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Automated Flow Cytometric Analysis of Cerebrospinal Fluid

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Background: **Recently, the UF-100 (Sysmex Corporation) flow cytometer was developed to automate urinalysis. We evaluated the use of flow cytometry in the analysis of cerebrospinal fluid (CSF).**

Methods: **UF-100 data were correlated with microscopy and biochemical data for 256 CSF samples. Microbiological analysis was performed in 144 suspected cases of meningitis.**

Results: **Good agreement was obtained between UF-100 and microscopy data for erythrocytes ($r = 0.919$) and leukocytes ($r = 0.886$). In some cases, however, incorrect classification of lymphocytes by the UF-100 led to underestimation of the leukocyte count. UF-100 bacterial count positively correlated ($P < 0.001$) with UF-100 leukocyte count ($r = 0.666$), CSF total protein ($r = 0.754$), and CSF lactate concentrations ($r = 0.641$), and negatively correlated with CSF glucose concentration ($r = -0.405$; $P < 0.001$). UF-100 bacterial counts were unreliable in hemorrhagic samples and in samples collected by ventricular drainage where interference by blood platelets and cell debris was observed. Another major problem was the UF-100 "bacterial" background signal in sterile CSF samples. *Cryptococcus neoformans* yeast cells and cholesterol crystals in craniopharyngioma were detected by the flow cytometer.**

Conclusions: **Flow cytometry of CSF with the UF-100 offers a rapid and reliable leukocytes and erythrocyte count. Additional settings offered by the instrument may be useful in the diagnosis of neurological disorders.**

Microscopic analysis has been the gold standard in examining cerebrospinal fluid (CSF)[1] cells and particles. However, it is imprecise, has wide interobserver variability, and is labor-intensive and time-consuming. Automation seems to be the answer to improving both the accuracy and the productivity of CSF analysis.

The Sysmex UF-100 (TOA Medical Electronics), a flow cytometer-based walkaway instrument that performs automated microscopic analysis, has recently been developed. Until now, the instrument has only been evaluated for urinalysis purposes *(1–4)*. Recently, preliminary data on CSF leukocytes have been published *(5)*. Because flow cytometry generally allows accurate and precise quantitative analysis of erythrocytes, leukocytes, and bacteria *(6, 7)*, we aimed to explore the possibilities of the instrument to analyze CSF. In this study, flow cytometric data from CSF were compared not only with Fuchs-Rosenthal chamber counting, but also with biochemical data (protein, glucose, and lactate) and with microbiological data (Gram stain and culture) from CSF.

Materials and Methods

PATIENTS AND SAMPLES

We studied 256 routinely collected CSF samples submitted to our laboratory. Each CSF sample was collected in a sterile container, aliquoted at the bedside in two to four fractions, and sent as urgent to different laboratory units (e.g., the emergency laboratory and, if necessary, the microbiology and/or cytology laboratory), depending on the diagnostic need. We obtained samples from 130 (50.8%) male and 126 (49.2%) female inpatients admitted to the Pediatrics (n = 98; 38.3%), Hematology/Oncology (n = 91; 35.5%), Emergency/Intensive Care (n = 27; 10.5%), Neurology (n = 25; 9.8%), and Neurosurgery (n = 15; 5.9%) Departments. The majority of the samples were lumbar puncture specimens (n = 174; 68.0%), and others were sampled by ventricular drainage (n = 82; 32.0%).

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[1] Nonstandard abbreviations: CSF, cerebrospinal fluid; CNS, central nervous system; WBC, white blood cell; and RBC, red blood cell.

Clinicians were asked for the indications for CSF analysis. Possible infection of the central nervous system (CNS; n = 120; 46.9%) was the most important indication, followed by neoplasm (n = 101; 39.4%), CNS damage (e.g., subarachnoidal hemorrhage; n = 22; 8.6%), autoimmune disease (e.g., multiple sclerosis and amyotrophic lateral sclerosis; n = 4; 1.6%), dementia and encephalopathy (n = 4; 1.6%), and other indications (n = 5; 1.9%). All analyses were performed within 8 h after collection. Because the UF-100 requires a minimum sample volume of 800 μL, samples were diluted four times in sterile 0.15 mol/L NaCl solution (B. Braun) *(5)*.

