DIFFERENCES IN THE CEREBROSPINAL FLUID INFLAMMATORY CELL REACTION OF PATIENTS WITH LEPTOMENINGEAL INVOLVEMENT BY LYMPHOMA AND CARCINOMA

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Running head

A different inflammatory background is seen in the cerebrospinal fluid from patients with lymphomatous meningitis and leptomeningeal carcinomatosis. These last patients have recurrent description of polimorphonuclear cells, a distinctive parameter not found in patients with lymphomatous meningitis and patients with cancer but without leptomeningeal carcinomatosis.

Abbreviations

CSF, cerebrospinal fluid; LC, leptomeningeal carcinomatosis; LyM, lymphomatous meningitis; PMN, polimorphonuclear; CNS, central nervous system; FCI, flow cytometry immunophenotyping; MRI, magnetic resonance imaging; DLBNHL, large B-cell non-Hodgkin lymphoma; mAb, monoclonal antibodies; EpCAM, epithelial-cell antigen molecule; FSC, forward scatter; SSC, side-scatter.

ABSTRACT

Dissemination of neoplastic cells into the cerebrospinal fluid (CSF) and leptomeninges is a devastating complication described in patients with epithelial-cell neoplasia (leptomeningeal carcinomatosis, LC) and lymphomas (lymphomatous meningitis, LyM). Information about the surrounding inflammatory-cell populations is scarce. In this study we describe the distribution of the main leukocyte populations in the CSF of patients with LC and LyM. Flow cytometry immunophenotyping was used to describe the distribution of the main leukocyte populations in the CSF from 77 patients diagnosed with neoplastic meningitis (LC, n=65; LyM n=12), and compared it with the CSF from 55 patients diagnosed with the same groups of neoplasia without meningeal involvement (solid tumors, n=36; high-grade lymphoma, n=19). Median (interquartile) rates of lymphocytes, monocytes and polimorphonuclear (PMN) cells were 59.7% (35-76.6), 24% (16-53) and 1.5% (0-7.6) in LC, and 98.5% (82.4-100), 1.5% (0-15.9) and 0% in LyM (p<0.001). No difference was observed between patients with breast (n=30) and lung adenocarcinoma (n=21), nor with different rates of malignant CSF involvement. Patients with lymphoma (with or without LyM) had a similar CSF leukocyte distribution, but patients with LC and patients with cancer without LC cancer had a distinctive PMN cells rate (p= 0.002). These data show that the CSF from patients with LC have a higher number of inflammatory cells and a different leukocyte distribution than the CSF from patients with LyM. Description of PMN cells is a distinctive parameter of patients with LC, compared with the CSF from patients with LyM and patients with cancer but without LC.

INTRODUCTION

Leptomeningeal tumor involvement defines the dissemination of neoplastic cells into the cerebrospinal fluid (CSF) and infiltration of the leptomeninges. It is usually found in the context of epithelial-cell solid tumors or melanoma (leptomenigeal carcinomatosis, LC), hematologic malignancies (leukemic or lymphomatous meningitis, LyM), and less frequently, in primary central nervous system (CNS) tumors[1,2,3]. Unfortunately, this neoplastic complication still leads to a very bad prognosis for the patient.

In recent years, several studies have shown that flow cytometry immunophenotyping (FCI) improves the sensitivity of classical cytology examination of the CSF. These works highlight the importance of the CSF FCI studies for early diagnosis of neoplastic CNS involvement in patients with aggressive lymphomas[4,5,6,7,8], but there are also promising results in epithelial-cell neoplasms[9].

