THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

OPTIMIZATION OF FLOW CYTOMETRIC MARKER EVALUATION IN HEMATOLOGICAL DISEASES

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INTRODUCTION

Laboratory analysis is necessary in the diagnostic and monitoring process of hematological diseases. The correct diagnosis and exact classification of leukemia types is obligatory for the selection of appropriate treatment protocols, which play an important role in the effectiveness of therapy. Besides the classical mophological and cytochemical examinations, the use of flow cytometric immunophenotyping with monoclonal antibodies is recommended. The flow cytometric method enables the identification of malignant lineage, usually by the detection of intracellular antigens present earlier than surface markers, as well as the determination of the level of differentiation among the leukemic cells. Leukemia associated immunophenotypes (LAIP) can also be identified, which are utilized in the follow-up of patients by detection of minimal residual disease (MRD) or relapse.

In my thesis I present the importance of cellular marker analysis in the diagnosis and monitoring of hematological diseases throughout my work in the last 6 years at the Univesity of Debrecen, using mainly flow cytometric techniques. During my work I carried out several other methods as well, including protein biochemical, microscopic and cell biological techniques in order to verify my statements. The presented results including new data help in the better understanding of hematological diseases and can be used in the diagnosis and follow-up of patients.

Cell surface and intracellular proteins

The cell membrane is a well structured and dynamic organization composed by the lipid bilayer and various protein components. The classification of membrane antigens based on the so-called cluster of differentiation organized them clearly for the everyday routine. Most

commonly the cell surface markers are determined during the phenotypic analysis of hemopoietic cells, even though more antigens are localized intracellularly in these cells. The most reliable tool for lineage assessment is based on the detection of intracellular antigens, since the earliest and most specific markers for different cell lineages are frequently absent form the cell surface but are usually detectable intracellularly.

Cellular protein analysis with flow cytometry

Labeling with monoclonal antibodies can be performed in a direct (one-step) or indirect (two-step) manner. The results of the anlysis can be converted to dot-plots or histograms. For the detection of intracellular markers, permeabilization of cells is necessary, which can influence the sensitivity and specificity of the measurement. Immunological detection of membrane proteins does not always correlate with their function. In these cases the use of a functional assay is more relevant. Cell marker analysis can be quantitated by flow cytometry. With the application of calibrating beads enumeration of antigens is possible. In case of paroxysmal nocturnal hemoglobinuria (PNH) the advantage of flow cytometry is, that even in non-hemolysing patients, small PNH clones can be detected and the proportion of type III (complete deficiency), type II (partial deficiency) and type I (normal) cells in each investigated population can be determined.

Immunophenotyping of hematological malignancies with flow cytometry

Multicolor immunophenotyping with flow cytometry was a checkpoint in the diagnosis of hematological malignancies, since morphological techinques are subjective and depend heavily on the diagnostic routine of the examiner. There are other advantages of flow

cytometry as well. Although microscopy gives accurate morphological information it is rather time-consuming with yet another drawback, the low number of cells observed (few hundred) compared to flow cytometry (from 10 000 for example in de novo cases to even 1 000 000 in MRD detection).

The aim of my study was to optimize cellular marker analysis in an objective way using flow cytometry with the intention of quantitating cell analysis wherever possible followed by the utilization of these techniques on clinical samples. My quest was also to find new diganostic and prognostic markers.

