Immunocytochemical Identification of Meningeal Leukemia and Lymphoma: Poly-L-Lysine–Coated Slides Permit Multimarker Analysis Even With Minute Cerebrospinal Fluid Cell Specimens

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Use of immunocytology for accurate identification of malignant cells in cerebrospinal fluid (CSF) has so far been hampered by high cell requirements of the immunologic methods hitherto used. In an attempt to minimize cell loss in cytopreparation, electrostatic binding of cells to poly-L-lysine (PLL)–coated multispot slides, followed by immunocytochemistry, was investigated. Using optimized conditions of cell attachment and fixation and performing all washing procedures on the slide made multimarker analysis possible even in paucicellular specimens, while preserving excellent cell morphology and yielding high sensitivity in the detection of antigens. In a study of 26 CSF specimens with inconclusive cytomorphology, comprising 335 single marker determinations, we were able to discriminate reliably between resting or activated benign cells and a wide range of types of malignant lymphoid cell. A definitive diagnosis was reached in all cases by one tap only. Malignant meningitis was ruled out in ten specimens and proved in 16, including five in which the type of malignancy could only be determined by immunophenotyping. We conclude that immunocytochemistry on PLL-coated slides constitutes the method of choice for immunologic cell differentiation in CSF, which allows equivocal morphologic findings to be clarified.

INVOLVEMENT of the central nervous system (CNS) can occur in all hematologic malignancies, although the incidence widely varies according to the type of leukemia or lymphoma and the treatment given. It is a complication of significant morbidity in neoplasias of high malignancy, including acute lymphoblastic leukemia (ALL) (although it is far less frequent with CNS prophylaxis[1-4]), blast crisis of chronic myeloid leukemia (CML),[5] acute myeloid leukemia (AML),[1,6-8] as well as intermediate and high-grade non-Hodgkin's lymphomas (NHL), as defined in the NHLPC-Project working formulation[9] and notably including Burkitt's lymphoma, diffuse histiocytic lymphomas of Rappaport, and lymphoblastic lymphomas.[10-14] In contrast, symptomatic CNS involvement is unusual in low-grade neoplasias, including, among others, extremely rare cases of B-cell chronic lymphocytic leukemia (B-CLL)[15-17] and multiple myeloma.[18] Finally, NHL, again mainly of Rappaport's diffuse histiocytic lymphoma type, may rarely develop primarily and exclusively within the brain parenchyma, and is called primary CNS lymphoma.[19-21] In acute leukemias, the leptomeninges appear to be the principal site of CNS involvement, whereas in NHL they are affected in ~70% of patients with secondary CNS involvement[11-14] and in an estimated one-third of patients with primary CNS lymphoma,[19-21] based on conventional morphologic assessment of cells in the cerebrospinal fluid (CSF).

As often noted, though rarely dealt with in more detail,[22] it may prove impossible to distinguish reliably between normal and malignant cells in the CSF and to identify, in cases with no known or with ill-defined underlying malignancy, the type of malignant cell by morphologic criteria alone. Immunocytology, on the other hand, although it is of proven value in classifying leukemias and lymphomas[23] and easy to perform with large cell specimens of bone marrow (BM) or blood, has so far been hampered in paucicellular CSF by high cell requirements of the methods hitherto used, including visual immunofluorescence,[24-26] immunocytochemistry,[27] flow cytometry,[28] and a latex sphere rosetting technique.[29] These approaches included repeated washing centrifugations to remove contaminating proteins, followed by incubation with antibodies either in suspension, again requiring repeated washing centrifugations, or on cytospin preparations. The low cell recoveries obtained using these methods required large volumes of CSF with high cell counts, or allowed only single antigens to be tested, such as terminal deoxynucleotidyl transferase (TdT),[24-26] immunoglobulin light chains,[27] or the common acute lymphoblastic leukemia antigen (CALLA),[28,29] each in a selected group of patients with known subclassified malignancy.

