The value of multiparameter flow cytometry of cerebrospinal fluid involved by leukemia/lymphoma cells*

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The usefulness of multiparameter flow cytometric (FC) analysis of cerebrospinal fluid (CSF) was evaluated in leukemia/lymphoma patients having central nervous system (CNS) involvement of the disease. In 12 specimens of 8 patients with different types of leukemia/lymphoma (one case of T-ALL, 3 cases of early B-cell ALL, one case of AML, and 3 proven or suspicious NHL cases) the presence of pathological clone in CSF has been confirmed or excluded. The phenotypic patterns of CSF cells were defined according to those of bone marrow (BM)/peripheral blood (PB) at diagnosis or during follow-up of the same patients. Furthermore, in one case of suspicious CNS infiltration of NHL, the pathological clone was characterized as a highly suspicious of solid tumor and was proved to be a lung cancer metastasis. The definition was made on the basis of CD45 (common leukocyte antigen) and other studied CD markers negativity. The exact comparison of immunophenotypic profiles of specimens from different sites (CSF, BM, PB) of the same patient has been performed and no phenotypic changes were found. In some CSF specimens, where no cells of suspicious pathological clone were detected, in 4-color analysis only normal lymphocyte population was found even in small cell samples (even if the cellularity was ‹ than $0.3x10^{-6}$). In these populations the high values of T-cells (CD3+) predominated and the high prevalence of CD4+ over CD8+ cells, and an almost total lack of B-lymphocytes was found.

Our results suggest that positive CSF immunology is auseful indicator of malignancy and reflects leptomeningeal involvement. Simultaneously we demonstrated that FC analysis of CSF in the aim to detect possible CSF seeding of leukemia/lymphoma is a reliable and quick technique.

Key words: flow cytometry, multiparameter analysis, cerebrospinal fluid, lymphocyte subpopulations, CNS tumors

Flow cytometry has become an important tool in the diagnosis and characterization of hematologic and lymphoid neoplasia [5, 6]. This technology serves as an excellent completion to microscope-based traditional diagnostic methods and adds distinctive capabilities that are unmatched by any other diagnostic methods [3, 9]. FC is very usefull for fluids investigation where cells are naturally suspended and is also useful in lymphoid tissues, from which single-cell suspensions can be easily obtained [1, 10]. The advantages of FC are largely based on its ability to analyze very rapidly, even in small samples, multiple cell properties simultaneously, including size, granularity, surface and intracellular antigens and their combinations [2]. The quantitative nature of the obtained data, both with regard to cell population distributions and to expression of individual antigens, offers objective criteria for interpretation of the results [4, 13, 15]. Examples of applications include the detection of clonal cells in B-cell malignancies, the recognition of antigenic expression anomalies in B- or T-cell malignancies, the identification of T-cell subtypes, solid cell tumors and their metastasis [3, 14, 16]. The unique attributes of FC allow for increased sensitivity in the detection of neoplastic cells [2].

The aim of our study was to demonstrate the implementation of FC analysis, especially that of multiparameter one, to CNS neoplasms examining the CSF samples. Results are proving that the method allows to discriminate lymphoproliferative disorders, metastasis of solid tumor and reactive lymphocytes.

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Table 1. Summary of clinical data and marker expression of 8 patients with malignant CNS neoplasms