MATERIALS AND METHODS

Reagents

Anti-CD3 PerCP, anti-CD5 PE, anti-CD14 FITC, anti-CD19 PerCP, anti-CD19 APC, anti-CD20 FITC, anti-CD33 PE, anti-CD34 PE, anti-CD34 PerCP, anti-CD41 PE, anti-CD42a FITC, anti-CD45 FITC, anti-CD45 PerCP, Calibrite, FacsLysing Solution, Quantibrite (Becton Dickinson, San Jose, CA), anti-CD5 PE-Cy5 (Immunotech, Marseille, France), anti-CD10 PE, anti-CD13 FITC, anti-CD56 PE-Cy5, anti-CD79α PE, anti-IgM PE, anti-MPO PE, anti-TdT FITC, anti-mouse FITC, Intrastain kit (Dako, Glostrup, Denmark), anti-CD55 PE, anti-CD59 PE (Pharmingen, San Diego, CA), anti-Zap-70 (clone 2F.3) (Upstate, Lake Placid, NY), anti-FXIIIA rabbit serum (CenteonPharma, ZLB Behring, Marburg, Germany), BSA, Ficoll Histopaque 1007, FITC-staining kit, Glycerol, HBSS, HEPES, Paraformaldehyde, Propidium iodide, Verapamil (Sigma, St. Louis, MO), Calcein-AM (MolecularProbes, Eugene, OR,USA), ECL Kit (Amersham, Buckinghamshire, UK), EDTA, NaCl (Spektrum-3D, Debrecen, Hungary), Fix&Perm kit (Caltag, Burlingame, USA),

Heparibene (Na-heparin) (Ratiopharm, Merckle, Germany), KCl (ScharlauChemie, Barcelona, Spain), MOWIOL 4-88 (Hoechst Pharmaceuticals, Frankfurt, Germany), SDS, TRIS (Bio-Rad, Hercules, CA), Triton-X 100 (Reanal, Budapest, Hungary), Vectastain ABC Kit (Vector Laboratories, Burlingame, CA).

Human samples, cell lines

Bone marrow specimens anticoagulated with heparin or EDTA and peripheral blood samples anticoagulated with EDTA (DE OEC-KEB 1034-2001) were used for various measurements. All leukemic samples were obtained at the onset of the diseases before any treatment was initiated. A few cell lines were also used, namely: human epidermoid carcinoma cell lines KBV-1, KB-8-5 (human MDR1+ cell lines) and KB3-1 (human MDR1- cell line).

Methods

Antigen detection using direct and indirect staining techniques

Expression of surface and intracellular markers were studied within 24 hours of sample collection. Surface staining of whole blood and bone marrow samples was carried out according to standard procedures. Whole blood or bone marrow samples were incubated by saturating concentrations of commercially available directly conjugated antibodies (FITC, PE, PerCP, PE-Cy5, APC) for 20 minutes at room temperature in the dark. Red cells were lysed by FACS lysing solution (Becton Dickinson Biosciences, San Jose, CA) and samples were washed (300x g, 5 minutes) in PBS and finally resuspended in PBS containing 1% paraformaldehyde (PFA). Generation and labeling of mouse monoclonal antibodies against

FXIII subunits was carried out in our department earlier utilizing a fluorescein isothiocyanate (FITC) labeling kit (Sigma, St. Louis, MO). For intracytoplasmic staining, the procedure described for the Intrastain kit was strictly followed. Surface staining was executed before permeabilization of cells. FXIII-A antibody was used at a 2 μg/mL final concentration with appropriately matched isotype control. Indirect intracytoplasmic staining was used to detect Zap-70 expression in B-CLL patients' samples by 3- and 4-color immunophenotyping. Within 24 hours of sample collection $1.5x10^5$ cells were incubated with 100 μL fixative (Caltag, Fix&Perm, Burlingame, USA) for 15 min., at 25°C, then the cells were washed in PBS. Afterwards 1.5 μg unstained antibody (anti-Zap-70, Upstate, Lake Placid, NY, clone 2F3.2 mouse IgG2a) was given to the cells together with the peremabilizing reagent (Caltag Fix&Perm), which lysed the RBC-s as well. After another washing step (PBS) 50x diluted goat anti-mouse-FITC antibody was added for 20 min. in the dark. Then surface staining was executed as desribed previously by direct labeling and fixation in 1% PFA. Labeled cells were kept at 4°C up to 24 hours until flow cytometric measurement, performed on a FACScan or FACSCalibur flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Data obtained on 10 000-100 000 cells in de novo leukemias or MRD detection and Zap-70 analysis were stored in list-mode data files and analyzed by mainly Cell Quest 3.2 (BD) or Paint A Gate software. The instruments were calibrated using Calibrite (BD) beads according to standard protocols. Blasts were gated according to their low CD45/side scatter (SSC) signals compared to normal leukocytes, while B-CLL cells were detected by the pathological CD5/CD20 coexpression from the lymphocyte gate based on forward scatter (FSC)/SSC. Results were given in either the percentage of positive cells for a given marker, or mean fluorescence intensity (MFI) units, or antibody binding capacity (ABC) units, or absolute cell count/μL.