As we show, this methodologic problem can be overcome by electrostatic binding of cells to poly-L-lysine (PLL)-coated multispot slides, allowing repeated washing centrifugations to be avoided, followed by sensitive immunocytochemistry.[30,31] The various steps of this procedure were modified to optimize cell recovery, light microscopic morphology, and antigen preservation. Its value was documented in a study on 26 CSF specimens, the morphologic cell assessment of which had been nondiagnostic.

MATERIALS AND METHODS

Patients

Twenty-six CSF samples were obtained from 24 patients for immunologic cell differentiation after morphologic assessment of May-Grünwald-Giemsa–stained cytocentrifuge preparations had failed to provide an unequivocal diagnosis. Details of patient history and presentation are shown in Table 1. No specimens were excluded from analysis for technical reasons except when contaminated by blood.

Preparation of PLL-Bound CSF Cells

The CSF samples were diluted with equal volumes of protein-free, HEPES-buffered (0.03 mol/L) minimum essential medium (MEM,

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Table 1. Past History and Major Clinical Data at Presentation in 26 Cases of Nondiagnostic Morphologic CSF Cell Assessment

Case No.	Sex/Age (yr)	Past History (Months Since Diagnosis)	Reasons for CSF Analysis	WBC/μL CSF	Vol (mL)	No. of Antigens Tested
1	F/9	ALL, subtype not determined (21)	Headache, vomiting, repeated Plc	16	3.8	9
2	M/9	ALL, acid phosphatase$^+$ (86); LM (2)	6th cranial nerve palsy	6	6.0	9
3	M/22	T-ALL (6)	Repeated Plc during CNS-PT	27	3.4	9
4	M/37	Lb-T-NHL (4)	Plc/convoluted nuclei previously	4	3.9	4
5	F/33	C-ALL (19)	No symptoms or signs, routine tap	1.6	2.1	2
6a	M/11	C-ALL (93); repeated LM	Repeated Plc	24	4.8	9
6b		See 6a: c-ALL LM (7)	Persisting Plc during CNS-T	11	2.7	9
7	F/20	Burkitt-type NHL (6)	Headache, cranial nerve palsies	5	1.9	9
8	M/34	AIDS; Burkitt-type NHL (2)	6th cranial nerve palsy	675	2.0	16
9	F/70	Polymorphic Cb-NHL (27)	3rd cranial nerve palsy	40	—	9
10	F/72	CLL, subtype not determined (31)	Seizures, headache, hemianopia	31	4.1	15
11	F/66	B-CLL (118)	Parinaud syndrome, repeated Plc	14	3.5	8
12	M/49	B-CLL (34); pneumococcal meningitis (3)	Plc persisting since meningitis	30	2.7	10
13	M/72	CLL, subtype not determined (87)	Amaurosis, papilledema	178	1.0	16
14	M/45	Mature-cell-type MM (31)	Blurred vision, diplopia	400	0.9	19
15	M/55	Unclassified low-grade NHL (8)	Ataxia, tetraparesis	200	2.4	18
16	F/51	Reticulum cell sarcoma (6)	Paraparesis, incontinence	4,400	0.6	16
17	F/52	Undifferentiated abdominal tumor (11)	Parahypesthesia, incontinence	71	1.2	17
18	M/56	Plasma cell dyscrasia suggesting MM (0)	Headache, 7th cranial nerve palsy	80	—	18
19	F/73	No known systemic malignancy	Parenchymal CNS tumor in CCT	3	9.0	9
20	M/65	No known systemic malignancy	Focal seizures, coma for 2 mo	19	3.6	13
21	M/22	No known systemic malignancy	Seizure, hypodense foci in CCT	40	1.5	7
22	F/2	No known systemic malignancy	Focal seizures, coma for 1 wk	90	0.7	16
23a	M/63	No known systemic malignancy	Vomiting, blurred vision, ataxia	154	1.4	15
23b		See 23a: primary meningeal Lb-T-NHL (8)	Restaging after 8 mo of CNS-T	11	2.4	9
24	M/66	No known systemic malignancy	Pain, monoplegia of lower limb	435	0.4	20