No.	Sex	Diagnosis	Data	Antigen expression	Specimen
1	m	T-ALL	13.5.2002	CD1,CD2,CD4+CD8 (dual), CD4,CD7,CD38,CD71,cCD3,nTdT,CD10	BM, PB
			17.6.2003	CD3,CD4,CD5,CD7,CD8 (no pathol.cells)	CSF
			21.10.2003	CD3,CD7,CD4,CD8 (remission)	BM
			23.10.2003	T-ALL CNS relapse, the same phenotype as at diagnosis in BM and PB (67%); residual lymphocytes (3%)	CSF
			28.4.2004	remission	BM
2	m	Early B-ALL	29.3.2004	HLA-DR,CD19,CD10,CD20,CD22	BM
			2.4.2004	No pathol. cells; CD3,CD4,CD8, CD45 (small amount of T-cells)	CSF
3	f	Early B-ALL	20.10.2000	CD10,CD19,HLA-DR,CD34, low CD45	BM
			21.1.2003	remission	BM
			24.1.2003	CD10,CD19,HLA-DR,CD34, low CD45	CSF
			14.7.2003	No pathol. cells	CSF
4	m	Early B-ALL	29.11.1995	*CD10,CD19,HLA-DR	BM
			17.10.1997	*CD10,CD19,HLA-DR	CSF
5	f	AML-M2	4.4.2003	CD13,CD33,CD11b,HLA-DR	BM
			22.4.2003	CD13,CD33,HLA-DR	BM
			13.8.2003	No pathol. cells; CD4,CD8,CD3,CD45 (small amount of T-cells)	CSF
6	f	B-NHL	22.10.2003	No cells	CSF
7	f	B-NHL	11.6.1999	CD19,CD22	CSF
			15.6.1999	CD19,CD20,kappa	CSF
			7.10.1999	No cells (after CHT+RT)	CSF
8	m	Susp.B-NHL	19.8.2003	CD4,CD8,CD3,CD45 (reactive T-cells) + CD45-negat. cells (lung cancer metastasis)	CSF

*one-color staining only

Material and methods

Patients and samples. Eleven CSF samples were obtained from 8 patients with suspect meningeal involvement, mainly of acute leukemia or non-Hodgkin's lymphoma. One patient had T-ALL and his BM and PB were immunophenotyped in detail in our laboratory at diagnosis, three patients had early B-ALL, one patient AML and 4 patients were suspect of CSF involved with NHL; in one of them the immunological diagnosis of solid tumor had been made. Three patients were children, five were adults. The number of CSF examinations per patient varied from one to three. Patients characteristics are listed in Table 1.

Methods. CSF samples obtained by lumbal puncture were stored in heparinized tubes and were processed within 1 to 2 hours. The aspirates for the immunophenotyping were washed in phoshate-buffered saline (PBS) and resuspended in PBS; simultaneously performed studies of BM or PB cells were measured after erythrocyte lysis (Optilyse B, Immunotech, Marseille, France). Antibodies directly labeled by 4 different fluorochroms (FITC, PE, ECD and PC5) were used in 2- or 4-combinations. Cells were stained using monoclonal antibodies directed against the following surface or cytoplasmic markers (used mainly from Immunotech, Marseille, France, or rarely from Caltag, CA, USA) – CD1, CD2, CD3, CD4, CD5, CD7, CD8, CD34, CD38, CD71, TdT, CD10, CD19, CD20, CD22, CD45, HLA-DR, IgM, kappa, lambda, CD13, CD33, CD11b. The analysis was performed by multiparameter flow cytometry using an EPICS ALTRA flow cytometer (Beckman Coulter) equipped by Expo 32 program for analysis. The labeling procedure, analysis and cellular fixation/permeabilization are given in detail in previous studies [7, 8]. The background fluorescence level for each specimen was established using cells incubated with the appropriate fluorochrome-coupled isotype control. Results of FACS analysis were available within 1 hour.

Results

In presented study the results of 21 immunophenotypic analyses in 8 patients with leukemia/lymphoma with suspecious CNS involvement are demonstrated. Eleven of them were analyses of CSF specimens, the rest were samples of BM or PB.

Table 2. Multiparameter flow cytometric analysis of CSF in the patient with early B-cell ALL; no cells of pathological clone are present; lymphocyte subsets values

CSF			Ly
1.	CD10/CD22/CD45	:	0/0/98
2.	CD4/CD8/CD45/CD3	:	46/53/100/93
3.	CD11b/CD19	:	1/1(1)

Table 3. Multiparameter flow cytometric analysis of CSF in the patient with AML M2; no cells of pathological clone are present; lymphocyte subsets values

CSF	
1. IK (F/PE/ECD/PC5)	0
2. CD4/CD8/CD45/CD3	74/25/100/93
3. HLA-DR/CD33/CD45/CD13	23/1/100/1

Table 4. Multiparameter flow cytometric analysis of CSF in the patient with metastatic solid tumor; lymphocyte subsets values