At the time of writing this paper, there is a growing interest in the immunologic response to cancer[10,11,12,13]. Immune cells should develop a cytotoxic activity that helps eliminate neoplastic cells, but tumor cells can also secrete cytokines, chemokines and growth factors that throughout the interaction with different cells of the tumor microenvironment, might contribute to angiogenesis and metastases development[11]. Most of the studies focus on solid tumors from different cell lines[11,14,15,16,17] and although many of them can spread into the CSF and meninges, information about the inflammatory populations in these loci is scarce[18]. The aim of our study was to perform an exploratory analysis of the CSF leukocyte populations in the CSF of patients diagnosed with leptomeningeal neoplastic infiltration by solid tumors and lymphomas. We did not find similar studies during the period of this research.

METHODS

Study design

Two different populations of patients were included. The CSF samples from patients with LC suspicion were provided by the prospective collection performed by 18 Spanish hospitals between 2010 and 2012 in the context of another study related with LC prognostic factors[19]. The CSF samples from patients with lymphoma were studied at diagnosis or relapse, as part of the staging of cases with high-risk of CNS involvement, even though they might not have any suggestive symptomatology.

The diagnosis of LC was based on CSF cytology or on suitable clinical symptoms together with magnetic resonance imaging (MRI) and/or biochemical CSF abnormalities[20]. Patients with melanoma, medulloblastoma, mesothelioma, acute leukemia, low-grade lymphoma and primary brain tumors, were not considered for this study. Immunodeficient patients (HIV or transplant recipients) were also excluded.

Local ethics committees of participating centers approved the study, and informed consent was obtained from each participant before their enrolment. All procedures were in accordance with the ethical standards of the Helsinki Declaration.

Patients and samples

Two-hundred and forty-six CSF samples from patients diagnosed with epithelial-cell solid tumors (n=207) and high-grade B-cell lymphoma (n=39) were evaluated. The final number of samples included in the study was 132 after excluding CSF samples with macroscopic blood contamination, and samples which showed erythrocytes in the cell-pellet after centrifugation. Seventy-seven CSF samples were from patients who were finally diagnosed with leptomenigeal involvement: 65 had LC and 12 LyM. The localization of the primary epithelial-cell tumor was: breast (n=30), lung (n=23), gastrointestinal (n=4), ovary (n=4), bladder (n=1), prostate (n=1) and kidney (n=2). Adenocarcinoma was the histology for 60/65 patients (92.3%). The histological diagnosis of patients with lymphoma was diffuse large B-cell non-Hodgkin lymphoma (DLBNHL, n=10), Burkitt cell lymphoma (n=1) and CNS primary lymphoma (n=1).

A group of 55 patients from this series in whom the diagnosis of neoplastic involvement was excluded, and did not develop LC or LyM during the following 12 months after the CSF study, were used for comparison of their CSF leukocyte populations. Thirty-six patients diagnosed with breast (n=19), lung (n=11), colon (n=4), prostate (n=1) and a tumor of unknown origin (n=1), were the control group of patients with LC. They all lacked infectious, inflammatory or autoimmune diseases. The diagnoses of the control group for patients with LyM were DLBNHL (n=15), Burkitt-cell lymphoma (n=2) and primary CNS B-cell lymphoma (n=2). Supplementary Material 1 shows a summary of the different groups of patients included in this study.

FCI Studies

FCI studies were performed on CSF samples obtained from a lumbar puncture (n=126) or from an Ommaya reservoir (n=6), and collected in tubes with EDTA and an immunofixative reagent (TransFix®,

Cytomark), to guarantee safe transportation[21] to the Fundación Jiménez Díaz (Madrid, Spain). Samples were processed with a median of 3 days from extraction with previously defined FCI protocols for processing CSF samples in search of hematological malignancies[22], or epithelial-cell solid tumors[9].