Quantitative antigen determination

Quantitive measurements were performed using commercially available calibrating beads (Quantibrite) according to the manufacturer's instructions. The number of PE molecules bound per cell was calculated from the mean fluorescence intensity (MFI) of the unknown cell population using a calibration curve created from given values for PE molecules per bead plotted against FL-2 MFI. Monoclonal antibodies in this study were monovalent and the PE:mAb ratio was 1:1, this was the basis of the antibody binding capacity (ABC) calculation.

Calcein assay

Mononuclear cells were separated using Ficoll (Ficoll Histopaque 1007, Sigma, St. Louis, MO) gradient according to standard procedures, then they were resuspended in 1 mL Hank's Balanced Salt Solution (HBSS). Cells were loaded with calcein-AM (200µM, 10 minutes, 37°C) following an incubation period with or without the inhibitor (verapamil: 75µM on 37°C for 5 minutes). After a centrifuge step propidium-iodide (0,01 mg/mL) labeling was carried out to detect the viability of the cells. Samples were stored at 4°C and measured within 24 hours on a Becton Dickinson FACScalibur or FACScan flow cytometer. For quality control of the test human KBV-1 (known MDR-positive cell line, cultured in zhe presence of vinblastin) and KB3-1 (known MDR-negative cell line, cultured without vinbalstin) cell lines were used. For culturing of cells DMEM containing 10% FBA was used in a 5% CO_2 containing thermostate.

Calcein assay combined with surface staining

Monoclonal antibodies against the pan B cell marker CD19 and T cell marker CD3, both conjugated with PE-Cy5, were used in our experiments together with calcein. The expression and multidrug resistance activity factor (MAF) values of normal B and T cells were studied selectively analyzing 20 samples.

Confocal microscopy

About 10^6 cells from a FXIII-A positive ALL patient were resuspended in 100 µL PBS and cytocentrifuged (Wescor, Logan UT, 1000 rpm, 5 min.) to a slide. Until further use, slides were stored at -20°C. Then cytospin preparations were thawed, fixed in 4% PFA for 10 min, and washed three times in PBS. FITC-conjugated anti-FXIII monoclonal antibody was dissolved at 15 µg/mL in PBS containing 1 mg/mL BSA (Sigma, Schnelldorf, Germany) and 0.1% Triton X-100 and added to the cells for 30 min at room temperature. During the last 5 minutes of incubation propidium iodide (PI) was added to the labeling solution at 0.5 µg/mL final concentration. Next, cytospins were washed three times with PBS containing 1 mg/mL BSA and 0.05% Triton X-100. Finally the samples were washed again in PBS and mounted in 10 µL Mowiol (0.1 M Tris-HCl, pH 8.5, 25 w/v % glycerol and 10% Mowiol 4-88, Hoechst Pharmaceuticals, Frankfurt, Germany). Labeling of suspended cells followed the same protocol, except for using 0.05% Triton X-100 during labeling and 0.01% for washing. 3 mL of buffer volumes and centrifugation at 300x g for 5 minutes was used for the washing procedure. The last pellet was mixed with 10 µL Mowiol and mounted on pre-cleaned microscopic slides. For CLSM, a Zeiss (Göttingen, Germany) LSM 510 systems and a C-Apochromat 63×/1.25 NA water immersion objective were used. Fluorescein was excited with a 488 nm Ar ion laser and detected through a 505-550 nm band pass filter. PI was

excited with a 543 nm HeNe laser and detected through a 560 nm long pass filter. Pinholes were set to obtain 1 μm optical slices and 512×512 pixel images were taken with pixel times of 6.4 μs, and 2× line-averaging. All images were obtained in multi-track mode to avoid crosstalk with other channels.

