Analysis of cerebrospinal fluid cells by flow cytometry: Comparison to conventional cytology

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Aims. This study compared the results obtained by basic immunophenotyping of cerebrospinal fluid (CSF) cells by flow cytometry (FC) to the results of conventional cytology and evaluated the possibility of detailed analyses of CSF B-cell subpopulations.
Methods. Samples from 42 patients were examined by conventional cytology (native and/or pre-centrifuged CSF) and FC. The results from 15 patients without evidence of organic neurological disease were used to estimate reference ranges.
Results. Pre-centrifugated CSF had significantly higher cell yield on the cytologic slide, but cell subpopulation percentages were altered; the percentage of lymphocytes was significantly higher and monocytes significantly lower compared to both native CSF slides and FC. The percentage of granulocytes was higher in FC compared to cytology. For leukocyte count, the following reference ranges were estimated for Fuchs-Rosenthal chamber (FR) counting and FC, respectively: leukocytes ≤4.7/μL and ≤2.5/μL, lymphocytes ≤4.1/μL and ≤1.8/μL, monocytes ≤1.2/μL and ≤0.9/μL, and granulocytes 0/μL and ≤0.2/μL. The following reference ranges were estimated for basic subpopulations: T-lymphocytes 84.1–100%, B lymphocytes 0.0–1.5%, NK cells 0.0–6.3%, NKT cells 0–9.5%, and CD3+CD4+/CD3+CD8+ 0.8–4.9. Using a volume of 1.2–2.4 mL, the number of B lymphocytes was too low (<20) in samples with ≤2.7 cells/μL in the FR.
Conclusions. Even normal CSF samples are amenable to basic mononuclear cell subpopulation analysis by FC. However, analysis of the B-cell subpopulations requires either a larger sample volume or selection of samples with ≥ 3 cells/μL.

Key words: cerebrospinal fluid, cytology, flow cytometry, B lymphocytes

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INTRODUCTION

Quantitative and qualitative cytological examination belongs to the basic diagnostic panel for cerebrospinal fluid (CSF) analysis[1-3]. In contrast, examination of CSF cells by flow cytometry (FC) is rare outside hemato-oncological indications[4]. By analyzing surface, and possibly also cytoplasmic, markers, FC has the potential to provide significantly more precise information on the cellular composition of the CSF (ref.[4,5]) compared to conventional cytology. Determination of various subpopulations of CSF cells and assessing their functional status in healthy subjects and neuroimmunological diseases has been the subject of several studies[6-8] and reviews[4,5,9]. However, low sample volume and low sample cellularity in non-infectious samples are limiting factors for routine use. For logistical reasons, it is usually complicated to use mononuclear cell separation or to freeze cells for later analysis.

The aim of our study was to introduce basic CSF mononuclear cell immunophenotyping by FC into routine practice. In addition, we assessed the possibility of a more detailed analysis of CSF B-lymphocyte subpopulations for use in CSF diagnostics outside hemato-oncology and in neuroimmunological research considering the crucial role B lymphocytes are known to play in the immunopathogenesis of multiple sclerosis (MS) (ref.[10]) and paraneoplastic syndromes[11]. We also focused on comparing the results to those of conventional cytological examination by cytocentrifugation of native CSF (ref.[12]) or CSF sediment after a pre-centrifugation step[13].

MATERIAL AND METHODS

Conventional cytology

Cell counting was performed microscopically using the Fuchs-Rosenthal (FR) chamber; hence, the microscopic cell count is hereafter denoted by FR.

Cytological slides of the CSF were prepared by cytocentrifugation at 20 *g* for 4 min in a Cytofuge 2 cytocentrifuge (Stat Spin, USA). Routinely, 0.2 mL of gently mixed CSF was applied to the cytocentrifugation chamber. When using pre-centrifugation (0.5–1.5 mL of native CSF, 400 *g*, 10 min), 0.1 or 0.2 mL of the cell sediment was applied after gentle resuspension.

Flow cytometry

Flow cytometric analysis was performed on the Navios EX flow cytometer (Beckman Coulter) using the following antibodies.

Basic CSF panel: CD45-FITC, CD3-PC5, CD19-ECD, CD56-RD1 (Cyto-Stat Tetrachrom, Cat. No. 6607073), CD4-APC-Alexa Fluor 750 (Cat. No. A94682), CD8-APC-AlexaFluor 700 (Cat. No. B49181), CD16-PE (Cat. No. A07766), CD14-Pacific Blue (Cat. No. B00846), Anti-HLA-DR-PC7 (Cat. No. B49180).

B-panel: CD45-Krome Orange (Cat. No. B36294), CD19-APC (Cat. No. IM2470), CD5-Pacific Blue (Cat. No. A82790), CD27-PC7 (Cat. No. B49205), CD38-PC5.5 (Cat. No. B49199), Anti-Kappa-FITC (Goat Polyclonal, Cat. No. C15623), Anti-Lambda-PE (Goat Polyclonal, Cat. No. C15189).

For red blood cell lysis in blood samples and blood-contaminated CSF samples, VersaLyse solution (Cat. No. A09777) was used.

Cell count was determined in the basic panel analysis by Flow-Count Fluorospheres (Cat. No. 7547053, Beckman Coulter).

A control using Flow-Check Pro Fluorospheres (Cat. No. A63493) was performed on a daily basis before sample analysis. Once per week, a control using Immuno-Trol Cells (Cat. No. 6607077, Beckman Coulter) was run as part of the routine operation of the cytometry laboratory.