Briefly, an aliquot of 100 mm^3 of the CSF sample was used for cell-count using the fluorescent dye DRAQ5 (Biostatus Limited) for DNA staining, and Perfect-Count microspheres (Cytognos SL, Salamanca, Spain). The remaining sample was centrifuged and the cell-pellet was stained. Since the samples were sent to a laboratory to exclude malignant infiltration, different panels of monoclonal antibodies (mAb) were used in each group of patients: a 2-color mAb combination directed against the epithelial-cell antigen molecule (EpCAM) were used in patients with epithelial-cell solid tumors[8], and a 6-color mAb combination was used in patients with lymphoma[21]. After incubation (20 minutes at room temperature in the darkness), samples were washed once, and the cell-pellet was resuspended in PBS. In samples from patients with solid tumors, an appropriate dilution of DRAQ5 was added and incubated for 10 minutes before acquisition of the whole volume of sample on a FACSCanto II flow cytometer (Becton Dickinson Biosciences [BDB]) using the FACSDiva software (version 6.1). The FSC area/FSC height dot-plot was used to help exclude aspirated air and doublets. Analyses were performed using the INFINICYT^{TM} software program (Cytognos SL, Salamanca, Spain). FCI categorized a sample as positive for malignancy when a cluster of cells had EpCAM expression and a hyperdiploid DNA content (solid tumors), or showed aberrant immunophenotypic features (lymphoma). After identification of neoplastic cells, lymphocytes, monocytes, and polimorphonuclear (PMN) cells were located on the basis of their forward (FSC) and side-scatter (SSC) characteristics. Lymphocytes were catalogued as FCS^{low}/SSC^{low}, monocytes as $FCS^{high}/SSC^{intermediate/high}$, and PMN cells as FSC^{low}/SSC^{high}. In order to check if this strategy for the identification of the leukocyte subpopulations (using only FSC/SSC data) was accurate enough, a second tube with 4 (n=6) or 6-color (n=16) mAbs directed against lymphocytes, monocytes and PMN was run in parallel on 22 samples from a subset of patients with LC that had a high CSF cell-count (Figure 1).

Statistics

The mean value and interquartile range were calculated for all parameters. General patient characteristics were compared among groups using $\chi^2$and the Mann-Whitney test. The Spearman correlation test was used to determine the correlation of the rate of each leukocyte population obtained using either the FSC/SSC dot-plot or specific mAbs.

The nonparametric Mann-Whitney test was used to compare the distribution of the CSF leukocyte populations, sample volume, cell-count and biochemical data between groups. A 2-sided *p*- value of <0.01 was considered statistically significant. Calculations were performed using the IBM SPSS Statistics software, version 20.0 (IBM Corporation, New York, USA).

RESULTS

1- General data

One hundred and thirty-two patients (LyM, LC and controls) were included in the study. No differences in the distribution by sex and age were observed. Also, the volume of samples received for FCI study, the absolute cell-count, and the levels of glucose and proteins were similar (Table I). However, the relative distribution between neoplastic and inflammatory cells was different. Patients with LyM showed more tumor cells and patients with LC showed a higher proportion of inflammatory cells.

2- Correlation of the CSF leukocyte populations using FSC/SSC *vs* specific mAb

A linear relationship was obtained after comparing the percentage for each leukocyte population using either identification with the FSC/SSC or with mAb. The Spearman correlation coefficient (*r*) was 0.99 for lymphocytes, 0.97 for monocytes and 0.91 for PMN cells (Figure 2). By using the INFINICYT™ software, a single file with the individual data entries from the 65 patients with LC was created and analyzed using only the FSC/SSC characteristics. Lymphocytes, monocytes and PMN cells were properly located in different clusters (Supplementary Material 2).

3- CSF leukocyte populations of patients with LC and LyM

3.a- Cell distribution

Lymphocytes were the predominant population in many patients with LyM and LC, although patients diagnosed with LC often presented a higher rate of monocytes. Moreover, PMN cells were only detected in cases of LC (45/65 samples), and the rate of PMN cells was >1% in 38 CSF samples. Statistically significant differences between LC and LyM were found in the rate of each population (p<0.001, Table II).