Abbreviations: ALL, acute lymphoblastic leukemia; LM, leukemic meningitis; NHL, non-Hodgkin's lymphoma (except in case 16, pathologists had used the Kiel classification scheme[32]); Lb-NHL, lymphoblastic NHL; c-ALL, common ALL; Cb-NHL, centroblastic NHL; CLL, chronic lymphocytic leukemia; MM, multiple myeloma; Plc, pleocytosis (>4 cells/μL CSF); CNS-(P)T, (prophylactic) CNS treatment; CCT, computed cranial tomography.

GIBCO, Paisley, England) pH 7.4, and centrifuged once at 150 g for 20 minutes. The sediments were resuspended in 20 to 200 μL MEM to yield a cell density of $\geq 1 \times 10^3$ cells/10 μL. For cell attachment, multispot slides with 21 spots separated by water-repellent dimethylpolysiloxane and each coated (overnight at 4°C) with 0.4 μg PLL of 18,000 molecular weight (mol wt) (Sigma, St. Louis), stored at −20°C, were used.[31] Ten microliters of cell suspension was added to each spot, and cells were allowed to settle down for 45 minutes at 4°C. These conditions were delineated as optimal from methodologic studies which included variations in slide-coating with PLL (0.05 to 1,000 μg of 18,000-, 180,000-, and 340,000-mol wt polymers applied to each spot), and in time (15 to 120 minutes) and temperature (4 and 20°C) of cell attachment.

After cell attachment, the cell monolayers were gently washed in PBS and fixed for seven minutes at 20°C with freshly prepared 0.05% glutaraldehyde (grade 1, Sigma) in phosphate-buffered saline (PBS). These conditions were previously determined to be minimal for preserving morphologic detail and do not adversely affect the epitopes recognized by the antibodies listed below, except for Ki-67, as determined by comparison with binding of these antibodies to unfixed cells.

Spots assigned for testing nuclear TdT or cytoplasmic immunoglobulin were then incubated for 30 minutes at 20°C with a 0.04% solution of the nonionic detergent Brij 56 (Sigma) to permeabilize the cell membranes. This provided higher sensitivity in detection of TdT[31] and cytoplasmic immunoglobulin (unpublished observations) than conventional alcohol fixation of dehydrated cells. To detect the nuclear Ki-67 antigen, which is exceptionally sensitive to glutaraldehyde, PLL-bound nondehydrated cells were fixed first with 0.025% glutaraldehyde for 1.5 minutes only and subsequently with acetone for five minutes at 20°C.

Immunocytochemistry

The following mouse monoclonal antibodies (MoAbs) and rabbit polyclonal antibodies were used for immunologic cell characterization: MAS 036b (anti-CD1a MoAb, Sera-Lab, Crawley Down, England); OKT3 (anti-CD3 MoAb, Ortho, Raritan, NJ); anti-Leu-3a (anti-CD4 MoAb, Becton Dickinson, Mountain View, CA); NEI-015 (anti-CD5 MoAb, NEN, Boston); anti-Leu-2a (anti-CD8 MoAb, Becton Dickinson); OKT10 (anti-CD38 MoAb, Ortho); affinity-purified rabbit anti-calf TdT Ab (Pharmacia/P-L Biochemicals, Uppsala, Sweden); NEI-034 (anti-CD10/CALLA MoAb, NEN); OKIa1 (anti-Ia/HLA-DR MoAb, Ortho); B1 (anti-CD20 MoAb, Coulter, Hialeah, FL); BA-1 (anti-CD24 MoAb, Hybritech, San Diego); rabbit anti-human immunoglobulin μ heavy chain, κ light chain, and λ light chain Abs (Dakopatts, Glostrup, Denmark); OKT9 (anti-transferrin receptor MoAb, Ortho); Ki-67 (MoAb reacting with a nuclear antigen in proliferating cells in G_1, S, G_2, and M phases of the generative cycle[33]; provided by Drs J. Gerdes and H. Stein, Berlin, FRG; OKM1 (anti-CD11b MoAb, Ortho); and anti-Leu-M5 (anti-CD11c MoAb, Becton Dickinson). Details of the specificity of these reagents were given in a recent review by Foon and Todd.[23]