Sample preparation

For FC analysis of the CSF, the available amount of sample (range 1.0 to 3.6 mL per panel; usually 1.2 or 2.4 mL) was used. After centrifugation in an Eppendorf tube at 400 *g* for 10 min, 1 mL of the supernatant was pipetted out for biochemical analyses and the remaining 0.2 mL washed by the addition of 1 mL PBS (NaCl 8 g/L, KCl 0.2 g/L, Na_2HPO_4 1.44 g/L, KH_2PO_4 0.24 g/L, pH 7.4) with 1% BSA (Serva, Cat. No. 11924.04). After gentle mixing and centrifugation, 1 mL of the supernatant was discarded and the remaining 0.2 mL made up to a total volume of 0.4 mL using PBS with 1% BSA or merged with the second sample aliquot. For the B panel, the washing step was repeated a second time. After incubating with antibodies (5 µL Cyto-Stat tetraChrome and 5 µL of every other separately purchased antibody for a basic panel; 4 µL of each antibody for B panel) for 25 min, red blood cells were lysed in blood-contaminated samples by adding 300 µL of VersaLyse for 25 min. Next, Flow Count Fluorospheres were added in an amount corresponding to 10% of the initial CSF volume used (e.g., 240 µL if standard amount of 2.4 mL was available) and the samples immediately analyzed. For B panel analysis, after incubation with the antibodies, samples were washed with 2 mL

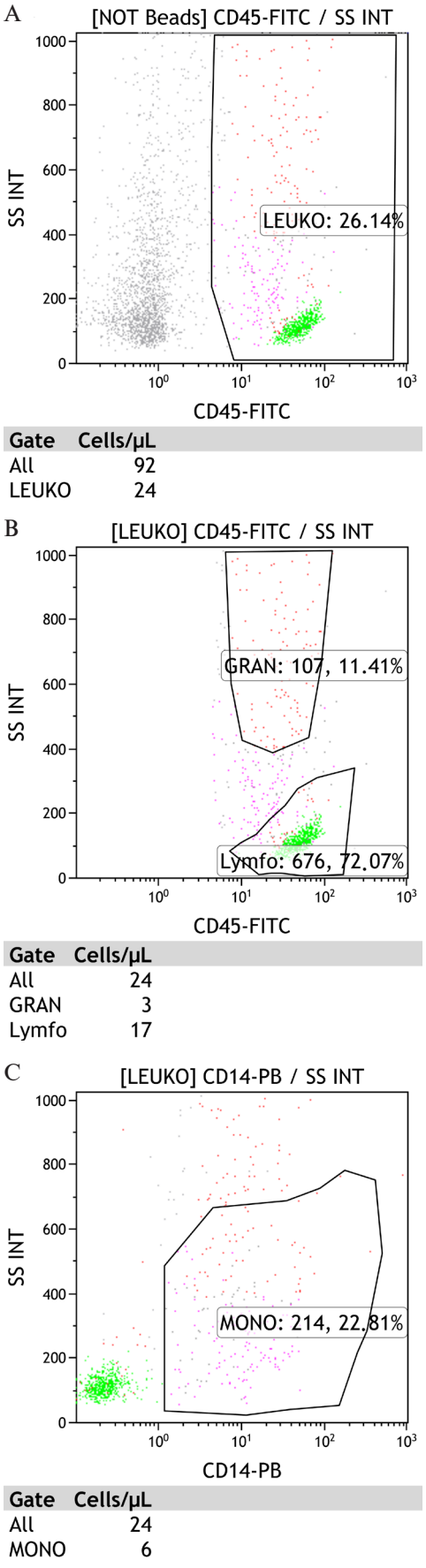

Fig. 1. CSF flow cytometry basic panel. **A.** Leukocyte gate (CD45 versus side scatter). **B.** Lymphocyte and granulocyte gate (CD45 versus side scatter). **C.** Monocyte gate (CD14 versus side scatter).

PBS containing 1% BSA, centrifuged for 7 min at 400 *g*, and 2 mL of the supernatant pipetted out and discarded. Finally, 300 µL of PBS containing 1% BSA was added.

For the analysis of blood (basic panel), 50 µL of EDTA-blood was incubated with antibodies (5 µL each) for 25 min. Next, 0.5 mL of the lysing solution (VersaLyse) was added for another 25 min. After vortexing, Flow Count Fluorospheres were added and the sample analyzed. For B panel analysis, 100 µL of blood was washed thrice in PBS with 1% BSA and centrifuged for 7 min at 400 *g*. After the last wash, 300 µL of PBS with 1% BSA was added and resuspended cells pipetted into the cytometric tube with antibodies (5 µL each). After 25 min, 1 mL of lysing solution (VersaLyse) was added for another 25 min, and the sample was centrifuged for 7 min at 400 *g*, decanted, washed with 3 mL of PBS with 1% BSA, centrifuged again for 7 min at 400 *g*, and decanted. Finally, 400 µL of PBS with 1% BSA was added and, after vortexing, the sample analyzed.

FC data analysis

FC data were analyzed using Kaluza software (Beckman Coulter). The gating strategy is outlined in Fig. 1. For CD19+ cell analysis, we used the mononuclear cell (MNC) gate. As a comparison, the analysis was also carried out with CD19+ cells out of the lymphocyte gate (Fig. 2).

Biochemical tests

CSF and serum albumin, IgG, IgM, IgA, and free light chains kappa (fKLC) and lambda (fLLC) were measured by nephelometry on a BN ProSpec analyzer (Siemens) according to the manufacturer's instructions. Oligoclonal IgG, IgM, and free light chains were analyzed as described previously[14,15].