CSF	Gate-1 lymphocytes (36%)	Gate-2 pathological cells (49%)
1. IK (F/PE/ECD/PC5)	2/0/0/1	0/1/0/0
2. CD4/CD8/CD45/CD3 T-cell lineage	67/23/84/72	2/0/3/2
3. CD20/CD19/CD45 B-cell lineage	2/2/85	0/0/3
4. CD14/CD13/CD45/CD33 Myeloid lineage	1/2/84/1	2/13/3/2

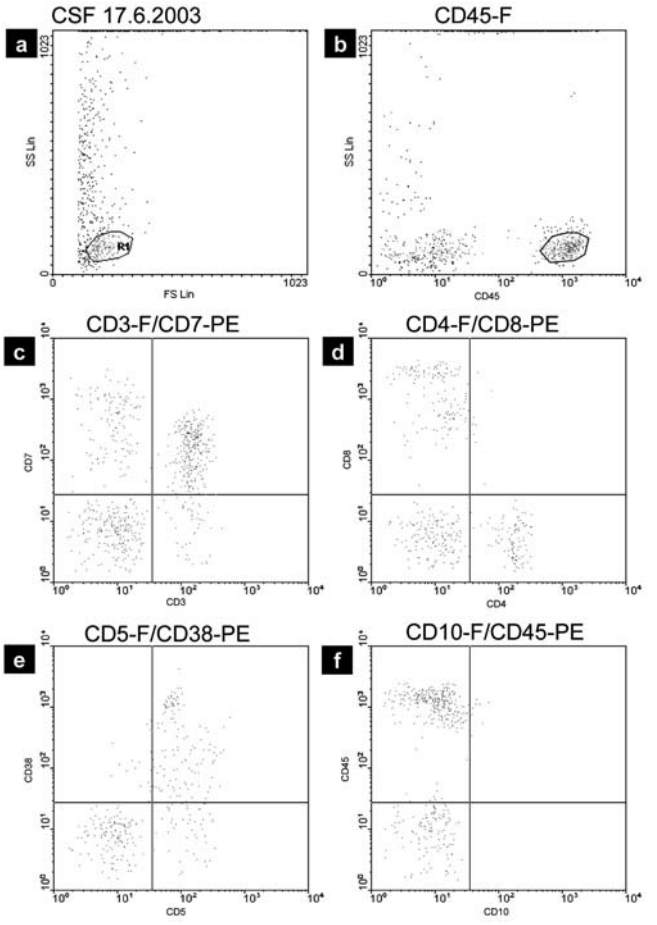

Figure 1. Immunological findings of patient No 1 in which suspecious T-cell leukemia relapse was not confirmed. Physical parameters are given in a, b and dot-plot quadrant graphs with markers defined T-ALL in c–f.

In the first part the results are given to demonstrate the comparison of immunophenotypic profiles of infiltrated CSF specimens with those of different sites of the same patient (BM, PB).

In T-ALL patient (No. 1) in BM and PB the exact phenotypic profile has been established at diagnosis and T-ALL of late cortical phenotype with expression of markers CD7+, CD5+, CD2+, CD1+, "dual" CD4+CD8, CD38, CD71 and coexpression of CD10 were found (Tab. 1). In cytoplasm the expression of CD3 marker and in nucleus TdT enzyme was detected. In this patient two CNS relapses were supposed. The first one was not confirmed by CSF cells immunophenotyping (Fig. 1) and in the second one (Fig. 2) the pathological cells of the same phenotypic features as those of cells in BM/PB at diagnosis were found. The CNS relapse preceded by 3 days BM phenotyping, in which the remission was present (Tab. 1).

From three patients with ALL of early B-phenotype in 4 suspicious specimens the CNS infiltration in CSF was confirmed twice. The phenotypic patterns of leukemia cells in CSF and BM in these cases displayed the same markers. Example is given in Table 1 and Figure 3 (patient No. 3). The distinct very low values of CD45 expression and very high values of CD10,CD19,CD34 and HLA-DR were detected. After 6 months CSF puncture was repeated and no pathological cells were present. In two patients with ALL of early B-phenotype no pathological cells in suspecious CNS relapse were detected; an example is given in Table 2 and Figure 4 (patient No. 2).