SYSMEX UF-100

The Sysmex UF-100 uses argon laser flow cytometry. The UF-100 measures the conductivity and analyzes the formed particles by electrical impedance for volume, measures forward light-scatter for size, and uses fluorescent dyes for DNA (phenanthridine) and membranes (carbocyanine). Pulse intensity and width of the forward scattered light and fluorescence light are measured. From these data, together with the impedance data, the formed particles are categorized by multiparametric algorithms on the basis of their size, shape, volume, and staining characteristics. The results are displayed in scattergrams, histograms, and as counts/μL. Because it was developed for urinalysis, the UF-100 automatically detects and counts red blood cells (RBCs), white blood cells (WBCs), bacteria, yeast cells, crystals, epithelial cells, small round cells, sperm cells, and casts. Particles that cannot be classified in one of the former categories are counted as "other cells".

BIOCHEMICAL INVESTIGATIONS

Biochemical indices were analyzed using commercially available reagents on a Hitachi 917 analyzer (Roche Diagnostics). Total CSF protein concentration was measured using a pyrogallol red assay (Sopachem). The CSF glucose concentration was measured by a hexokinase method (Roche). The L-lactate concentration in CSF was assayed using lactate oxidase (Roche).

MICROSCOPIC ANALYSIS

Manual microscopic examination was performed in Fuchs-Rosenthal counting chambers. For each sample, at least 20 random microscopic fields were examined at ×400, and the mean number of cells/mm^3 was calculated.

WBC differentiation was performed on all CSF samples with >5 WBC/μL and on all samples suspected for CNS neoplasm. Therefore, CSF samples were prepared by cytocentrifugation (Cytospin 3; Shandon Scientific Limited) of 25–150 μL of CSF, depending on cell content, onto a glass microscope slide at 400*g* for 5 min. After modified Wright staining, slides were examined by light microscopy under immersion oil at ×500 magnification. Differentiation was performed on 100 cells, if present.

MICROBIOLOGICAL INVESTIGATIONS

Gram staining and CSF culture were performed for 144 samples (56.3%) for which clinical requests for microbiological investigation had been received. CSF specimens were centrifuged (1000*g* for 10 min), the supernatant was removed until the remaining volume was ~250 μL, and 15 μL was placed on two different slides for a conventional Gram stain and a methylene blue stain. Slides were examined by light microscopy under immersion oil at ×1000 magnification for detection and morphology of organisms. For the CSF culture, 15 μL of the centrifuged sample was inoculated on 50 mL/L sheep blood agar and chocolate agar, and 150 μL was inoculated into 10 mL of a thioglycolate broth. Agar plates were incubated at 35 °C in 5% carbon dioxide and examined daily for 3 days. Broth cultures were incubated at 35 °C and examined daily for 7 days.

PERFORMANCE AND INTERFERENCE STUDIES

To evaluate the UF-100 bacterial count, we analyzed physiological saline solutions (5 mL) containing one colony from patient isolates of *Escherichia coli* (n = 3) and *Streptococcus agalactiae* (n = 3). Platelet-rich plasma, obtained after centrifugation of sterile citrated blood for 10 min at 200*g* (n = 3; mean platelet count, 485 × 10^3/μL), was used to study suspected interference with the bacterial count from platelets. Three physiological saline solutions (5 mL) containing one colony of *Cryptococcus neoformans* were used to evaluate the UF-100 yeast cell count.

STATISTICS

Data are presented as medians and interquartile ranges (25th-75th percentiles). Statistical differences were evaluated using the Wilcoxon test. Agreement between automated cell counts and microscopic data was examined by Spearman rank analysis. $P < 0.05$ was considered statistically significant.