FXIII-A ELISA

Detection of FXIII-A in cell lysates from ALL samples was carried out as published earlier by our institute's other work group. Briefly, to avoid platelet contamination lymphoid cells were washed three times in PBS containing 20 mM of EDTA at 2200 g for 4 minutes. The exact cell count was measured before sonication in order to calculate the amount of FXIII-A/cell. In case of ALL, B-CLL and separated monocyte samples, solubilization of cells was carried out with sonication at 4°C for 3x30 seconds. Afterwards two FXIII-A antibodies (one capturing monoclonal and another peroxidase conjugated against a different epitope) were incubated together with diluted cell lysates on a stretavidine coated ELISA plate for one hour. Then the plate was washed with PBS and the reaction was stopped by sulfuric acid. Absorbance values were detected at 450 nm-s using a Labsystem Multiscan microplate program.

Western blot

Blasts were washed three times in PBS containing 20 mM of EDTA (2200 g, 4 minutes). At the end 100 μL SDS PAGE sample buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 0.1% bromphenol blue, 4.5% mercaptoethanol amine) was added and the mixture was boiled for 5 minutes. Samples (containing about the same amount of cells/well) were loaded onto 7.5% SDS polyacrylamide gel and electrophoresed under reducing conditions at 40 mA. Samples

were electroblotted to Immobilon P membrane (Millipore, Bedford, MA). Nonspecific binding was blocked using Tris-buffered saline (0.5 M NaCl, 20 mM Tris-HCl, pH: 7.5; TBS) containing 3% gelatin for 1 hour at room temperature and an overnight incubation at 4°C in the blocking buffer. Blots were then incubated in a polyclonal rabbit antiserum against FXIII-A (Centeon Pharma, ZLB Behring, Marburg, Germany) for 1 hour (1:5000 dilution in TBS containing 1% gelatin) and subsequently washed three times in TBS containing 0.05% Tween-20. Detection of the primary antibody was carried out using biotinylated anti-rabbit IgG, avidin H and biotinylated peroxidase complex (Vectastain ABC kit, Vector Laboratories Inc., Burlingame, CA). Reaction was visualized by enhanced chemiluminescence using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Buckinghamshire, UK) as described by the manufacturer. Platelet lysates and purified FXIII-A were used as positive controls and B-CLL cell lysates were utilized as negative controls.

RESULTS

Studies in PNH using normal cells as standard

From January 2001 to December 2004 I studied 80 hemolysing patients' blood sample with my colleagues, but only 4 turned out to have PNH. In all four patients' history abdominal pain, vomiting, darker urine and fever could be explored. The laboratory tests showed unconjugated hyperbilirubinemia, higher LDH activity and hemolysis. RBCs and leukocytes were subclassified in cell types I, II and III based on their MFI after direct immunostaining. Type III cells in case of CD55 and CD59 staining are defined as having a lower MFI than 10. In case of type II cells MFI was 34±11 and in type I cells MFI was 280±150. Upon analysis of CD14 type II were not detected, type III cells had an MFI 39±36, while type I cells

2060±1000 respectively. The average of the percent negative cells in the case of RBCs was one third of that obtained in case of monocytes and granulocytes. This phenomenon is well known in PNH patients and can be explained by the premature destruction of these PNH erythrocytes. The size of the PNH clone can better be assessed by immunophenotypic analysis of monocytes and granulocytes rather than of erythrocytes.

PgP and MDR detection

In order to investigate different lymphocyte subsets alone, B and T cells can be gated and selectively analyzed for calcein efflux. Since calcein is a very strong fluorescent dye that causes intensive fluorescence spillover at the FL-2 channel, PE labeled antibodies can not be used. However by application of an appropriate compensation calcein assay can be combined with surface antibody staining by using a PerCP or PE-Cy5 conjugate, both of which are detectable at FL-3 channel (>650 nm). Analyzing 20 normal blood samples MAF was found to be higher in normal T cells compared to normal B cells.