3.b- Localization of the primary epithelial-cell neoplasia

Patients with the 2 most frequent tumors (breast and lung adenocarcinoma) showed no differences in their CSF leukocyte distribution (Supplementary Material 3), but both groups had statistical differences as compared to the CSF from patients with LyM (p<0.001).

3.c- Rate of malignant infiltration of the CSF

We also attempted to establish whether the differences in the distribution of the CSF leukocytes of patients with LyM and LC could be related to the quantity of malignant cells (Table III). FCI detected lymphoma in the CSF of all patients with LyM (range of infiltration 3-99%), but in patients with diagnostic criteria of LC, FCI detected malignant epithelial-cells in 54/65 samples (range FCI^{pos}: 0.2-92%). In patients with LC, no differences in the rate of the CSF leukocyte populations were found between FCI^{pos} and FCI^{neg} samples. There were, however, statistically significant differences in the absolute number of monocytes, and differences in the number of lymphocytes had a p significance <0.05. A comparison of the CSF from patients with LC FCI^{pos} samples and patients with LyM showed statistically significant differences in their leukocyte distribution and absolute numbers of monocytes and PMN cells (p<0.001).

4- CSF leukocyte distribution of patients with LC and LyM and their respective control group

In order to determine whether the differences between patients with LC and LyM were related to their primary systemic disease, we compared each group of patients with their respective control group; patients diagnosed with lymphoma or epithelial-cell neoplasia but without infiltrative meningeal disease. As expected, statistically significant differences were observed in the total CSF cell-count between patients with and without CNS involvement. However, within patients diagnosed with lymphoma, no differences were observed between patients with and without LyM, neither in the distribution nor in the absolute number of lymphocytes, monocytes and PMN cells. On the other hand, statistically significant differences were observed in the rate of PMN cells (p<0.01) found in patients with solid neoplasia plus LC and patients with solid neoplasia without LC. The absolute number of lymphocytes, monocytes, and PMN cells also showed statistically significant differences between the two groups of patients with cancer (Table II).

DISCUSSION

The inflammatory cell infiltrate surrounding tumors is progressively gaining importance, not only for a better understanding of the pathophysiology of the disease, but also for future possible design of immune-based cancer therapies. Preliminary studies suggest that, depending on the infiltrate-cell composition, immune cells can either inhibit (T-helper 1 and cytotoxic T lymphocytes, type 1 macrophages, and dendritic cells) or facilitate tumor growth (T-helper 2 lymphocytes, neutrophils and type 2 macrophages)[10,11,15].

In an attempt to reach a better comprehension of the role of the CSF inflammatory cell environment in leptomeningeal disease, we described the major leukocyte populations in the CSF of patients with LyM and LC. FCI offers an accurate diagnosis of leptomeningeal neoplastic infiltration[4-9], and allows the simultaneous identification of the main non-malignant reactive leukocyte populations; that is lymphocytes, monocytes and PMN. It was outside the scope of this study to evaluate less frequent cell subsets such as plasma and dendritic cells or lymphocyte subpopulations. The good correlation found after the identification of the main populations using either their forward and scatter size characteristics or their monoclonal antibody profile, provides consistency of the results obtained.

The present study shows that the CSF inflammatory-cell background is different in patients with LyM and LC. Interestingly, no differences were found between patients with breast and lung adenocarcinoma with different rates of infiltration. This observation needs further validation in larger studies including patients with epithelial-cell tumors from different localizations, and patients with non-epithelial tumors not included in our series (i.e primary brain tumors, melanoma or mesothelioma). However, the statistical differences found in this exploratory study with a p value <0.001, gives a reasonable confidence that these results would also be reproducible in larger series of patients. In our work, the inflammatory cell response seemed to be much more evident in the CSF of patients with LC: the number of leukocytes was higher than in LyM, and the distribution of these leukocytes was different from what is expected in a healthy subject (predominance of lymphocytes over monocytes and absence of PMN cells)[5,23]. In contrast, the CSF from patients with LyM exhibited more lymphoma cells and less infiltrate, thus in these patients it

would be necessary to analyze the lymphocyte subpopulations in order to evaluate whether the lymphoma remains unnoticed to a specific T-cell recognition in the CSF[18,24].