Binding of the above primary antibodies was determined by double-sandwich immunocytochemistry[31] with peroxidase-labeled goat anti-mouse or goat anti-rabbit and swine anti-goat immunoglobulin Abs (Tago, Burlingame, CA) as second and third layer,

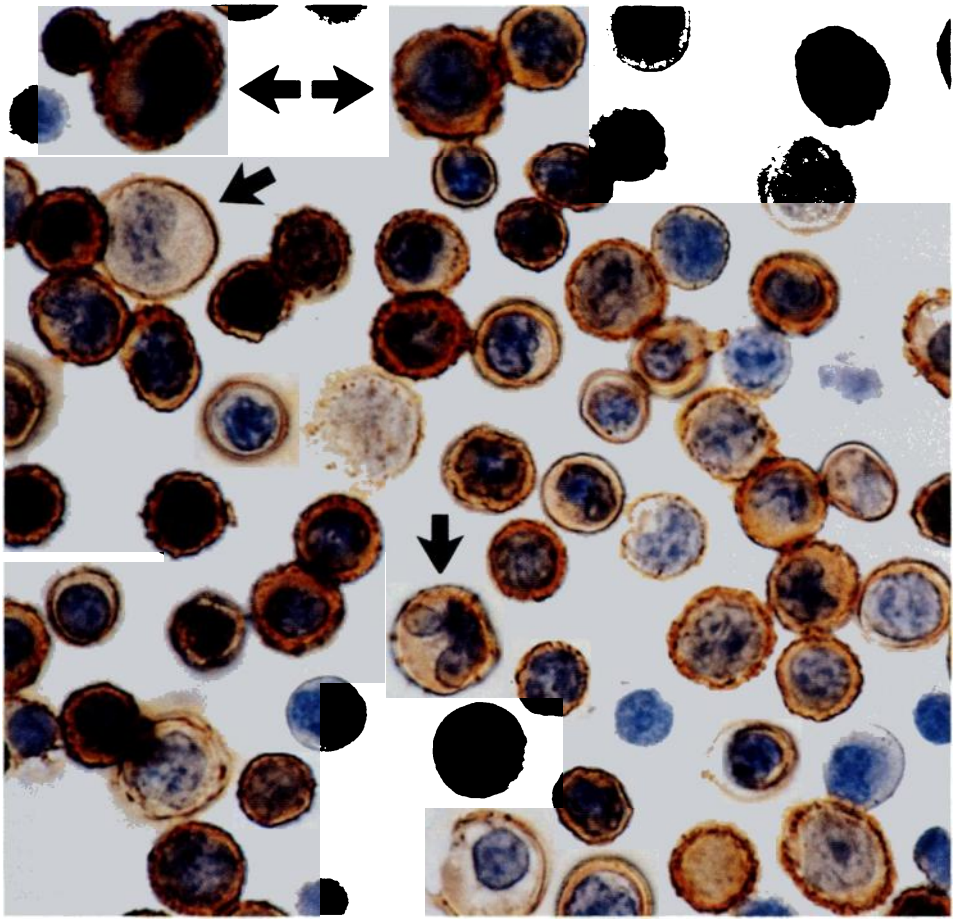

Fig 1. Case 2: Benign mature/activated T cell pleocytosis. CD5-positive small T lymphocytes and larger, blast-like activated T cells (arrows) with few negative lymphocytes interspersed (aminoethylcarbazole used as chromogen). High density and uniform distribution of cells on the PLL-coated slide obtained with a specimen containing only 6 cells/μL and allowing nine antigens to be tested.