FXIII-A detection in AML

We found that in case acute monocytic and myelomonocytic leukemias FXIII-A was more sensitive than CD14 in detecting leukemic blasts. In disorders where more mature monocytes are found like chronic myelomonocytic leukemias FXIII-A and CD14 labelings were identical. It is very important to note that in case of FXIII-A the intensity of staining is enhanced in malignant cells unlike other cytoplasmic markers like myeloperoxidase. When AML M7 cases were studied we found that FXIII-A labeling is more specific than the generally utilized platelet markers. Conventionally used platelet/megakaryocyte markers, such

as CD41 (GpIIb), CD42a/b (GpIX/GpIb) often display false-positivity in case of non-megakaryocytic leukemias. In de novo M7 cases, fairly high GPIIb and GPIX expression was detectable on various cell subsets while FXIII-A expression was much lower. When samples were washed in EDTA containing buffer and reanalyzed we found that FXIII-A and platelet glycoprotein markers match. This means that FXIII-A depicts the true megakaryocyte fraction in these cases.

FXIII-A detection in ALL

In bone marrow samples derived from de novo acute leukemic patients we found FXIII-A positivity in 40% of acute lymphoblastic leukemia patients by 3-color flow cytometry. In these cases FXIII-A positivity was coexpressed with CD45dim/CD34+ labeling and B-cell markers like CD19, and cytoplasmic CD79a. This finding was unexpected since normal peripheral blood lymphocytes as well as normal lymphoid precursors in the bone marrow are devoid of FXIII-A labeling. Thus, human peripheral blood taken from a healthy donor was labeled with magnetic CD34 CliniMacs reagent and CD34+ progenitor cells were separated using a CliniMacs instrument. To determine sample purity, 100 000 events were analyzed by flow cytometry. The purity of the CD34+ cells was 98% after magnetic separation in the leukapheresis product. Normal B-lymphocyte precursors - CD34 and cyCD79a - positive events were found in 3%. In these normal B-lineage committed cells no FXIII-A could be detected. In addition, FXIII-A was undetectable in lymphocytes derived from chronic lymphocytic leukemia. To further verify the presence of FXIII-A in ALL blasts cell lysates were prepared. To avoid platelet contamination, lymphoid cells were washed in PBS containing EDTA. Two methods were used to identify FXIII-A in these cells, Western-blotting and ELISA. The exact cell count was determined before sonication in order to

calculate the amount of FXIII-A content/cell. By Western-blotting it was found that under reducing conditions FXIII-A appeared as a 82 kD protein band in ALL blasts but not in mature B-cells derived from patients with CLL. The band obtained in case of ALL blasts however seemed much fainter than that obtained for platelets. It is known that platelets contain very large quantities of FXIII-A. Thus, we tried to compare the results to that of monocytes that were purified. By ELISA we found 3.1 ± 1.2 fg/blast (range: 2.0-4.8 fg/blast) that is 10% of the quantity seen in monocytes. Finally, we used confocal laser scanning microscopy to localize FXIII-A on cytospins and in cell suspensions. In both sample types FXIII-A appeared with a similar staining pattern to normal monocytes and nuclei were devoid of FXIII-A staining. The FXIII-A+ lymphoblasts were negative for the platelet markers GPIIb and GPIX.

Optimization of Zap-70 analysis in B-CLL

Zap-70 is an intracellular protein detectable by flow cytometry. Different anti-Zap-70 antibody clones, both conjugated and unconjugated are commercially available. The use of different permeabilizing kits can alter the results as well. Moreover, analysis methods for the calculation of Zap-70 positive B cells show great variability with all these methods containing subjective elements. In the first studies T and NK cells, considered as positive cells were used as internal positive controls, other groups used isotype control in the experiments, while normal B cells serving as internal negative control were also utilized for Zap-70 detection in B cells. All this data show the need for standardization of these procedures. For optimizing the method 10 normal samples were analyzed. To accurately determine Zap-70 expression in normal B cells the vertical cursor was set at different places and the effect of its position on the test was examined. The higher the Zap-70 negative T/NK cell percent the lower the Zap-

