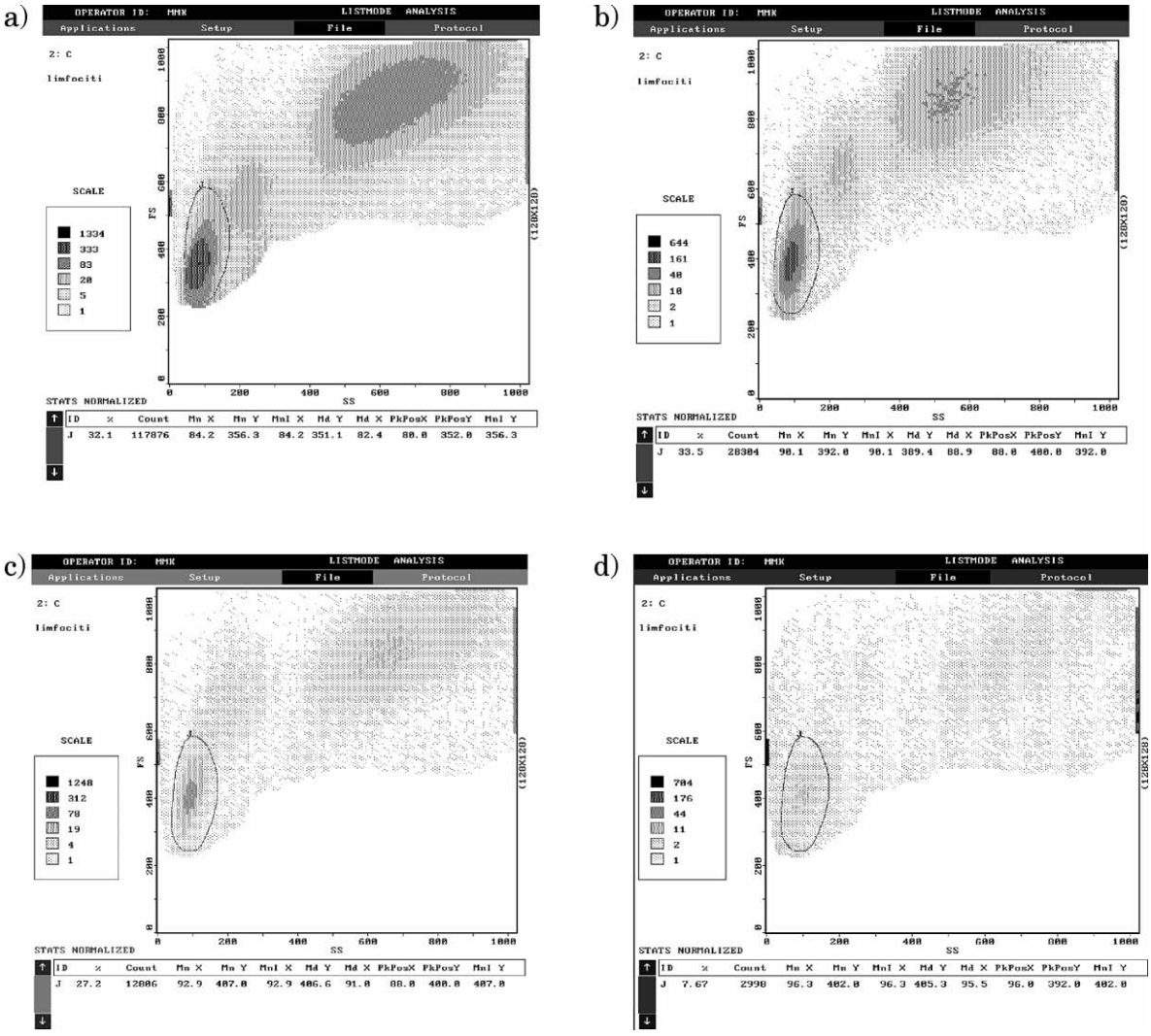

Fig. 4 (a-d). Linearity of the flow cytometry immunophenotyping (FCI). Relative lymphocytes number (%) on measuring instrument (Coulter EPICS-XL): a) in a sample with normal leucocyte number and b) samples diluted with PBS buffer: a) the ratio of 1:5, c) the ratio of 1:10 and d) the ratio of 1:50.