Patients

A total 42 samples were evaluated: 10 patients with multiple sclerosis (MS), 3 patients with clinically isolated syndrome (CIS) not fulfilling recent MS diagnostic criteria[16], 1 patient with neuroborreliosis, 4 patients with non-inflammatory neurological diseases (vascular, n=1; cognitive deficit, n=1; polyneuropathy, n=1; primary CNS lymphoma, n=1), and 9 patients with unknown or uncertain neurological diagnosis. In addition, 15 patients without evidence of organic neurological disease served as a control group.

Statistical analysis

Statistical analyses were carried out using MedCalc® Statistical Software version 19.6 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2020). Data are presented as medians with interquartile range (IQR) and range. A robust method[17] was used to estimate reference values.

Ethics approval

The project was approved by the Ethics Committee of the University Hospital Ostrava (no. 237/2020). Lumbar puncture was carried out exclusively for diagnostic reasons. Patients signed informed consent with the use of the waste samples for research purposes.

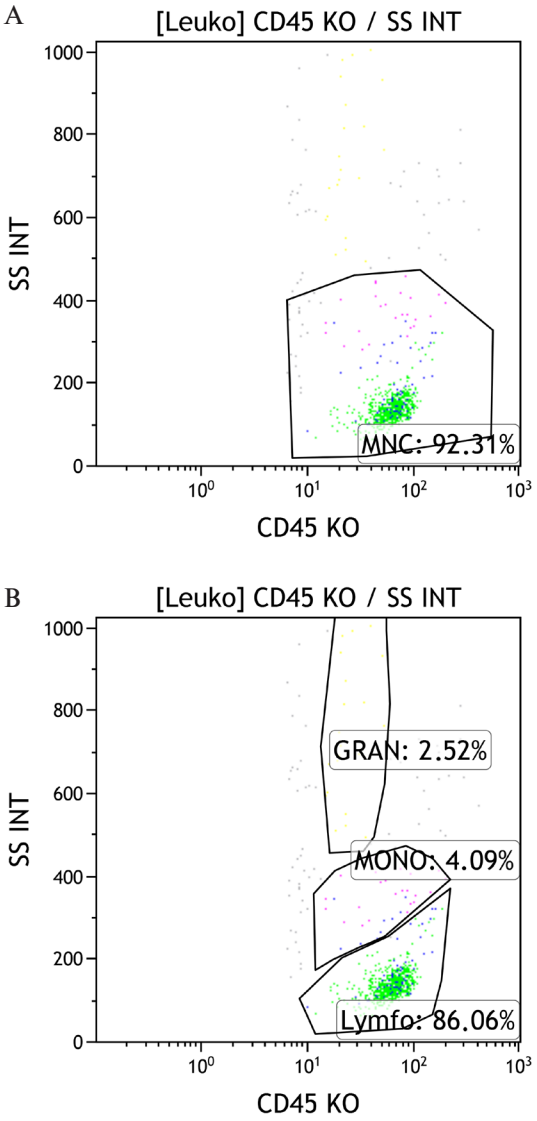

Fig. 2. CSF flow cytometry B panel. **A.** Mononuclear cell gate (CD45 versus side scatter). **B.** Lymphocyte, monocyte, and granulocyte gate (CD45 versus side scatter).

RESULTS

The CSF cell concentration in our series varied between 0.0 and 44.3/µL (FR: median 1.8; IQR 0.7–3.0/µL) and 0.1 and 78.1/µL (FC: median 0.7; IQR 0.5–1.5/µL; Table 1). FR values were significantly higher than FC values (Wilcoxon paired test, 11 positive and 29 negative differences, P=0.008). The number of lymphocytes analyzed in the FC basic panel varied between 59 and 17,787 (median 504; IQR 261–747). The number of analyzed lymphocytes was < 100 in only four samples (cell counts 0.0, 0.7, 2.0, and 4.3/µL in a CSF volume of 0.8–1.2 mL).

Table 1. CSF cell concentrations and number of cells available for analysis.

Diagnostic group	n	Cells/μL (FR)	Cells/μL (FC)	Number of cells on slide (native CSF)	Number of cells on slide (pre-centrifuged CSF)
MS (group 1)	10	2.8 (0.7–3.7; 0.7–44.3)	2.2 (0.6–4.5; 0.2–78.1)	105 (48–175; 1–231)	300 (132–387; 32–556)
Symptomatic controls (group 6)	15	2.0 (0.8–2.3; 0.0–4.3)	0.7 (0.5–1.2; 0.1–3.1)†	6.5 (3–21; 0–100)	132 (63–309; 3–457)
CIS (group 2)	3	1.7 (0.4–2.4; 0.0–2.7)	0.3 (0.3–0.5; 0.3–0.6)	66 (16–110; 0–124)	89 (22–133; 0–147)
Neuroborreliosis (group 3, myelitis)	1	37.7	29.6	>260	Not performed
Non-inflammatory CNS diseases (group 5)	4	1.7 (0.7–2.8; 0.3–3.3)	1.1 (0.9–2.7; 0.7–4.2)	1.5 (1–67; 1–131)	322 (223–386; 155–418)
Unknown or uncertain diagnosis (group 9)	9	1.0 (0.7–2.1; 0.7–9.0)	0.6 (0.4–0.7; 0.2–2.4)	23 (8–40; 2–109)	97 (57–119; 53–225)
Difference between groups*; MS versus controls**		NS; *P*=0.429	*P*=0.038; *P*= 0.078	NS; *P* = 0.071	NS; *P* = 0.100
Total	42	42	41	39	33

Data are presented as median (interquartile range; range) unless otherwise noted. * Kruskal-Wallis test. **Mann-Whitney test. †n=14.
MS, multiple sclerosis; CIS, clinically isolated syndrome; FC, flow cytometry; FR, Fuchs-Rosenthal chamber; NS, non-significant result. Diagnostic group numbers correspond to the standard description of the neuroimmunology study group in University Hospital Ostrava and Faculty of Medicine, University of Ostrava.