Results

RBCs AND WBCs

The distributions of automated (UF-100) cell and particle counts are summarized in Table 1. Median UF-100 RBC and WBC counts were 6 RBCs/μL (interquartile range, 2–78 RBCs/μL) and 2 WBCs/μL (interquartile range, 0–7 WBCs/μL), respectively. After logarithmic transformation, good agreement ($P < 0.001$) was found between

Table 1. Distribution of automated (UF-100) RBC, WBC, bacterial, epithelial cell, and crystal counts (cells/μL).

Percentile	RBC	WBC	Bacteria	Epithelial cells	Crystals
10	1	0	2	0	0
25	2	0	7	0	1
50	6	2	18	1	3
75	78	7	79	2	9
90	917	24	1170	4	24

UF-100 and microscopic counts for RBCs ($r = 0.919$; Fig. 1A) and WBCs ($r = 0.886$; Fig. 1B).

Neutrophils and monocytes were properly classified as WBCs. In 3 of 38 cases with >5 WBCs/mL and >10% lymphocytes in the microscopic WBC differentiation, lymphocytes were erroneously classified as other cells by the UF-100, leading to an underestimation of the WBC count. In these cases, lymphocytes were easily identifiable on the UF-100 scattergram (phenanthridine fluorescence vs forward scatter) as a cluster located between the bacteria and WBC area. In one case of acute lymphatic leukemia with leptomeningeal involvement, lymphoblasts were correctly classified as WBCs.

BACTERIA

The median UF-100 count in the bacterial channel was 18 bacteria/μL (interquartile range, 7–79 bacteria/μL). Remarkably, a "bacterial" background signal was detected by the instrument in CSF samples with negative bacterial cultures. The median count in the bacterial channel was low in samples collected by lumbar puncture (10 bacteria/μL; n = 174), but was higher in samples collected by ventricular drainage (125 bacteria/μL; n = 82; $P < 0.0001$). The median UF-100 bacterial channel count was also higher in hemorrhagic (>100 RBCs/μL) CSF samples (226 bacteria/μL; n = 60) than in those with ≤100 RBCs/μL (11 bacteria/μL; n = 196; $P < 0.0001$).

After logarithmic transformation of all variables, the UF-100 bacterial channel count correlated well with the UF-100 WBC count ($r = 0.666$; $P < 0.001$; Fig. 2A), CSF total protein ($r = 0.754$; $P < 0.001$; Fig. 2B), and CSF lactate ($r = 0.641$; $P < 0.001$), whereas CSF glucose negatively correlated with UF-100 count in the bacterial channel ($r = -0.405$; $P < 0.001$).

Bacterial cultures were positive for 7 of 144 CSF specimens (4.9%) and showed coagulase-negative staphylococci (n = 4), *S. viridans* (n = 2), and *Propionibacterium* spp. (n = 1). The latter microorganism was considered a contamination (Gram staining negative; UF-100, 21 bacteria/μL). The former microorganisms were isolated from