Immune phenotyping of CSF (patient No. 5) with suspicious AML infiltration of CNS did not confirm the presence of pathological clone. In multiparametric 4-color analysis only asmall amount of normal T-lymphocytes was detected (Tab. 3, Fig. 5).

In some CSF examinations, the involvement of CNS by malignant cell clones was not confirmed. Samples had very low cellularity (even < than $0.3x10^{-6}$) and in 4-color staining only normal T-cells could be defined. High expression of CD3+ T cells with high predominance of CD4+ over CD8+ T-cells were stated in all cases but one, in which the proportion CD4/C8 was equal. B-lymphocytes were absent or very

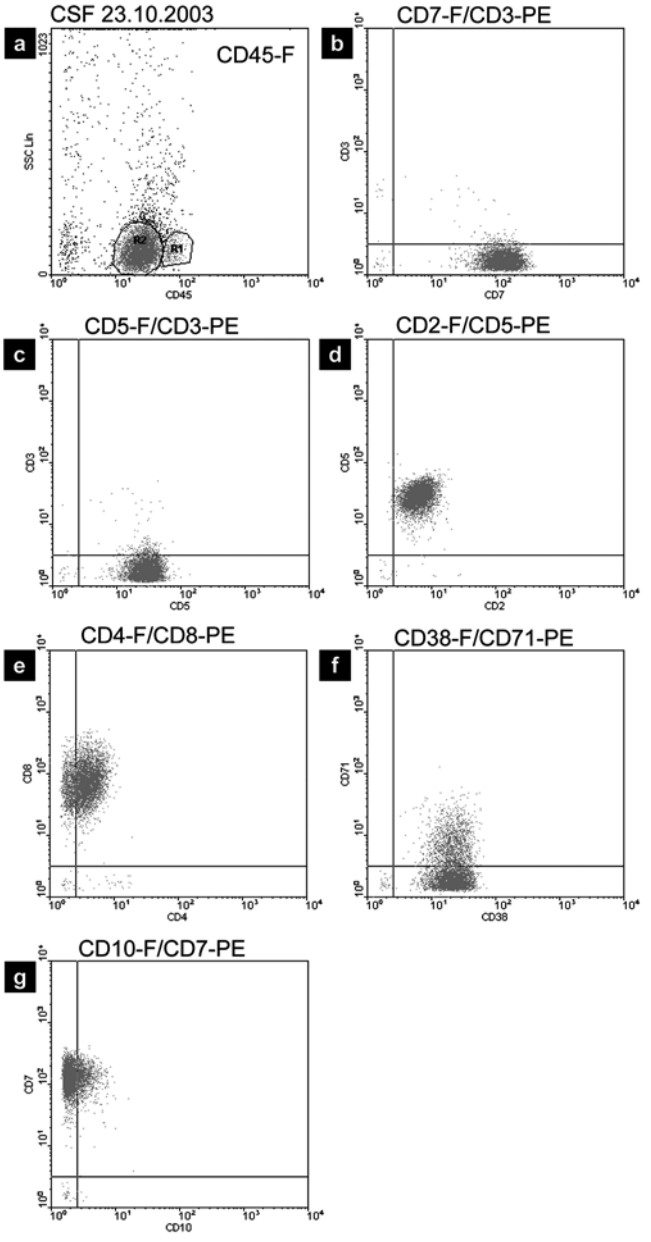

Figure 2. Immunological parameters defining relapse in a patient with T-ALL (patient No. 1). In a SSC with CD45 residual normal lymphocyte population (R1) and pathological clone (R2) are shown. Dot-plots in quadrantes with markers defined T-ALL are given in double combinations in b–g.