TABLE 2
COMPARISON OF CYTOMORPHOLOGY DIAGNOSIS AND RESTRICTION OF IMUNOGLOBULINS LIGHT CHAINS DETERMINATED BY FLOW CYTOMETRY IMMUNOPHENOTYPING (FCI) ACCORDING TO THE CHOICE OF BOUNDARY VALUES OF THE RATIO OF LIGHT CHAINS WHICH DETERMINE THE RESTRICTION

Cytomorphology (N=237)	restriction of imunoglobulins light chains determinated by FCI (kappa or lambda type)			
	Negative		Positive	
	Criterion values of the ratio of light chains			
	kappa≤3 lambda lambda≤ 1kappa	kappa≤5.5 lambda lambda≤1.4 kappa	kappa>3 lambda lambda>1 kappa	kappa>5.5 lambda lambda>1.4 kappa
Benign, reactive lymphoid proliferations (N=9)	12 (5.0%)	23 (9.7%)	37	26
B-NHL (N=59)	19	28	140 (59.0%)	131 (55.3%)
Other neoplasms (N=29)	6 (20.7%)	10 (34.5%)	23 (79.3)	19 (65.5%)

the acceptable range (1.12–1.98) as well as instrument accuracy calculated as absolute bias of all parameters (1.9–2.0). Accuracy of all measured parameters on Coulter EPICS-XL was within the acceptable limits of deviation for the measuring instrument (±10%):0–1% for relative number of lymphocytes, 7–7.5% for absolute lymphocytes number and 7–8% for total number of cells on a homogeneous specific cell population (population size of lymphocytes). Linearity of the FCI in diluted sample (PBS buffer) with the ratio of 1:50 was followed by non-linear downregulation of relative lymphocyte number (Figure 4 a-d).

Clinical evaluation

The final diagnosis of benign, reactive lymphoid proliferations or B-NHL was obtained by standard cytological and histological methods. In total population of respondents statistical significances in expressions of cellular antigens CD3, CD5, CD22, CD23, CD19 and CD5 on B-cells (CD5+CD19+) between patient´s with final diagnosis of benign, reactive lymphoid proliferations and patient´s with diagnosis of B-NHL were found (Table 1). The ratio of imunoglobulins light chains was defined as percentage of the dominant type of imunoglobulins light chain divided by the percentage of non-dominant type of imunoglobulins light chain on B lymphocytes (CD19+). Equivalent areas under the Receiver-Operating Characteristic curves (ROC – curves) (0.73 kappa/ lambda compared to 0.74 lambda/kappa) confirmed the identical diagnostic value of both imunoglobulins light chain ratios in differentiation of benign, reactive lymphoid proliferations and B-NHLs. Comparison of the restriction of imunoglobulins light chains determinated by FCI and cytomorphology diagnosis showed that their compatibility depends on the choice of criterion values of the light chains ratio which determine the restriction (Table 2). Diagnostic sensitivity (82%), specificity (72%), positive predictive value (PPV, 93%), negative predictive value (NPV, 48%) and prevalence (72%) for FCI was calculated according to the matrix of shares of all classified data of retained neural network (Table 3).

Discussion and Conclusion

Flow cytometry immunophenotyping (FCI) as a fast, objective and quantitative multiparametric method contributes to diagnosis, classification and monitoring of various hematological diseases (leukemias, lymphomas, myelomas). Today, leukemia and lymphoma immunophenotyping represents an extremely important complement to cytomorphology in the diagnosis and monitoring of hematopoietic malignancies, because of data that form a specific immunophenotypic antigen profile of cell lineage and its maturation stage[8–22,52]. Sample preparation for FCI analysis must consider the type of specimen submitted and the number of cells available for analysis. Peripheral blood, bone marrow or tissue specimens should be processed to contain a suspension of the cells of interest at a concentration optimal for monoclonal staining[23]. Although cytological specimens, such as FNAs, are cell suspensions and therefore ideal material for FCI, they are rarely used for FCI, because of the problem to obtain ideal number of 10000 cells in a tube for FCI analysis (cells concentration in the sample ≈ 0.5–5.0x10^6/mL)[9]. Insufficient number of cells could be a consequence of inadequate or poor FNAs of lymph node, as well as selective cells loss during the cell preparation process[23]. It has been proved, however, that the quality of FNAs of lymph nodes depends on the appropriate leukocyte number in analytical sample, achievement that is more a rule than an exception[35].