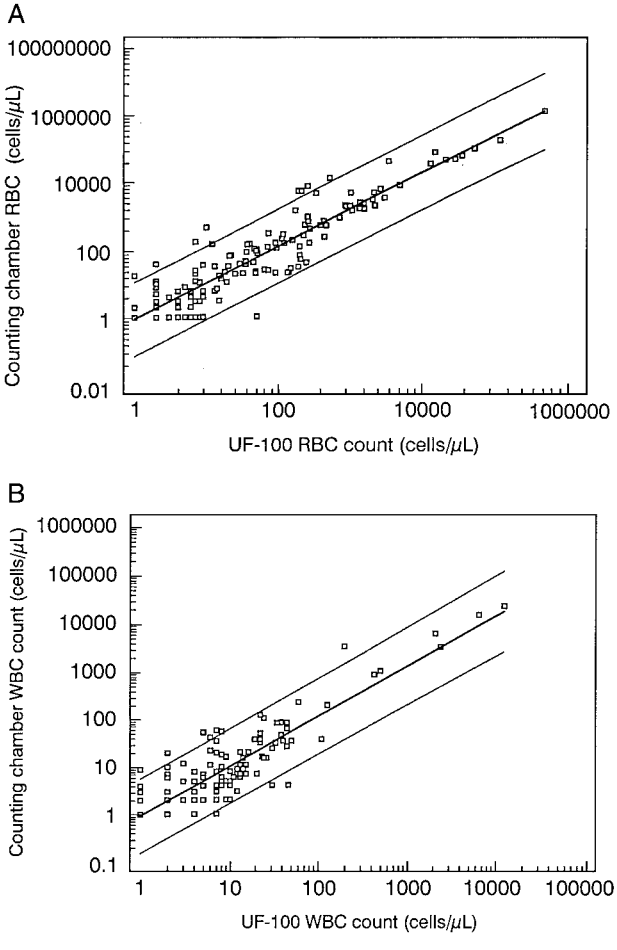

Fig. 1. Comparison between flow cytometry (UF-100) and microscopic counts of RBCs (*A*) and WBCs (*B*).

Significant agreement ($P < 0.001$) was obtained by Spearman rank analysis for RBCs ($r = 0.919$) and WBCs ($r = 0.886$).

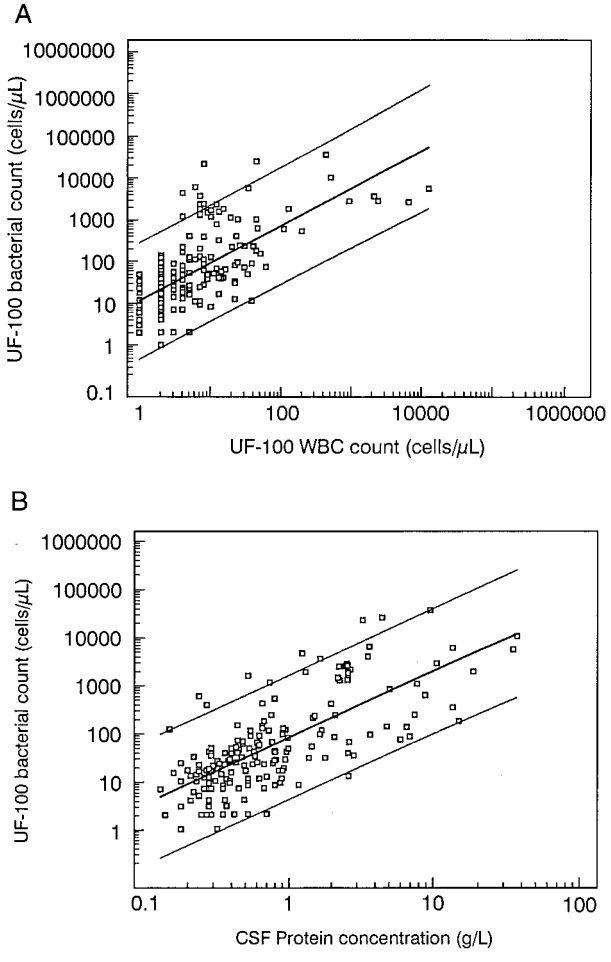

Fig. 2. Correlation between UF-100 bacterial counts and UF-100 WBC counts (*A*) or CSF total protein (*B*).

UF-100 bacterial counts are shown on the *y-axis*; UF-100 WBC counts (*A*) and CSF total protein (*B*) are shown on the *x-axis*. Shown are data points, the linear regression curve (*thick solid line*), and the 95% confidence intervals (*thin solid lines*). Spearman correlation: (*A*), $r = 0.666$; $P < 0.001$; (*B*), $r = 0.754$; $P < 0.001$.

patients with a CSF shunt infection and were associated with positive Gram staining and high UF-100 bacterial counts (>1000 bacteria/μL) in four of these samples. In two of the samples that cultured positive, however, Gram staining was negative, whereas the UF-100 counted 76 and 86 bacteria/μL, respectively.