In any case, this different distribution of the inflammatory cells observed in the CSF from patients with LyM and LC could be useful as a quick guide when diagnosing neoplastic meningitis without known primary neoplasia. A normal distribution is more often seen in lymphoma, and few lymphocytes, many monocytes and PMN cells as part of an important inflammatory-cell counterpart, are more often seen in epithelial-cell tumors. Unfortunately, although these are easy clues, they are not diagnostic on their own and cannot help locate the primary tumor.

Several reasons, not only related to the origin of the neoplasia (lymphoid or epithelial), might explain these differences in the CSF inflammatory cell compartment from patients with LyM and LC. Patients with LyM were studied at diagnosis of NHL hence they did not previously receive any therapy. In contrast, most of the patients with LC had received one or more lines of therapy for systemic disease, and the development of LC was usually associated with concomitant advanced systemic cancer[1,2,20]. Probably, this clinical scenario might explain why the CSF leukocyte distribution in patients with LyM was analogous to what we found in patients with lymphoma without CSF involvement, and is described in normal CSF samples[23].

Another relevant observation from our study is that more than 58% of the CSF from patients with LC had PMN cells. This population was never detected in the CSF from patients with LyM nor their respective control group. In a larger series of patients[5], low percentages of PMN were detected in 9% of samples with LyM. This supports our finding that detection of PMN is much more frequent in patients with LC than LyM. Patients with LC and patients with solid neoplasia without CSF involvement showed a high rate of monocytes in the CSF, not correlated to an inversion of the lymphocyte/monocyte ratio in peripheral blood (data not shown), but the rate of PMN cells was the only significant distinctive parameter between them. It has to be established whether this reveals a progressive gradient of damage of the blood-brain-barrier[25] (finding PMN cells in latest stages), or it also implies an active recruitment promoted by the tumor or surrounding cells. Until then, the description of PMNs in the CSF of a patient with cancer should point out several non-neoplastic conditions, and also LC. In those frequent situations

with negative cytology or FCI findings, PMN cells might add information to the other valuable, but not specific, indicators for leptomeningeal disease in CSF: i.e glucose and proteins levels.

Speculations

In summary, a different inflammatory background is found in the CSF from patients with LyM and LC. Patients with LyM had a similar CSF leukocyte distribution as their control group, but patients with LC showed a recurrent description of PMN cells when compared with patients with LyM and patients with epithelial-cell cancer but without LC. Further studies are warranted to determine the significance of these exploratory findings.

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LEGENDS FOR FIGURES

Figure 1: Identification of the leukocyte populations on the basis of their forward (FSC) and side-scatter (SSC) characteristics (a), and their antigenic profile (b,c,d). Lymphocytes are colored in red, monocytes

in blue and PMN in green. PerCP-Cy5-5 (Peridinin chlorophyll-cyanin5-5), APC (allophycocyanin) and PE (phycoerythrin) are the fluorochromes attached to the monoclonal antibodies.

Figure 2: Linear correlations between rates of each leukocyte populations identified on the basis of their forward (FSC) and side-scatter (SSC) versus identification using monoclonal antibodies. PMN cells: polimorphonuclear cells.

LEGENDS FOR SUPPLEMENTARY MATERIAL

Supplementary material 1: Summary of the different groups of patients included in the study.

Supplementary material 2: The automatic population separator diagram was used to check the precise separation of all the leukocyte populations after distinguishing the sum-up of the 65 flow cytometry files from patients with LC in a single file. After integrating the parameters of size and complexity, this diagram effectively separates in different clusters the lymphocytes (colored in red), monocytes (colored in blue) and PMN cells (colored in green).