TABLE 3
MATRIX OF SHARES OF ALL CLASSIFIED DATA OF RETAINED NEURAL NETWORK

	Benign, reactive lymphoid proliferations	B-NHL
Benign, reactive lymphoid proliferations	18	21
B-NHL	7	95

In order to evaluate the usefulness of FNAs for FCI analysis, we critically analysed and reviewed our institutional experience in the feasibility of FCI of all FNAs submitted to our Flow unit during an eight-year period (2000–2008). It is evident that during this period the percent of adequate FNAs for FCI constantly increases during the years indicating better experience in sampling and aspirations of lymph nodes. The high feasibilities of FNAs for FCI analysis ranged from 78 to 93% during the entire period proved that FNAs are an appropriate material for FCI analysis. FCI linearity lime (0.35x10^9/L leucocytes in the sample) is a part of the literature of well-known range of acceptable leukocytes number in FNAs of lymph nodes[31–33]. Unacceptable FNAs samples had a leucocytes number less than acceptable, and the conclusion about lowest linearity limit of the FCI (0.24x 10^9/L) as an acceptable cell number in FNAs of lymph nodes (4075 cells) was confirmed in the literature (5000 cells *per* sample required for FCI analysis)[32,33,36,37].

Over the years, standardization of FCI technique has lead to improvements in performing FCI analysis. In attempts to assist with standardization, the United States--Canadian Consensus and The National Committee for Clinical Laboratory Standards (NCCLS) guidelines provided recommendations for FCI in hematopathology, but each laboratory is ultimately responsible for validating its own procedures[7,27–30]. The United States-Canadian Consensus guidelines strongly recommend that every attempt should be made to derive useful information from any specimen submitted[27]. Each specimen must be considered a unique case and that is why for FCI analysis preparation of a single cell suspension is crucial. FCI analysis and the data interpretation rely on standardization and validation of the measuring instrument, the reagents and the procedure[34,51]. In our laboratory validation of measuring instrument and daily validation of its optical alignment and fluidics system was assessed with

FlowCheck Fluorospheres (uniformly sized beads run at a constant PMT voltage on a daily basis), whereas the reagents were validated with Immuno-Trol Cells Kit, and the procedure with Cyto-Trol™ Control Cells Kit. The mean forward scatter (FS) and side scatter (SS) channel numbers and percent of variation coefficients (% CV) were recorded. The acceptable ranges for each parameter were established by first running the beads 20 times over a 5 day time period at the same PMT setting. Levy–Jennings graphs were then used to plot the daily obtained values.