70 positive normal B cell percent. Based on these comparisons the most authentic results were obtained at a cursor position where 5% of the T/NK cells showed negativity (analysis was also permormed at 1% and 2% T/NK cell negativity cursor position). Here the average of (normal) B cell positivity was 3.36%±2.32 that showed good correlation with the literature and no false positivity was seen. Another way for calculating Zap-70 expression is by using normal B cells as internal negative control. However, in most B-CLL patients' sample all B cells show an aberrant CD5/CD20/CD23 coexpression together with the absence of normal B cells. Due to this, mixing normal B cells from a healthy donor blood sample may be needed to perform the analysis. Afterwards a normal CD5 negative CD19 positive B cell population will be present in the sample to which we can set the position of the vertical cursor. In our experiments we found that results assessed by both above mentioned analysis (using T/NK cells as positive or using normal B cells as negative control) techniques showed highly similar results.

Minimal residual disease detection by flow cytometry after monoclonal antibody therapy in ALL

A 15-year old female with common-ALL, treated according to ALL-BFM 95 experienced bone marrow (BM) relapse 48 months after diagnosis. Salvage therapy according to ALL R-87 was accompanied by corticosteroid psychosis and avascular necrosis of the right femoral head. A second BM relapse occurred 67 months later. At this time point CD10bright/CD19/TdT coexpression of leukemic lymphoblasts, infiltrating the initial BM sample in 76% (3432/µL), provided a useful aberrant immunophenotype for identification of MRD. After induction treatment (Day 25) these cells decreased below detection limit of flow cytometry ($<10^{-3}$). The CD20 coexpressing (CD10/CD/19/CD20+) lymphoblasts, representing

59% (1853/μL) of the initial leukemic population, disappeared by Day 25 and reappeared along with a small, increasing ratio of CD10/CD19/TdT-positive cells after consolidation, i.e. prior to rituximab treatment. Application of 2 courses of rituximab resulted in a delayed decrease in the CD10/CD19/TdT-positive subpopulation, although CD20-positive cells completely vanished (both from BM and peripheral blood) after the first infusion and never reappeared. At Month 13, a more immature CD19/TdT-positve/CD10/CD20-negative lymphoblast population was present at 12% (879/μL) in the sample. Relapse was obvious on MGG-smears. A monoclonal, incomplete, patient-specific T cell receptor (TCRδ) gene rearrangement was identified at second relapse and it was used as a target sequence to monitor MRD by RQ-PCR at the Genomics Center of the Biochemistry Department. MRD level in follow-up samples was compared to the tumor load of that initial sample. MRD level decreased slowly and fell below quantitation limit by Month 6, i.e. before the first rituximab treatment and remained undetectable until Month 10. At this data point MRD level by RQ-PCR started to rise and by Month 13 the tumor load was 20 times higher than at Day 1. Due to the previous corticosteroid side-effects, FLAG-IDA induction treatment was applied resulting in a third complete remission. Since a further intensification of conventional chemotherapy was limited by severe myelosuppression and the patient was MRD positive, two courses of rituximab, a monoclonal anti-CD20 antibody (MabThera®, Roche, Hertfordshire, UK, 375 mg/m^2, 4 weeks apart), intercalated in interim maintenance treatment, were applied as post-consolidation therapy, without complications. 13 months later a third BM relapse occured. The patient, having consented to hematopoietic stem cell transplantation, was referred to the regional transplantation center. She received a repeated course of FLAG-IDA and died of fulminant pulmonary aspergillosis in massive pancytopenia 14 months after the second relapse.

DISCUSSION

Sensitivity, specificity and predictive value are cardinal issues in laboratory testing. The use of unsuitable methods can lead to false positive and/or false negative results. Non-specific reactions due to incorrect labeling of cells, instead of true marker positivity have to be identified. While interpreting the results sensitivity, specificity and whenever possible predictive value was assessed. The multicolor immunophenotyping with flow cytometry is a recommended tool in the diagnosis of malignant and pre-malignant hematological diseases besides the classic morphological methods. A reproducible and well standardized method in the diagnostic process, which influences the treatment selection heavily, is inevitable. Besides the correct diagnosis there is also a need for detection of prognostic factors in clinical oncology. My studies comprised of: diagnosis with detection of aberrant phenotypes, prognostic marker identification (MDR, Zap-70) and monitoring (MRD). My aim was to optimize flow cytometric techniques suitable for the above mentioned processes and to reach the most ideal sensitivity and specificity. I also tried to improve the objectivity of flow cytometric measurements throughout my work.