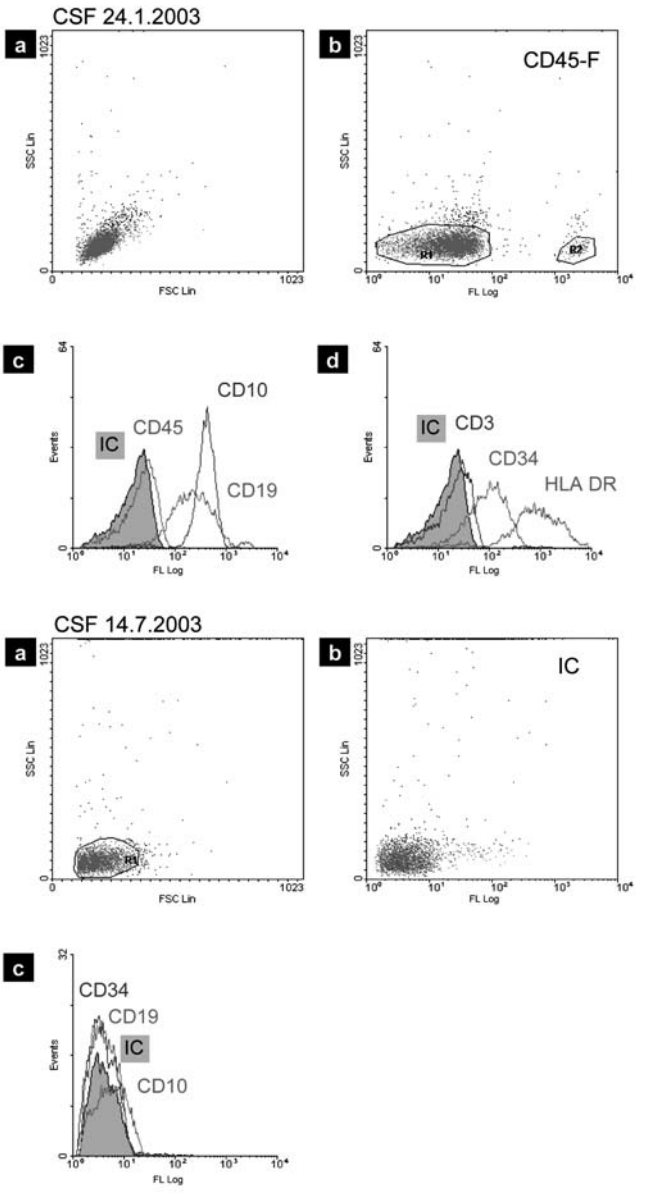

Figure 3. Immunological parameters defining relapse and remission in a patient (No. 3) with ALL of early B-phenotype. At first date relapse values are given, physical parameters of CSF cells in aand b, and comparative histograms – abscissa – intensity of fluorescence, ordinate – number of cells, show values of pathological markers in c and d. At second date CNS remission was found in CSF, no cells with markers of the pathological clone were present (SSC and FSC values are given in a, negative isotype control in b and markers examined in c).

scarce. The percentage marker values are given in Table 2, 3 and 4.

Furthermore, we have shown that the FC analysis enabled to differenciate between NHL and solid tumor CNS involvement. In patient No. 8 suspecious CNS infiltration of NHL was not confirmed. In his CSF sample, 2 different cell populations were detected. 36% were T-lymphocytes (CD3+,CD4+,CD8+) which could represent a reactive component of T-cell population. Second population (49%) was represented by cells of non-leukemia phenotype, the cells were negative for both, CD45 (pan-leukocyte marker) and for any of studied markers. Therefore, they were with high probability classified as solid tumor cells. The diagnosis was definitely proved at autopsy as metastatic large-cell lung carcinoma (Tab. 4, Fig. 6).