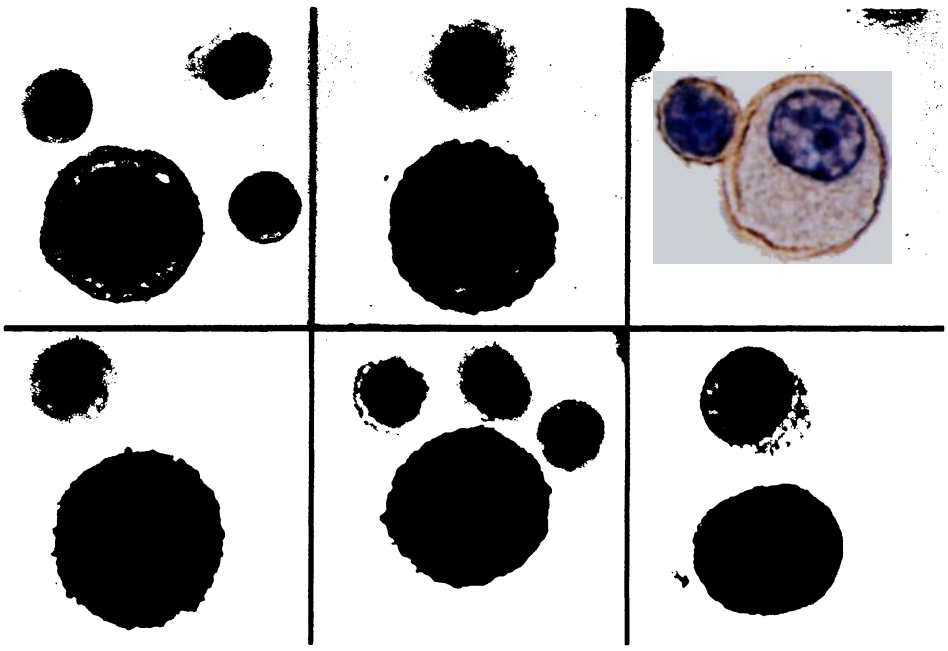

Fig 2. Case 18: Polyclonal population of activated B cells and plasma cells in the CSF of a patient with facial palsy since 4 weeks, correcting a diagnosis of multiple myeloma. Upper panel: Surface staining of Ia (HLA-DR) on a B immunoblast (left) as well as on a plasmablast (middle), and of CD38 on a plasma cell (right). Lower panel: Cytoplasmic staining of immunoglobulin κ light chains at stages of plasma cell development identical to those shown in the upper panel. The expression of immunoglobulin κ chains in some cells and λ chains in others proved their benign nature (negative plasma cell is apparent beside a positive plasma cell in right lower inset).