In the past few decades flow cytometry has emerged as a valuable, reliable and relatively cheap diagnostic tool in the detection of deficient expression of the glycosylphosphatidyl-inositol (GPI)-anchored proteins in PNH patients. The advantage of flow cytometry is, that even in non-hemolysing patients, small PNH clones can be detected and the proportion of type III, type II and type I cells can be determined. Previous reports did not express the heterogeneous expression of the GPI-anchored proteins compared to normal cell populations. We recommend a new parameter, the MFI rate, which can be obtained by dividing the MFI of the PNH type II or type III cells by the MFI of the respective cell population obtained from a normal sample. The smaller the MFI rate, the better the discrimination between the GPI-

negative and the normal cells can be achieved. Our results are in accordance with previous ones describing that anti-CD55 does not delineate the RBC population as well as anti-CD59 does.

Calcein–acetoxymethylester, the hydrophobic derivative of calcein enters the cells passively, fluorescent calcein is produced by cellular esterases. Then calcein is actively extruded from resistant cells by MDR proteins. The fluorescent signal in the cells is reduced when MDR transport proteins are active, but in the presence of inhibitors e.g. verapamil calcein is accumulated in the cells. If these transporters are not present or do not work the hydrophilic calcein is trapped in the cell. This provides a basis for quantitiave analysis. Results were expressed as multidrug resistance activity factor (MAF) calculated by the formula [FI(Vp+) - FI(Vp-)]/FI (Vp+) x 100 that measures the transport activity of both Pgp and MRP1 (MDR proteins), and the results were correlated with the level of extrusion of Pgp/MRP1 substrate cytotoxic drugs. Calcein assay can be combined with surface staining, this way the specificity of the test is better. Nevertheless, we have to take into consideration the fluorescent spillover.

Chronic lymphoproliferative diseases (CLPD), such as B-CLL are a heterogeneous group of diseases. The course can vary from indolent forms to progressive disease: the majority of patients survive without any treatment for decades, while others die within a few years despite agressive chemotherapy. Identification of prognostic factors whic are able to predict disease outcome in an early phase i.e. at the time of diagnosis is crucial to select patients with poor prognosis and to initiate early chemotherapy. Zap-70 showed a strong correlation with the immunoglobulin variable heavy chain (IgV(H)) gene mutational status in expression profile analyses. It is important to note, that correlation of Zap-70 expression and prognosis is not absolutely clear. Zap-70 is an intracellular marker that can be detected by flow cytometry following permeabilization of cells. The field of analysis in case of Zap-70 expression shows also great variability. In order to induce the sensitivity of the assay 100 000 cells were

detected in the measurements. The most widely accepted 20% positivity cut off was used to discriminate between Zap-70-positive and –negative groups. In the analysis the position of the vertical cursor is crutial being the basis of Zap-70-positive B cell calculation, which was set at different positions based on normal T/NK or normal B cells. The conclusion of the analysis was that very similar results were obtained using the T/NK cells as internal positive controls (with a vetrical cursor position, where 5% of these cells were negative), or normal B cells as internal negative control, which had to be added to the patients' sample.