Supplementary material 3: Comparison of the CSF leukocyte populations between patients with breast and lung adenocarcinoma. Results are expressed as median and interquartile range (in brackets).

Figure(s)
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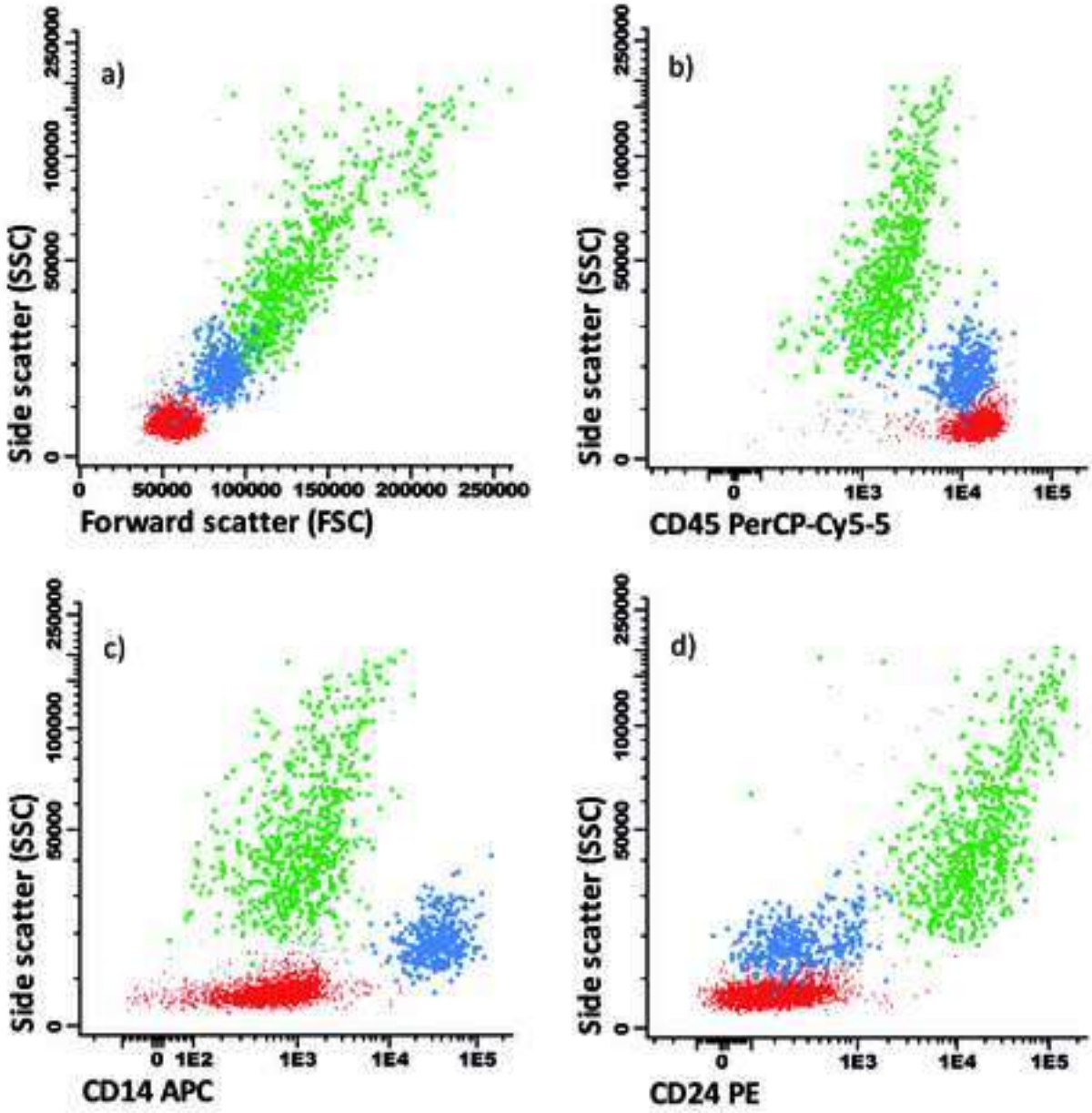