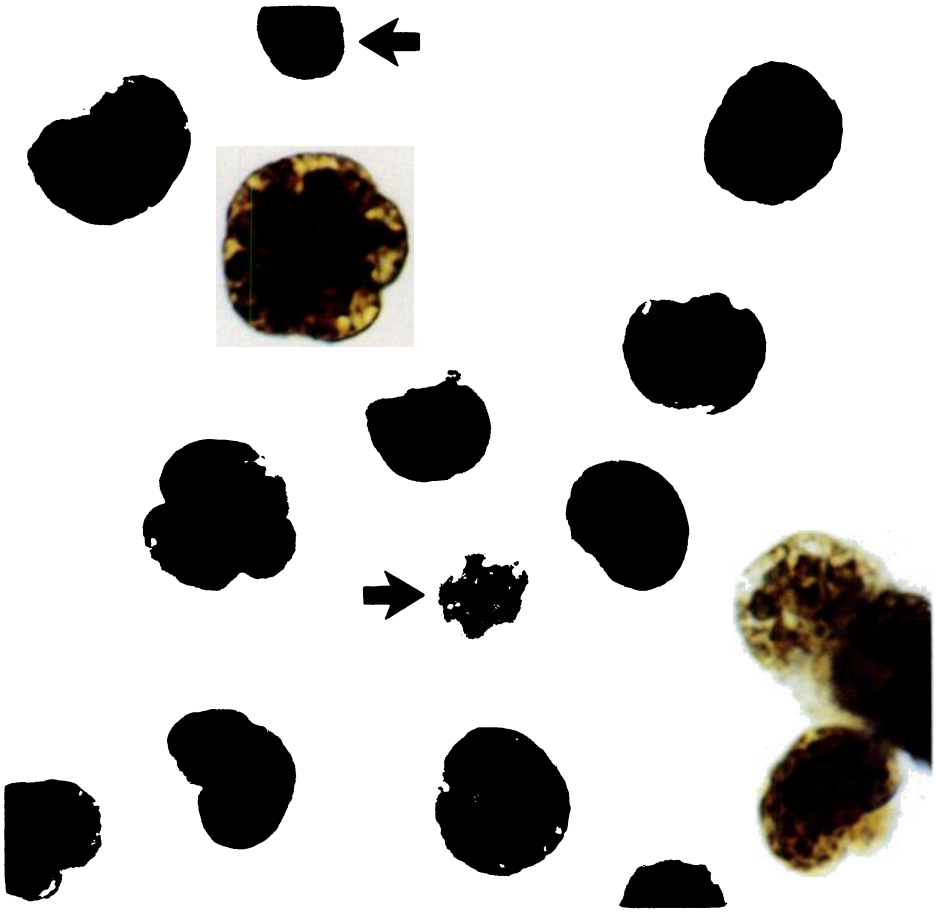

Fig 3. Case 14: Leptomeningeal involvement in plasmablastic transformation of multiple myeloma. Nuclear staining of the proliferation-associated Ki-67 antigen (negative small lymphocytes, arrows), indicating a high proliferation rate (diaminobenzidine used as chromogen).

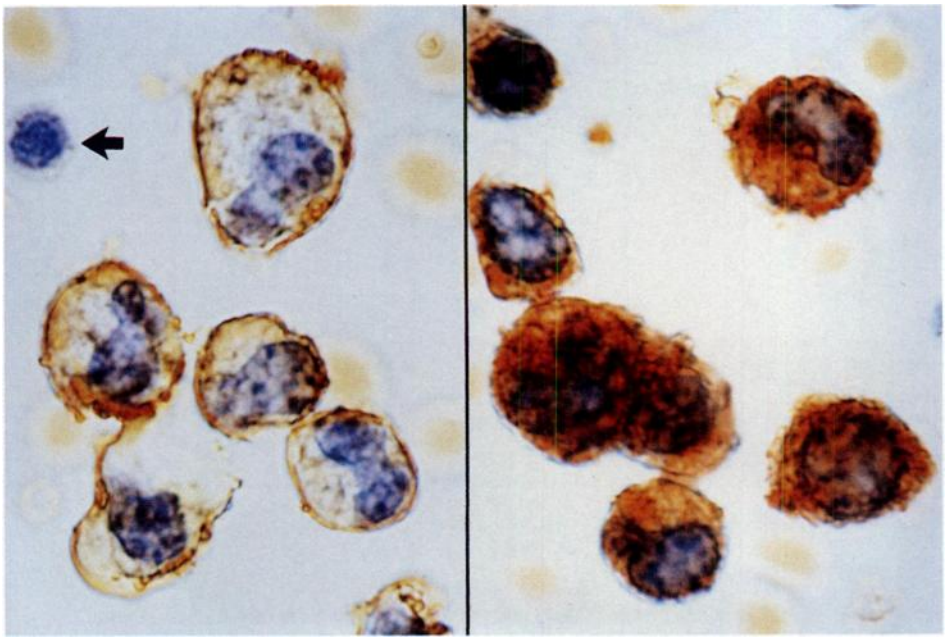

Fig 4. Case 17: Leptomeningeal involvement in lymphoblastic B-NHL, correcting a previous histologic diagnosis of "undifferentiated, probably epithelial tumor." Surface (left) and cytoplasmic staining (right) of immunoglobulin μ chains (negative small lymphocyte, arrow). Morphologically, cells had been assessed as "possibly malignant cells, possibly activated monocytes."