The expression of FXIII-A in tissue specimen has been investigated in multiple disorders. Based on the results of normal cells it was presumable, that malignant counterparts of megakaryocytes and monocytes also express FXIII-A. This has first been shown by immunomorphological techniques in case of acute monocytic leukemias. Today the generally accepted methodology of leukemia phenotyping is multi-color flow-cytometry. We found that in case acute monocytic and myelomonocytic leukemias FXIII-A was more sensitive in identification of leukemic blasts than the generally used monocyte marker CD14. When acute megakaryocytic leukemia cases were analyzed we found that FXIII-A labeling was more specific than the conventionally used platelet markers, which can lead to false-positive results not only in case of non-megakaryocytic leukemias, but also for example in case of extracorporeal circulation and reperfusion. Adhesion of circulating microparticles, a very heterogeneous group of cell fragments, to white blood cells can also be the cause of false platelet marker positivity. In de novo AML M7 cases a simple washing procedure with EDTA containing buffer lead to identical FXIII-A and platelet glycoprotein marker expression. This means that in these cases FXIII-A expression specifically shows the true megakaryocyte fraction. Similarly, in normal samples, this washing procedure was useful to eliminate false platelet marker positivity.

Normal lymphoid cells do not synthesize or contain either subunit of FXIII as has been shown previously. To our surprise in de novo B-acute lymphoblastic leukemic patients' bone marrow samples FXIII-A positivity was detecable in 40% by flow cytometry. A false-positive reaction due to platelet satellitism was excluded using various methods, such as confocal microscopy, ELISA and Western blotting. When comparing the amount of FXIII-A in lymphoblasts to normal monocytes it was found that the quantitiy is not negligible. By confocal laser scanning microscopy FXIII-A could be localized in the cytoplasm with a similar condensed staining pattern to normal monocytes and peritoneal macrophages, but nuclei were devoid of FXIII-A staining. The function of FXIII-A in the cytoplasm is still uncertain. Its role in phagocytosis, and in the alternative activation of macrophages has been demonstrated. In addition, a proangiogenic effect of activated FXIII has been described. Acute leukemias are usually associated with aberrant phenotypes confined to the original lineage or exhibiting lineage infidelity. These aberrant phenotypes are important because, with rare exceptions, single leukemia specific antigen detection cannot be routinely applied. LAIP is highly important in disease monitoring and treatment follow-up. Previous reports have emphasized the relevance of intracellular staining in the flow cytometric study of acute leukemias. Here, we present data that underlines the importance of the investigation of FXIII-A in subtypes of AML where its sensitivity and specificity surpasses that of several presently used markers. FXIII-A is eventually such an intracytoplasmic marker that can easily be utilized in the identification of aberrant phenotypes rendering it a valuable diagnostic tool in acute leukemias not only in the myeloid lineage but also in the B-lymphoid lineage as well.

Flow cytometry is not only suitable for diagnosis but also for the monitoring of hematological diseases, which is equally important as well. The aim of these studies is to find the small amounts of residual pathological cells in the body (MRD). More events need to be characterized for MRD detection than at the time of diagnosis, which elevates the sensitivity

of the examination. This way even 1 leukemic cell may be identified next to 100 000 normal cells. Detection of LAIP at time of diagnosis is cardinal, because these aberant phenotypes are searched in the course of MRD detection. In case of B- and T-ALL LAIP can be identified in 95% of samples. We followed a cALL case, where the previous severe drug side-effects and relapses lead to a unique therapeutical choice, which was rarely used in childhood ALL before. Rituximab is a monoclonal antibody directed against the CD20 B cell surface antigen, which is widely used in CLPD and was also tried out in adult B-ALL. Rituximab lead to the complete elimination of the CD20-positive leukemic subpopulation, but did not eradicate the more immature CD20-negative lymphoblasts. The Q-PCR method detected a different MRD clone than flow cytometry, so it is advisable to use both techniques in the follow-up procedure until the exact predictive value of both methods is analyzed.

For the diagnosis and monitoring of hematological diseases flow cytometry is one of the most objective and quantitable method available today. This can be achieved by analyzing more events, using multicolor staining and enumerating fluorescence intesities.

Concluding remarks

Flow cytometric results, especially those expressed in a quantitative form, combined with multiparametric analysis, are extremely valuable in the diagnosis and subclassification of malignant hematological diseases. The results of the prognostic factors detectable by flow cytometry are available for the clinician already at the same time as the immunophenotypic analysis.

PUBLICATIONS INCLUDED IN THE THESIS:

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