Table 2. Percentages of cells determined by conventional cytology using native or pre-centrifuged CSF and by flow cytometry (FC).

	n	Slides (native CSF)†		Slides (pre-centrifuged CSF)‡		*P**
		Median (IQR), %	Range, %	Median (IQR), %	Range, %	
Lymphocytes	15	86.5 (68.2–94.3)	43.5–95.9	93.7 (87.3–96.1)	83.0–99.1	0.002
Monocytes	15	9.5 (5.9–31.8)	3.7–56.5	5.4 (2.8–10.8)	0.9–15.1	<0.001
Neutrophilic granulocytes	15	0.0 (0.0–0.0)	0.0–7.0	0.0 (0.0–0.9)	0.0–5.6	0.438
	n	Slides (native CSF)†		FC		*P**
		Median (IQR), %	Range, %	Median (IQR), %	Range, %	
Lymphocytes	22	81.5 (60.6–94.6)	23.1–98.7	80.7 (70.6–86.9)	40.7–98.1	0.783
Monocytes	22	16.0 (5.7–37.6)	0.9–76.9	14.8 (7.3–26.8)	1.1–46.4	0.322
Neutrophilic granulocytes	15	0.0 (0.0–0.0)	0.0–7.0	7.8 (1.5–10.7)	0.0–17.5	<0.001
	n	Slides (pre-centrifuged CSF)‡		FC		*P**
		Median (IQR), %	Range, %	Median (IQR), %	Range, %	
Lymphocytes	31	92.7 (87.0–96.1)	70.0–99.1	83.7 (69.9–89.5)	40.7–95.9	<0.001
Monocytes	31	6.1 (3.9–9.9)	0.9–20.0	12.6 (7.4–25.9)	2.9–44.9	<0.001
Neutrophilic granulocytes	15	0.0 (0.0–0.8)	0.0–5.9	7.5 (3.1–10.6)	0.0–21.0	<0.001

*Wilcoxon paired test. †In two samples, not all cells were evaluated. For analysis of the slide cellularity, numbers of evaluated cells (231 and 260) are used instead. ‡ In four samples, not all cells were evaluated. For cellularity analysis, numbers of evaluated cells (316, 300, 410, 418) were used.

We compared the number of cells available on cytological slides using native CSF versus CSF sediment after pre-centrifugation in 30 samples for which both slides were prepared. We confirmed considerably higher cell numbers on slides from CSF sediment (median 140, IQR 76–300, range 0–457 cells) compared to slides from native CSF (median 15, IQR 3–37, range 0–229 cells; *P*<0.001). Unlike the earlier study[13], we demonstrated significant changes in the relative distributions of individual cell populations (Table 2).

The results of the FC analysis in the MS group and control group and the reference values obtained using the robust method recommended for a low number of measurements[17] are presented in Table 3.

In 16 samples, we were able to perform parallel CSF and blood FC analyses. A significant correlation was found between CSF and blood percentages of CD4+ Th lymphocytes (Spearman ρ=0.668, 95% confidence interval [CI] 0.257–0.874, *P*=0.005), CD8+ Tc lymphocytes (ρ=0.621, 95% CI 0.180–0.854, *P*=0.010), and the

Table 3. Results in the multiple sclerosis (MS) and control groups and estimated reference ranges.

	MS		Control		Estimated
	Median (IQR)	Range	Median (IQR)	Range	reference range
Cells/μL by FR	2.8 (0.7–3.7)	0.7–44.3	2.0 (0.8–2.3)	0.0–4.3	0.0–4.7
Cells/μL by FC	2.2 (0.6–4.5)	0.2–78.1	0.7 (0.5–1.2)	0.1–3.1	0.0–2.5
Lymphocytes/μL by FR	2.5 (0.7–3.0)	0.3–42.0	1.0 (0.4–2.2)	0.0–3.7	0.0–4.1
Lymphocytes/μL by FC	1.9 (0.3–3.7)	0.1–76.6	0.7 (0.4–1.0)	0.1–2.0	0.0–1.8
Monocytes/μL by FR	0.3 (0.0–0.7)	0.0–2.3	0.3 (0.0–0.7)	0.0–1.0	0.0–1.2
Monocytes/μL by FC	0.2 (0.1–0.3)	0.0–0.9	0.1 (0.0–0.1)	0.0–1.3	0.0–0.9
Granulocytes/μL by FR	0.0 (0.0–0.0)	0.0–0.7	0.0 (0.0–0.0)	0.0–0.3	0.0–0.3
Granulocytes/μL by FC	0.1 (0.0–0.1)	0.0–0.2	0.0 (0.0–0.1)	0.0–0.3	0.0–0.2*
CD3+ (%)	92.6 (90.8–94.1)	85.9–94.6	94.0 (91.8–95.4)	81.1–97.0	84.1–100
CD3+ CD4+ (%)	69.86 (63.5–78.5)	29.2–84.6	70.4 (60.7–74.7)	47.5–80.9	47.9–92.2
CD3+ CD8+ (%)	24.6 (18.1–29.2)	11.9–62.9	23.8 (20.1–26.3)	14.0–37.3	8.3–37.3
IRI	2.71 (2.28–4.35)	0.47–7.09	2.72 (2.36–3.64)	1.44–5.76	0.8–4.9
CD3+ DR+ (%)	11.9 (3.6–15.5)	2.6–29.0	8.5 (5.6–11.7)	3.2–28.3	0.0–21.9
CD3+ CD4- CD8- (%)	3.5 (2.3–4.7)	1.5–6.6	3.2 (2.1–7.1)	1.4–14.8	0.0–13.1
CD19 (%)	1.2 (0.4–3.8)	0.0–7.2	0.5 (0.2–0.7)	0.0–1.7	0.0–1.5
NK (%)	1.3 (0.9–2.8)	0.3–3.7	1.5 (1.0–2.6)	0.0–7.4	0.0–6.3
NKT (%)	1.2 (0.5–5.2)	0.1–12.7	2.1 (1.4–4.1)	0.2–10.5	0.0–9.5
CD14+ DR+ (%)	59.7 (51.9–65.2)	34.2–81.4	52.2 (34.5–63.9)	12.9–77.8	4.9–93.5
CD14+ CD16+ (%)	20.9 (11.4–27.1)	3.1–39.3	25.9 (18.3–38.0)	9.5–83.2	0.0–52.2