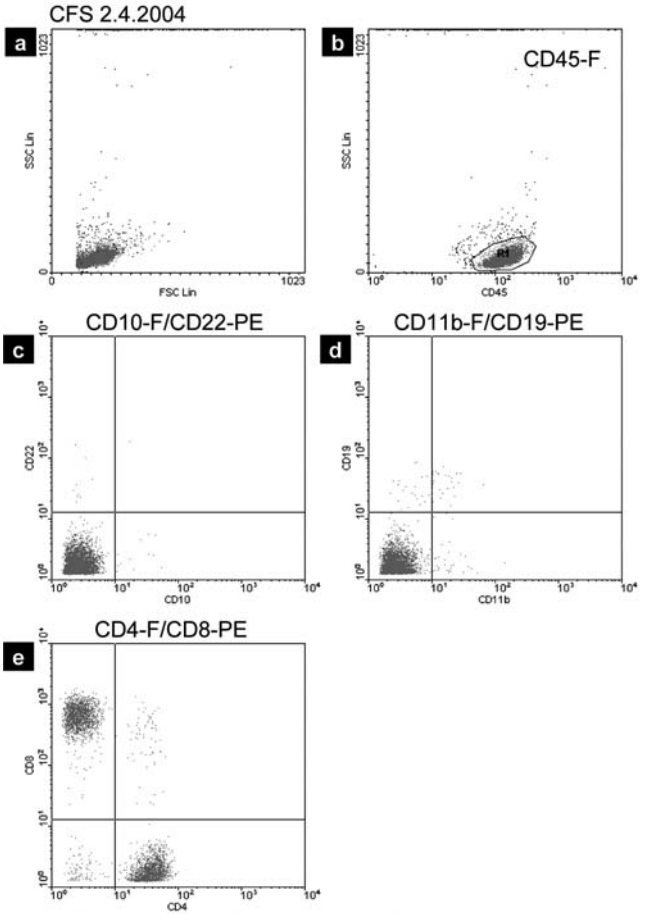

Figure 4. Immunological parameters defining CNS remission in patient (No. 2) with ALL of early B-cell phenotype. No cells of pathological clone were detected (physical parameters are shown in aand b and markers examined in c–e).

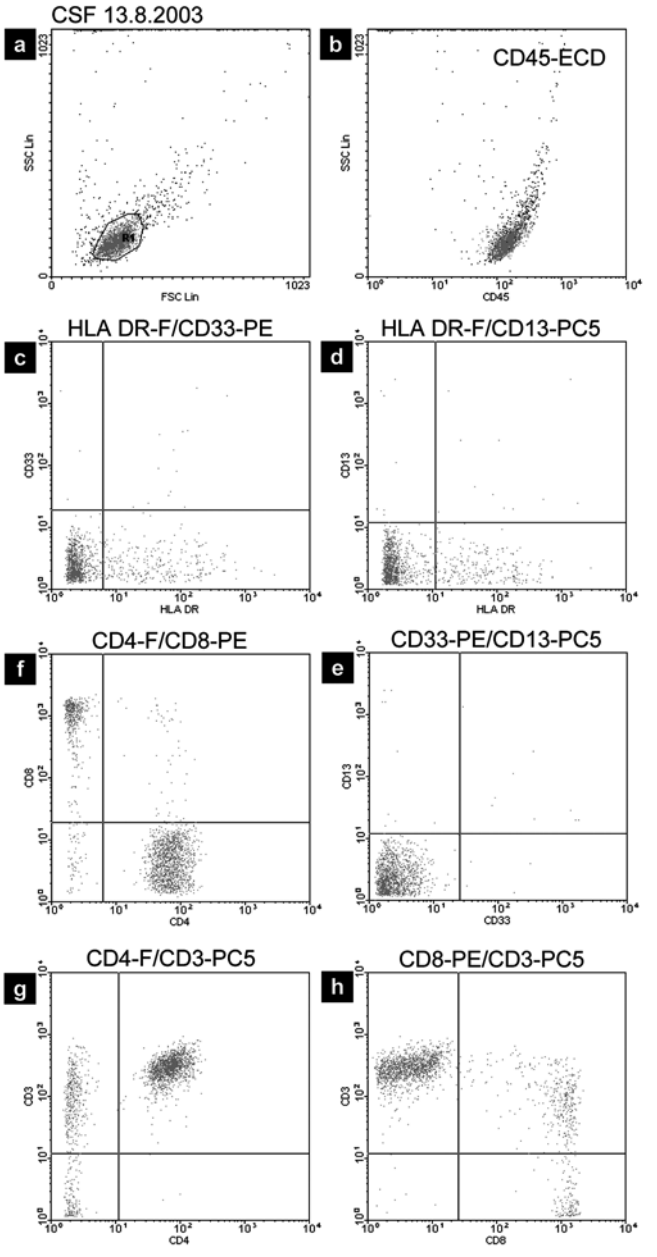

Figure 5. Flow cytometric immunophenotypic values of CSF (patient No. 5) with suspicious AML infiltration of CNS did not confirm the presence of myeloid pathological clone. In 4-color analysis only asmall amount of T-lymphocytes was detected (physical parameters are shown in aand b and markers examined to confirm/exclude pathological clone are given in double combinations in c–h).