One specimen, obtained by lumbar puncture from a 1-year-old infant with acute bacterial meningitis, showed gram-positive diplococci, and the UF-100 counted 2352 bacteria/μL, but the culture remained negative after 7 days. For nonhemorrhagic lumbar puncture samples with negative bacterial cultures, we found UF-100 counts in the bacterial channel that were 0–93/μL. In the samples with positive CSF culture, the observed UF-100 counts were 76–5298 bacteria/μL.

CRYSTALS AND YEAST CELLS

Median count in the UF-100 crystal channel was 3 crystals/μL (interquartile range, 1–9 crystals/μL). A higher crystal count was associated with a high RBC count. In one of two patients with a crystal count >100/μL, a craniopharyngioma was diagnosed and typical cholesterol crystals were found in CSF.

In none of the patient samples were UF-100 yeast cell counts above the manufacturer-defined cutoff value (10 cells/μL). However, *C. neoformans* yeast cells were correctly categorized by the UF-100 in three physiological saline solutions to which one colony of *C. neoformans* was added (mean yeast cell count, 28 cells/μL).

INTERFERENCE STUDIES

Interference studies focused on the UF-100 bacterial count. In 58 of 64 (90.6%) CSF samples with a bacterial count above the 75th percentile, Gram stain and/or culture remained negative (Table 2). Forty-three of these 58 samples (74.1%) were collected by ventricular drainage, 47 (81.0%) were hemorrhagic (>100 RBC/μL), and 35 (60.3%) were collected by ventricular drainage and hemorrhagic; only 3 (5.2%) were collected by lumbar puncture and contained ≤100 RBCs/μL. In 12 of these 58 samples (20.7%), however, cytological and biochemical indices strongly suggested bacterial CNS infection (WBC count >10/μL; CSF protein >1 g/L; CSF glucose <2.5 mmol/L), despite negative Gram stain and culture.

We assumed possible interference of cell debris and/or RBCs in the UF-100 bacterial count. In sterile dipotassium EDTA-treated blood (diluted 1:100–1:1000), UF-100 bacterial counts were high (>100 bacteria/μL). Further analysis of platelet-rich plasma (n = 3) showed that platelets were categorized exclusively as bacteria by the instrument.

Table 2. Study of samples with a UF-100 bacterial count above the 75th percentile and with negative Gram stain and culture.

Ventricular drainage[a]	>100 RBCs/μL[b]	Meningitis suspected[c]	n[d]	%
+	+	+	7	12.1
+	+	−	28	48.3
+	−	+	3	5.2
+	−	−	5	8.6
−	+	+	2	3.4
−	+	−	10	17.2
−	−	+	0	0.0
−	−	−	3	5.2

[a] Sample collected by ventricular drainage.
[b] More than 100 RBCs/μL counted with the UF-100.
[c] Bacterial meningitis cytologically and biochemically strongly suspected (>10 WBCs/μL, CSF protein >1 g/L, and CSF glucose <2.5 mmol/L).
[d] Number of cases among 58 samples.

Discussion

We evaluated the use of an automatic flow cytometer (Sysmex UF-100) in the routine analysis of CSF. Generally, good agreement was obtained between RBC and WBC counts by the UF-100 and the counting chamber. Although no major discrepancies were observed, UF-100 counts, especially the high RBC and WBC counts, were lower than the microscopic counts. This finding has also been observed by Ziebig et al. *(5)*. Comparison with counting chamber techniques, the "gold standard", is difficult because the latter techniques have several methodological steps that may contribute to imprecision and inaccuracy. Especially in the high RBC and WBC range, accuracy of microscopic counting can be poor.

As reported previously *(5, 8)*, we also experienced errors with the UF-100 in the correct allocation of some lymphocyte populations, leading to an underestimation of the WBC count. These lymphocytes are clearly visible on the UF-100 scattergram (phenanthridine fluorescence vs forward scatter) as a yellow cluster of elements between the bacteria and WBC area. Because the UF-100 is developed for urinalysis, flow cytometric gating for the detection of WBCs is focused on the neutrophils. Therefore, manual microscopic reanalysis is mandatory to detect lymphocytes in CSF. In most cases, and especially when low cell counts are encountered, automatic flow cytometry offers a rapid and reliable RBC and WBC count.