* After exclusion of an outlier according to the Tukey test

IQR, interquartile range; FR, Fuchs-Rosenthal chamber; FC, flow cytometry; IRI, immunoregulatory index.

immunoregulatory index (ρ=0.735, 95% CI 0.377–0.902, *P*=0.001). A weaker correlation was found for the percentage of NK cells (ρ=0.524, 95% CI 0.039–0.810, *P*=0.037). No significant correlations were found between CSF and blood values for other parameters. On the other hand, significant differences between CSF and blood were found for the percentages of lymphocyte and monocyte subpopulations using the paired Wilcoxon test. In CSF, there were significantly higher proportions of CD3+ lymphocytes (median 94.5% versus 72.9%, *P*<0.001), CD3+ DR+ lymphocytes (median 8.8% versus 5.8% of CD3+ cells, *P*=0.034), and CD16+ monocytes (median 18.6% versus 6.9%, *P*=0.002) than in blood. In contrast, proportions of CD19+ lymphocytes (median 0.5% versus 12.2%, *P*<0.001), CD3-CD16/56+ cells (median 1.1% versus 11.8%, *P*<0.001), and DR+ monocytes (median 58.3% versus 87.1%, *P*<0.001) were significantly lower in the CSF than in the peripheral blood. The immunoregulatory index was significantly higher in CSF than in blood (median 2.5 versus 2.2, *P*=0.005).

A reliable comparison of B-lymphocyte subpopulations in CSF and blood could not be made due to the low number of samples (only five samples with ≥ 10 CD19+ MNCs in the CSF available for comparison). The mean kappa:lambda ratio for CD19+ cells was higher in the CSF (median 2.79, range 0.64–4.33) than in blood (median 1.47, range 1.19–1.56). The proportions of CD5+, CD27+, and CD38++ cells were also higher in CSF (median 35.5%, 75.0%, and 36.4%, respectively) versus blood (1.9%, 23.2%, and 0.7%, respectively).

In a small group of patients, we were unable to find significant differences between MS patients and controls using the basic CSF panel. A tendency for a higher cell count in the CSF and a higher relative proportion of B lymphocytes and DR+ monocytes was observed. In conventional cytology, the only significant difference was the presence and proportion of plasma cells on cytological slides using CSF pre-centrifugation sample (absence of plasma cells in 12 controls versus their presence in 3/7 MS patients in a proportion of 1.0%, 1.5%, and 2.2%; *P*=0.0166). A similar, but non-significant, result was obtained for slides prepared from native CSF (absence of plasma cells in 6 controls versus their presence in 2/6 MS patients in a proportion of 0.4% and 0.9%).

The B panel was used in 14 CSF samples (4 MS patients, 1 patient with neuroborreliosis, 2 patients with non-inflammatory CNS diseases, 4 symptomatic controls, and 3 patients with unknown/uncertain diagnosis). In two symptomatic controls and one patient with an unknown diagnosis, the number of B lymphocytes available for analysis was < 10; these three samples were excluded from further analysis. The numbers of B lymphocytes available for analysis in the basic panel and the B panel significantly correlated. Both of these parameters also correlated with CSF cell count. More detailed analysis showed that the B panel can be reasonably applied in CSF samples with lymphocyte count ≥2.7/μL (≥8 lymphocytes in FR). In samples with lower lymphocyte counts, <20 B lymphocytes were detected in the basic FC panel in all 29 cases. In 13 samples with ≥2.7 lymphocytes/μL, ≥20 B lymphocytes were detected in 6 samples (46%). The highest CSF cell concentration in samples with <20 B lymphocytes detected by FC was 9/μL.