Discussion

Flow cytometry is a very suitable method for demonstrating pathological clone in cell suspensions according to their surface and/or cytoplasmic markers, mainly in their combinations. Four different markers can be detected at the same run. Our study demonstrated that the method could contribute to improving accuracy and precision in the diagnosis and classification of lymphomas and lymphoproliferative disorders and some solid tumors. Up to now, FC analysis has predominantly been used to demonstrate malignant clones in peripheral blood and bone marrow suspensions [2, 11]. Recently it was shown that FC is a reliable and quick method which allows not only the confirmation of meningeal involvement, but also a classification of the malignant clone according to its immunological properties [14].

DONSKOY et al [4] pointed out that phenotypically defined forms of ALL (almost of exclusive expression of cytoplasmic Ig heavy chain mu or surface CD22, CD23, and/or IgM marker, which normally characterize the most developmentally mature members of the B-cell series in BM) invade and persist in the CNS. Thus, immunophenotyping of BM blasts at diagnosis could delineate the ALL cases with biological predisposition to CNS involvement in individual cases of ALL [4].

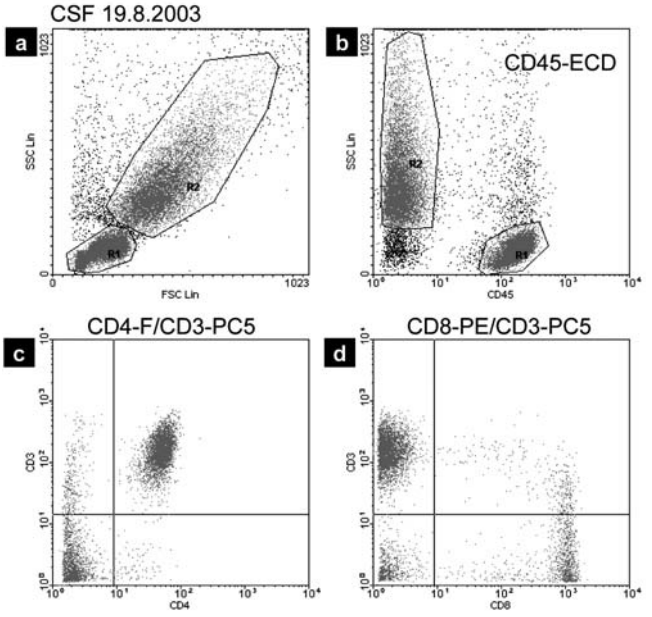

Figure 6. In cerebrospinal fluid (patient No. 8) two different cell populations were detected. 36% created T-cell lymphocytes – R1. Second population (49%) – R2 represented cells of non-leukemia phenotype (CD45-negative). They did not express any of the studied markers (physical parameters are shown in a and b, markers of 3-, 4-color analysis in quadrants c and d).

It was shown in our study that the immunophenotypic profiles of cells in CSF were in a very good agreement with those of cells in BM/PB at diagnosis or follow-up (patients No. 1, 2, 4). These findings are in line with results of BANGERTEN et al [1], who found out that discordant antigen expression in samples from different body sites within the same patient was a rare event. They studied 29 patients with NHL using FC. From these patients, 60 simultaneous specimens taken from different sites were examined. The results showed that in 26 of 29 patients, the immunophenotype in different specimens was identical. We found that CNS relapse could not necessary coincide with BM infiltration (patient No. 1).

The number of cells in normal CSF is very low, therefore only a few investigators have employed healthy individuals as controls to patients groups [12]. In their study the comparison with T-cell population of CSF and PB in healthy donors has been performed and much higher values of CD3+ and CD4+ T-cells in CSF than in PB were found. In our study we showed that multiparametric FC analysis overcomes these difficulties and we managed to show the lymphocyte values of some patients samples in which even very low cell numbers were present. In samples negative for suspicious pathological clone there were the very high values of T-cells (CD3+ with high CD4/CD8 ratio) and an absence of B-cells (CD19+). The results were similar to that obtained by others [12, 16].

In conclusion: Multiparameter flow cytometry is the method for reliable and precise single-cell definition of pathological clone in cerebrospinal fluid and is able to distinguish leukemia/lymphoma and solid tumor cells from healthy lymphocytes.

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