The advantage of FCI regarding cytomorphology is the possibility to detect monoclonal B-cell populations that by cytomorphology may be interpreted as reactive, based on morphologically relatively unique population of lymphocytes[19,24–26]. Dual- or three-color combinations of monoclonal antibodies (MoAb) and setting an electronic gate only around specific cell population (lymphocytes) has established better determination of imunoglobulins light chain expression, while the simultaneous expression of two or more antigens (coexpression) or cell size on specific cell population (lymphocytes) indicated changed immunophenotype of B cells[38–40]. The increased number of B lymphocytes in the benign, reactive lymphoid proliferations could suggest the existence of an immune response involving interactions between T and B cells[41]. In the literature there are specified limit values of the immunoglobulin light chains ratio to set monoclonality (kappa/lambda, lambda/kappa) from 1.4 to 6.0, but only few papers have explored the diagnostic importance of their determination[42–47]. The ratio of immunoglobulins light chains kappa/lambda less than 0.7 or greater than 5.5 in references is proven as optimal for distinguishing malignant lymphomas of benign, reactive lymphoid proliferations[42]. Therefore, this criterion values were also used in this paper. According to them, compatibility of cytomorphology diagnosis and restriction of imunoglobulins light chains determinated by flow cytometry immunophenotyping (FCI) is 55.3% (57.1% Ibrahim I et al, 1989)[48]. In 23 samples (9.7%) a negative outcome of both methods was established. Within the group of cytomorphology benign, reactive lymphoid proliferations of unknown origin (N=49), in 26 of them (53.1%) the restriction of imunoglobulins light chains was observed consistent with the known literature value of 63%[47]. By applying a stronger criteria for monoclonality (kappa>5.5 lambda; lambda>1.4 kappa) the numbers of cytomorphology benign, reactive lymphoid proliferations of unknown origin (26 instead of 37) and cytomorphology B-NHLs (131 instead of 140) reduced. That is why sensitivity decreased, but specificity and reliability of FCI increased. Setting clear monoclonality criteria is difficult due to sample heterogeneity[49,50].

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IMUNOFENOTIPIZACIJA UZORAKA DOBIVENIH CITOLOŠKOM PUNKCIJOM LIMFNOG ČVORA METODOM PROTOČNE CITOMETRIJE

S A Ž E T A K

Imunofenotipizacija metodom protočne citometrije ima važnu ulogu u diferencijalnoj dijagnostici limfadenopatija. Standardizacija metode protočne citometrije podrazumijeva korištenje predloženih analitičkih protokola i procedura koji osiguravaju pouzdanost analitičkih rezultata i u uzoraka koji nisu rutinski. U Zavodu za kliničku kemiju Kliničke bolnice »Merkur« imunofenotipizacija metodom protočne citometrije je akreditirana metoda prema HRN EN ISO 15189 »Medicinski laboratoriji – Posebni zahtjevi za kvalitetu i osposobljenost«. Svrha ovog rada je kritička analiza imunofenotipizacije ne-rutinskih uzoraka dobivenih citološkom punkcijom limfnog čvora metodom protočne citometrije, a u kojih je postojala sumnja na B staničnu neoplazmu (B-NHL). Tijekom osmogodišnjeg razdoblja u Kliničkoj bolnici »Merkur« ukupno je učinjeno 1295 citoloških punkcija limfnog čvora, od toga je 245 uzoraka punktata limfnog čvora u kojih je postavljena sumnja na B-NHL imunofenotipizirano metodom protočne citometrije. Limfociti su izdvojeni na gradijentu gustoće (metoda Boyuma i sur.). Određena je prosječna prihvatljivost uzoraka punktata limfnog čvora za imunofenotipizaciju metodom protočne citometrije (86%) koja je tijekom razdoblja iznosila od 78 do 93%, kao i prihvatljiv broj stanica u uzorku punktata limfnog čvora (4257 stanica). Analizom ekspresija staničnih površinskih biljega CD3, CD22, CD23, CD19 i CD5 pozitivnih limfocita B (CD5+CD19+) utvrđena je statistički značajna razlika u ekspresiji ovih biljega u benignih, reaktivnih limfadenopatija nepoznatog uzroka i u B-NHL. Podudarnost citomorfološke i imunofenotipizacijske dijagnoze ovisila je o odabiru graničnih vrijednosti omjera lakih lanaca imunoglobulina koji određuju monoklonalnost. Prema matrici udjela svih klasificiranih podataka zadržane neuronske mreže izračunate su dijagnostička osjetljivost (82%), specifičnost (72%), pozitivna prediktivna vrijednost (PPV, 93%), negativna prediktivna vrijednost (NPV, 48%) i prevalencija (72%) metode protočne citometrije. U zaključku, metoda protočne citometrije je pouzdana metoda za imunofenotipizaciju uzoraka punktata limfnog čvora, a u kojih je postavljena sumnja na B staničnu neoplazmu (B-NHL).