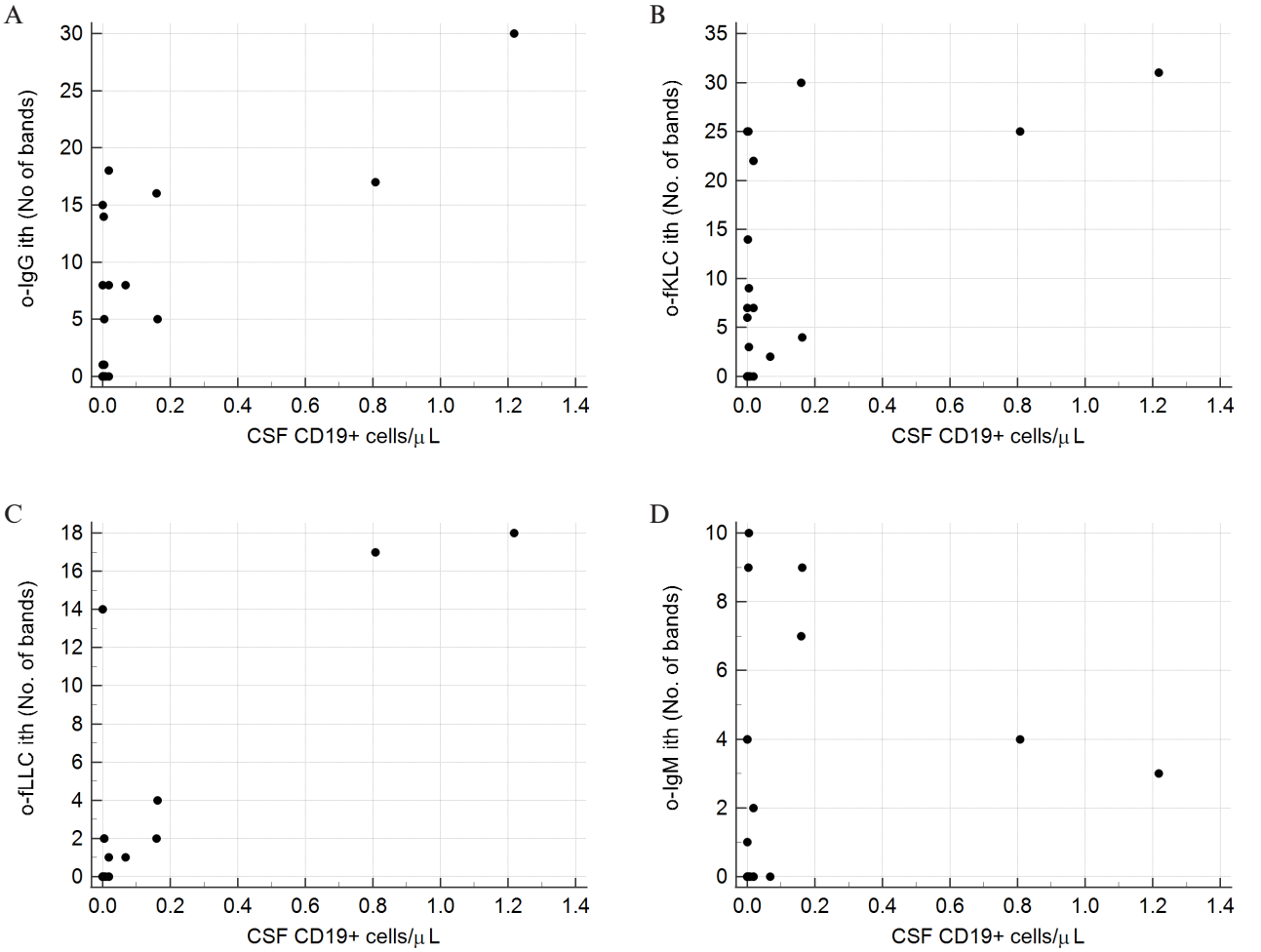

Fig. 3. Concentration of B lymphocytes in the CSF versus the number of CSF-restricted oligoclonal bands. **A.** IgG. **B.** fKLC. **C.** fLLC. **D.** IgM.

Percentage of CD19+ cells in CSF (out of lymphocytes) in the basic panel and B panel significantly correlated (n=14; ρ=0.669, 95% CI 0.214–0.885; *P*=0.009). This correlation was even more pronounced if only samples with at least 10 B lymphocytes available for analysis in the B panel were evaluated (n=11; ρ=0.797, 95% CI 0.378–0.945; *P*=0.003). For the analysis of B-lymphocyte subpopulations, the MNC gate was used. Using the lymphocyte gate, the median number of B lymphocytes available in the CSF represented 83% of the median CD19+ cells out of all MNCs (median 20 versus 24 cells in 11 samples with ≥10 CD19+ MNCs). In the blood, this difference was smaller (median 4448 vs. 4528 cells in 9 samples, i.e., 98%). CD5+ B cells are partly represented by larger cells and can escape analysis when a conventional lymphocyte gate is used[18].

The concentration of CD19+ lymphocytes in the basic panel significantly correlated with the number of CSF-restricted IgG bands (n=37, ρ=0.429, 95% CI 0.122–0.661, *P*=0.008), fLC bands of both types (n=36, ρ=0.408 and 0.539, 95% CI 0.092–0.650 and 0.255–0.737, *P*=0.0135 and 0.0007 for fKLC and fLLC, respectively), the concentration of intrathecally produced IgG and IgA calculated according to Reiber[19] (n=37, ρ=0.496 and 0.468, 95% CI 0.205–0.707 and 0.169–0.687, *P*=0.002 and 004 for IgG and IgA, respectively), and the fKLC index and the concentration of locally produced fKLC according to Reiber et al.[20] (n=24, ρ=0.410 and 0.511, 95% CI 0.008–0.698 and 0.135–0.758, *P*=0.046 and 0.011 for fKLC index and Reiber's $fKLC_{loc}$, respectively), and a possible correlation was observed for the fLLC index (n=24, ρ=0.345, 95% CI -0.068–0.657, *P*=0.099). On the other hand, in some patients with a pronounced intrathecal antibody response, the proportion of CD19+ cells was equal to zero (Fig. 3). In the case of intrathecal IgM synthesis, the correlation with CSF B-lymphocyte concentration was not significant (n=37, ρ=0.271 and 0.302, 95% CI -0.058–0.547 and -0.025–0.570, *P*=0.105 and 0.069 for the number of CSF-restricted IgM bands and Reiber's formula, respectively). A higher relative proportion of B lymphocytes in CSF was observed in patients with positive oligoclonal IgG, IgM, and fLC tests. Only in the case of oligoclonal fKLC positivity, however, did the difference reach significance (Mann-Whitney test, *P*=0.0262), apparently due to the low number of samples analyzed.