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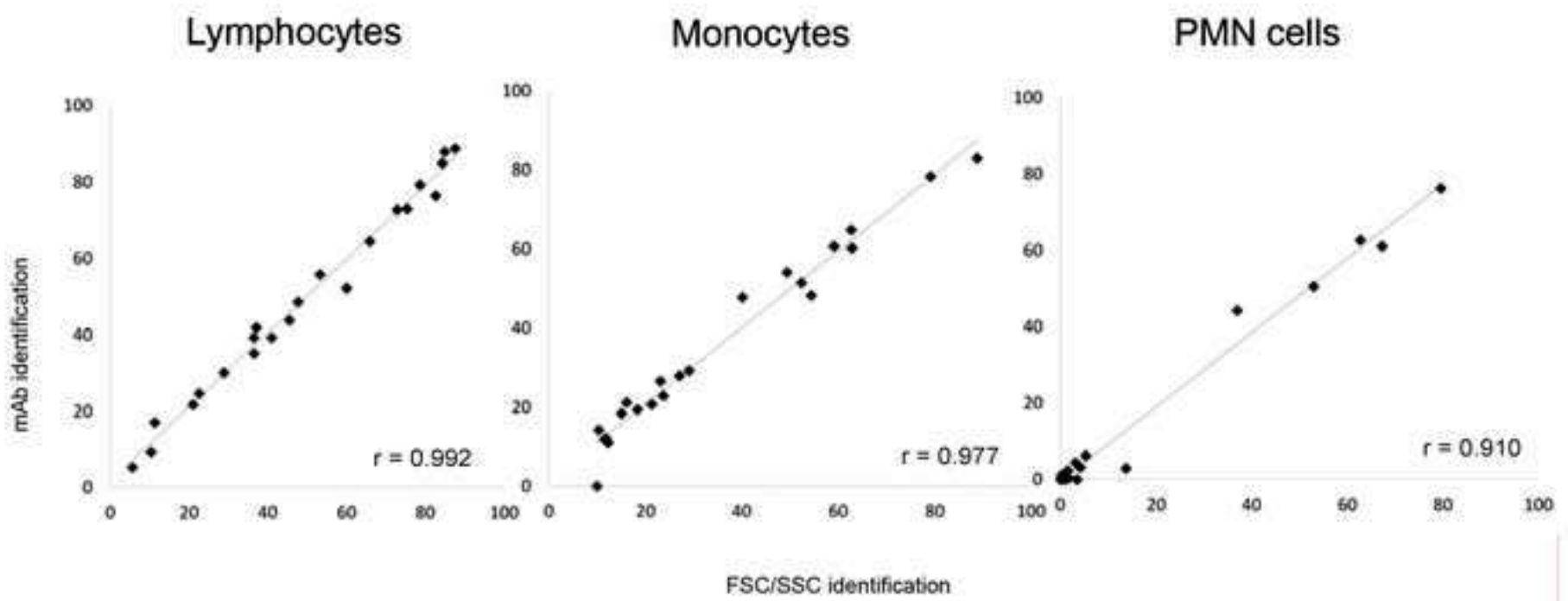

Table(s)

Table I

				Cerebrospinal fluid characteristics					
	Sex		Age (years) Median (IQR)	Volume (cm3) Median (IQR)	Absolute cell-count /mm^3 Median (IQR)	Glucose mg/dl Median (range)	Proteins mg/dl Median (range)	Tumor cells/mm^3 Median (IQR)	Inflammatory cells/mm^3 Median (IQR)
	Male	Female							
LyM	8	4	59 (51-70.5)	1.9 (1.2-3.0)	9.0 (2.8-44.5)	42 (20-85)	70.5 (22-225)	4.6 (1.1-38.2)	2.34 (0.8-3.4)
LC	26	39	59.5 (48.7-67.2)	2.5 (2.1-2.9)	8.3 (3.1-33)	57 (3-142)	75.5 (12-743)	0.36 (0-2.8)	7.96 (2.5-17.6)
P	0.117		0.702	0.157	0.613	0.267	0.831	0.002	0.031

General patient data and cerebrospinal fluid characteristics.