Rapid flow cytometric counting of bacteria might be of interest in CSF analysis, where the ability to make a prompt tentative diagnosis of meningitis has important implications for the management and outcome of patients. Conventional biochemical and microbiological techniques often lack power to assess bacterial CNS infection rapidly. In ~25% of cases of acute bacterial meningitis, no bacteria are seen in the initial CSF Gram stain, and in 30–40% of these cases, other common indirect indices such as CSF glucose, CSF total protein, and CSF lactate are not diagnostic *(9–11)*. Preanalytical use of antimicrobial agents can produce false-negative CSF cultures *(12)*. In addition, detection of bacterial DNA by PCR is not always a good alternative for the diagnosis of bacterial meningitis because inhibitory factors in the

CSF can possibly interfere with this technique *(13)*. In contrast, direct bacterial counting by flow cytometry should be less affected by such interfering and inhibiting factors. However, in CSF samples, a background signal is measured in the UF-100 "bacteria" channel. This is a major point of concern for flow cytometric CSF analysis because this body fluid is generally believed to be sterile in physiological conditions.

In this study, we found high UF-100 bacterial counts in all cases with positive CSF culture except in one case of sample contamination. In one case of acute bacterial meningitis, Gram staining showed gram-positive diplococci and the UF-100 bacterial count was high, whereas the culture remained negative after 7 days, probably because of antibiotic treatment before lumbar puncture. Moreover, the UF-100 bacterial counts correlated well with indirect indices of bacterial CNS infection, such as WBC count, CSF total protein, CSF glucose, and CSF lactate.

High UF-100 counts in the bacterial channel were encountered in hemorrhagic samples and in samples collected by ventricular drainage despite negative Gram stain results and culture. Although the cytological and biochemical indices strongly suggested bacterial infection in some of these samples, we postulate that cell fragments probably interfere in the UF-100 bacterial count. Cell fragments and bacteria share similar flow cytometric characteristics (e.g., low forward scatter, and low phenanthridine and carbocyanine fluorescence). Moreover, we demonstrated that blood platelets were exclusively categorized as bacteria by the UF-100.

Other indices provided by the UF-100 instrument, because it has been developed for urinalysis, are yeast cells, crystals, epithelial cells, small round cells, sperm cells, and casts. Although we observed zero to minimal counts of these various elements in CSF, they played an important role in selected cases of our study.

Cryptococcosis, a CNS infection with the yeast *C. neoformans*, can occur sporadically in patients who are not immunocompromised, but it is a leading mycological cause of morbidity and mortality among AIDS patients *(14)*. Laboratory diagnosis of meningitis with *C. neoformans* can be made by microscopic CSF examination in an India ink preparation for the presence of capsulated yeast cells, but this diagnosis is often missed in patients without apparent HIV infection. The UF-100 correctly categorized cryptococcal yeast cells in samples to which *C. neoformans* was added. Although we could not include CSF samples from patients with cryptococcosis in our study, the latter observation might have important implications in the rapid diagnosis of cryptococcosis, especially in patients without apparent HIV infection.

Cholesterol crystals were correctly detected by the UF-100 in a postoperative ventricular fluid of a child suffering from craniopharyngioma (Rathke's cleft cysts), a tumor of the suprasellar area. The finding of cholesterol crystals in CSF is a key feature in the diagnosis of spontaneous rupture of a craniopharyngioma cyst *(15)*.

In conclusion, the UF-100 analyzer is a useful additional tool for CSF examination, especially in the emergency setting. It provides rapid (36 s), reliable data on the RBC and WBC content in CSF. Flow cytometric bacterial counts, however, should be interpreted with caution in the case of hemorrhagic and ventricular fluid samples, and the background bacterial signal in sterile CSF is a major point of concern. Future clinical studies will be needed to evaluate the use of CSF flow cytometry in the diagnosis of neurological disorders.

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