The kappa:lambda ratio for CD19+ cells did not seem to correlate with the free kappa:lambda ratio in the CSF (n=6 samples with ≥10 CD19+ MNCs in the CSF:

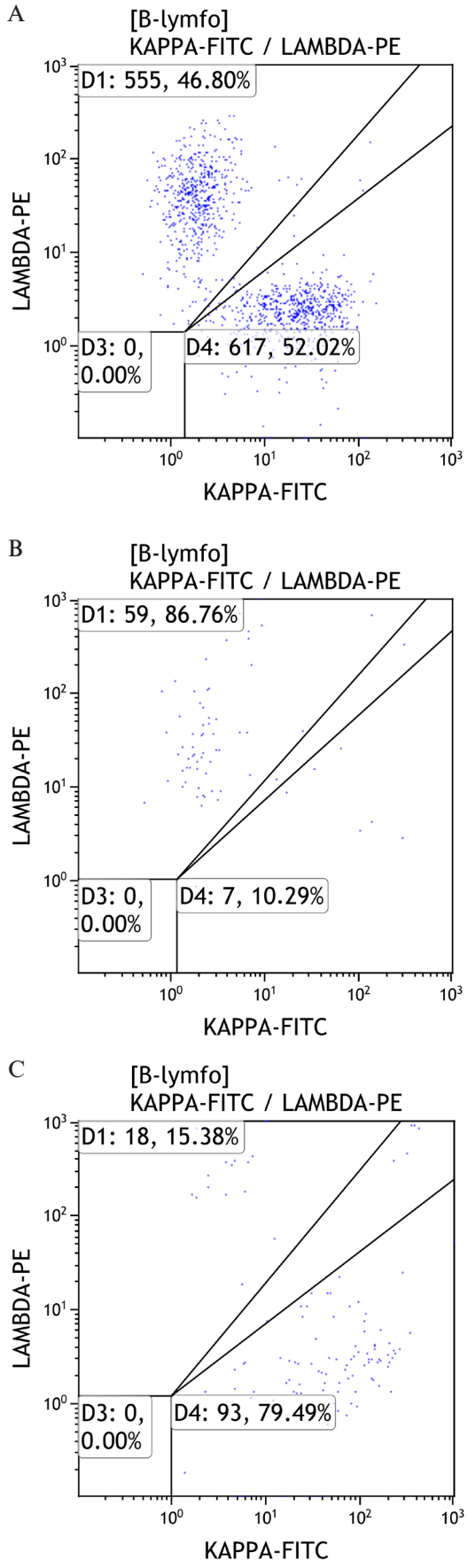

Fig. 4. Estimation of the kappa:lambda ratio on the surface of CSF B lymphocytes. **A.** Neuroborreliosis, kappa:lambda ratio 1.11. **B.** Primary CNS lymphoma, kappa:lambda ratio 0.12. **C.** Multiple sclerosis, kappa:lambda ratio 5.17.

ρ=-0.522, 95% CI -0.937–0.503, *P*=0.288). Rathbone et al.[21] found a significant correlation only when analyzing the kappa:lambda ratio for CSF plasmablasts.

In MS patients, we observed a tendency for a higher kappa:lambda ratio for CD19 cells and higher proportion of CD38++ B lymphocytes. However, due to the low number of samples examined no significant difference could be found. No clear differences were observed in the proportion of CD5+ B cells and CD27+ B cells, though the number of patients was very small. In a patient with suspected primary CNS lymphoma, the kappa:lambda ratio was abnormally low (Fig. 4). In contrast, in two out of four MS patients, this ratio exceeded 4.0. If evaluated in isolation, such a finding may have led to an incorrect suspicion of malignant lymphoproliferative disease. This potential pitfall has already been reported in the literature[22].

DISCUSSION

Processing CSF samples for FC in the CSF laboratory has the principal advantage of substantially reducing the amount of sample needed. Supernatant obtained after centrifugation is used for biochemical analyses (total protein, lactate, glucose, albumin, immunoglobulins, oligoclonal bands, etc.), whereas sediment after resuspension and washing is used for FC. The parameters of centrifugation should be considered and then kept constant. In the literature, centrifugation G-force varies from 200 *g* (ref.[23]) up to 500 *g* (ref.[24]), or even 1050 *g* (ref.[25]). In our study, we followed the recommendations for CSF biobanking[26] although no data are available on how various CSF analyses could be affected by centrifugation parameters.

In agreement with an earlier publication[9], we preferred careful pipetting of the supernatant (leaving the cell sediment in a volume of 200 µL in the Eppendorf tube) over simple decantation. The CSF sediment obtained in such a way can then be used for either cytocentrifugation or FC. We suppose that parallel preparation of cytological slides by cytocentrifugation of both native CSF and CSF sediment after pre-centrifugation can be useful for CSF samples with low cell counts (≤4/µL). In case of a lack of material, we prefer the sediment after pre-centrifugation because the cell yield from native CSF is often very low. In samples with borderline or elevated cell counts (>4/µL), the number of cells on routine cytological slides is usually sufficient for evaluation. It would be desirable to examine the effect of longer cytocentrifugation than used in our laboratory (4 min) mainly for logistic reasons (time saving during night and weekend laboratory service). In addition, resuspension of the CSF sediment in, or adding a drop of, protein-rich medium that can have cytoprotective effects[1,27] should be considered. This is clearly preferable to direct collection of CSF in protein-containing medium[28], as the supernatant collected in that way cannot be used for other CSF analyses.