IQR: interquartile range. LyM: lymphomatous meningitis. LC: leptomeningeal carcinomatosis.

Table(s)

Table II

		Lymphoma control group (n=19)	*p**	LyM (n=12)	*p***	LC (n=65)	*p****	Epithelial-cell control group (n=36)
Lymphocytes	%	97.0 (72.5-100.0)	0.722	98.5 (82.4-100.0)	<0.001	59.7 (35.0-76.6)	0.159	66.2 (59.5-78.3)
	cells/mm^3	0.99 (0.4-2.9)	0.273	1.86 (0.74-3.3)	0.347	3.41 (0.65-12.0)	<0.001	0.66 (0.33-1.1)
Monocytes	%	3.0 (0.0-21.5)	0.661	1.5 (0.0-15.9)	<0.001	24.0 (16.0-53.0)	0.840	31.0 (18.8-40.0)
	cells/mm^3	0.15 (0.0-0.4)	0.770	0.06 (0-0.3)	<0.001	2.2 (0.53-6.9)	<0.001	0.3 (0.15-0.6)
PMN	%	0.0 (0.0-0.0)	1,000	0.0 (0.0-0.0)	<0.001	1.5 (0.0-7.6)	0.002	0.0 (0.0-1.3)
	cells/mm^3	0 (0.0-0.0)	1,000	0 (0.0-0.0)	<0.001	0.12 (0-0.5)	<0.001	0 (0.0-0.0)

Comparison of the distribution of the CSF leukocyte populations among different groups of patients.
Results are expressed as a median and interquartile range (in brackets).
p *: comparison between patients with lymphoma without LyM and patients with LyM
p **: comparison between patients with LyM and LC
p ***: comparison between patients with LC and patients with epithelial-cell tumors without LC.
PMN: polimorphonuclear; LyM: lymphomatous meningitis; LC: leptomeningeal carcinomatosis.

Table(s)

Table III

		LyM (n=12)	*p**	LC FCI^{pos} (n=54)	*p***	LC FCI^{neg} (n=11)
Lymphocytes	%	98.5 (82.4-100.0)	<0.001	57.8 (35.2-76.8)	0.930	60.0 (35.0-71.9)
	cells/mm^3	1.86 (0.7-3.3)	0.163	5.1 (1.2-12.7)	0.02	0.75 (0.1-12.8)
Monocytes	%	1.5 (0.0-15.9)	<0.001	25.5 (17.1-57.1)	0.298	23.4 (15.0-37.5)
	cells/mm^3	0.06 (0-0.3)	<0.001	3.43 (0.6-9.3)	0.004	0.56 (0.2-9.3)
PMN	%	0.0 (0.0-0.0)	<0.001	1.6 (0.0-7.1)	0.702	1.4 (0.0-22.5)
	cells/mm^3	0 (0.0-0.0)	<0.001	0.12 (0-0.6)	0.669	0.1 (0-0.6)

Comparison of the cerebrospinal fluid leukocyte populations between patients with LyM and patients with LC (FCI^{pos} vs FCI^{neg}). Results are expressed as median and interquartile range (in

*p** : comparison between patients with LyM and patients with LC and a positive detection of malignant cells in the CSF using FCI.

*p*** : comparison between patients with LC with or without a positive detection of malignant cells in the CSF using FCI.

PMN: polimorphonuclear; LyM: lymphomatous meningitis; LC: leptomeningeal carcinomatosis; FCI: flow cytometry immunophenotyping.

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