Compared to cytological slides prepared from native CSF, slides prepared from CSF sediment after pre-centrifugation had a significantly greater proportion of

lymphocytes. Interestingly, we found no significant difference in the proportion of lymphocytes between routine cytological slides and FC, whereas cytological slides containing pre-centrifuged CSF samples had a significantly greater percentage of lymphocytes. It is conceivable that monocytes are selectively damaged by the double centrifugation. On the other hand, the relative proportion of granulocytes was significantly higher in FC than cytology for both native CSF and CSF sediment. To determine whether FC provides a more accurate estimate of the granulocyte percentage compared to conventional cytology, or whether other cells or non-cellular material with high side scatter values that bind the CD45 antibody non-specifically are incorrectly classified as granulocytes, it would be necessary to define the granulocyte population by means of surface markers instead of the CD45 versus side scatter plot. However, the clinical relevance of analyzing granulocytes in the CSF by FC is doubtful.

Our small study also showed that the percentages of lymphocytes and monocytes vary widely and are affected by the method of slide preparation. Hence, borderline deviations from various "normal" ranges adopted from the literature should not be considered as definitively abnormal.

Determination of the CSF cell concentration by FC can suitably complement microscopic counting. Although some losses during the washing step are unavoidable, the analysis of many more cells in a substantially larger CSF volume is a significant advantage.

A volume of 2.4 mL was found to be sufficient for basic CSF FC analysis of samples with normal cellularity (<5 cells/μL). In our study, this volume was not available for any analyzed sample containing <100 lymphocytes. For B-lymphocyte subset analysis in such samples, at least twice the volume (4.8 mL) is desirable. In samples with pleocytosis (≥5 cells/μL), 1.2 mL is sufficient for basic analysis. For B-cell subset analysis, at least twice this volume is optimal (i.e., 2.4 mL, possibly even more in samples with <10 cells/μL).

When deciding whether a more detailed analysis of B cells in CSF should be attempted, we should take into account the amount of sample available and the FR cell count. In addition, the absolute count of B lymphocytes available for FC analysis in the basic panel can be used; however, this can present a logistical problem due to further time delay. Therefore, we consider the rationale to decide on B cell analysis already after FR cell counting according to the following rules:

a) In the case of <3 cells/μL, only the basic panel is performed using 2.4 mL CSF; analysis of B-lymphocyte subsets may be attempted if there is at least 4.8 mL of CSF available.
b) In the case of 3 to 9 cells/μL, the basic panel is performed using 2.4 mL CSF; analysis of B-lymphocyte subsets is attempted if at least 2.4 mL CSF is available. However, whenever possible, a larger volume should be used (up to 4.8 mL).
c) In the case of >9 cells/μL, both the basic panel and B-lymphocyte subset analysis can be used if there is at least 1.2 mL CSF available for each panel. If possible, a larger volume (2.4 mL) should be used for B-lymphocyte subset analysis.

For the sake of completeness, samples with pronounced lymphocytic pleocytosis (>100 lymphocytes/μL) can be processed by FC without previous concentration using a volume of 0.1–0.2 mL for each separate flow cytometric analysis. In the case of pronounced granulocytic pleocytosis (usually corresponding to purulent infections or, more rarely, the acute phase of non-purulent infection, or even a reaction to serious CNS tissue damage), we do not consider CSF FC by the presented panels to be useful.

Although the CSF cell composition is considered to not simply reflect that of blood[8], parallel analysis of CSF and blood can be useful considering the correlations we found in the representation of NK cells and T_h and T_c lymphocytes. Moreover, several studies have shown various alterations in lymphocyte subpopulations in peripheral blood in MS before treatment, as well as depending on treatment[29-31].

Differences in leukocyte subpopulations among patients with MS, other inflammatory diseases, non-inflammatory neurological diseases, and controls have to be studied in a larger group of subjects. Among the tested markers, we have not found a robust parameter that could reliably distinguish MS patients and subjects without organic neurological disease. It can be assumed that mainly B lymphocytes seated within the CNS tissue contribute to intrathecal antibody production, whereas the contribution of CSF B cells to this production is relatively small. This may be the reason for only weak correlations between CSF cellular parameters and tests of the intrathecal antibody response, as well as MS activity detected either clinically or by imaging (magnetic resonance) or laboratory (neurofilaments in CSF and/or serum/plasma) biomarkers. The relationship between MS activity and CSF biomarkers of intrathecal inflammation is seemingly much more complicated than in the case of biomarkers of tissue damage. Despite this, further research on the former group of biomarkers is important to understanding MS immunopathogenesis.

Our design of the B panel is based on Clavarino et al.[32]. For more detailed study of B-cell subpopulations, it is necessary to consider a limited amount of sample resulting in possible implementation of no more than two different B panels. Instead of kappa/lambda, the second panel could include, for example, CD3 and CD20 to assess the controversially discussed minor population of CD20+ T lymphocytes, or CD21 and IgD (ref.[33]), IgD and IgM, or IgG and IgA.

The main weakness of our study is the low number of samples examined. In particular, correlations between CSF and blood leukocyte subsets should be tested separately in the control group and MS group. Another weakness was the insufficient standardization of CSF volume for analysis. However, such standardization is hardly possible in routine practice due to the large variability in CSF volumes sent to the laboratory. The reproducibility of the evaluation was also not assessed, but may be important,

mainly for markers with more or less continuous expression (e.g., HLA-DR, CD5).

CONCLUSIONS

Despite the weaknesses mentioned above, we think that our study confirmed the potential of FC as a method for obtaining accurate information on the CSF cellular composition. In addition, the study indicated some aspects that should receive adequate attention in future research.

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