respectively. After each 30-minute incubation, cells were washed by simply dipping the slides into PBS. The enzyme reaction was performed using 3-amino-9-ethylcarbazole or 3,3-diaminobenzidine as chromogens. The slides were then rinsed for five minutes in distilled water (necessary to obtain uniform nuclear counterstaining in nondehydrated nonpermeabilized cells), stained with Mayer's acid hemalum, and mounted with phosphate-buffered glycerol. Four hundred to 1,000 cells were evaluated per antigen. The various types of cell were differentiated by morphology, myeloperoxidase content, and their composite immunophenotype.

Comparison With Immunocytochemistry on Cytospin Preparations

Nine CSF samples containing an excess number of cells (samples 8, 13 through 17, 22, 23a, and 24) were used to evaluate cell recovery, cytomorphology and antigen labeling obtained with PLL-bound nondehydrated cells and with conventional air-dried cytospin preparations processed in parallel. In the latter approach, cells were washed twice in MEM, centrifuged onto glass slides using a Shandon-Elliott cytocentrifuge, and fixed with methanol (20 minutes, 4°C) or acetone (five minutes, 20°C).

RESULTS

Technical Aspects

Unlike findings obtained with tissue sections,[34] maximal cell recovery and optimal morphology were achieved when slides were coated with small amounts of low-mol-wt PLL. In contrast, with increasing amounts of medium and high-mol-wt PLL, gradually increasing degeneration of cells was observed, eventually resulting in cell lysis and loss.

Washing the cells after their electrostatic binding to the PLL-coated slide sufficiently eliminated adsorbed CSF proteins, including immunoglobulin, to prevent background staining and saved both time and cells as compared with repeated tube washings required in conventional approaches. Figure 1 shows the uniform distribution and the high density of cells obtained on PLL-coated slides even with paucicellular specimens. As shown in Table 1, 16 of 26 CSF samples contained ≤40 cells/μL, including five with ≤6 cells/μL. Reproducibly high cell recoveries allowed 15 to 20 antigens to be tested in cases of ill-defined or unknown primary disease (samples 15 through 18, 23, and 24) and up to nine antigens in specimens containing ≤6 cells/μL, followed by an evaluation of ≥400 cells in each of 335 marker determinations. In contrast, cytospin preparations in all nine cases tested in parallel contained considerably smaller and moreover varying numbers of cells, often nonuniformly distributed on the slide, and larger fractions of difficult-to-evaluate damaged cells.

Considerably higher sensitivity in the detection of antigens was obtained with PLL-bound cells not subjected to the artifact of dehydration and fixed minimally with glutaraldehyde, as compared with air-dried cytospin preparations fixed

Table 2. Results of 335 Marker Determinations in 26 CSF Specimens With Inconclusive Cytomorphology

	Positive Lymphoid Cells (%)																				
Case No.	CD1a	CD3	CD4	CD5	CD8	T10 CD38	TdT	CALLA CD10	Ia	CD20	CD24	s-μ	s-κ	s-λ	c-μ	c-κ	c-λ	TfR	Ki-67	CD11b	Non-Ly (%)
1	0	—	51	89	41	—	0	0	17	—	1	—	—	—	—	—	—	—	—	7	0
2	0	—	46*	88	23	—	0	3	32	—	10	11	—	—	—	—	—	—	—	—	2
3	0	—	60*	85	10	—	0	1	6	—	—	2	—	—	—	—	—	—	—	7	8
4	0	>85	—	>85	—	—	0	—	—	—	—	—	—	—	—	—	—	—	—	—	—
5	—	—	—	45	—	—	44	—	—	—	—	—	—	—	—	—	—	—	—	—	10
6a	0	39*	20*	52	17	—	28	42	30	—	—	2	—	—	—	—	—	—	—	—	2
6b	0	—	45	—	33	—	—	14	—	—	14	0	—	—	0	—	—	—	2	2	5
7	—	22	—	21	—	—	—	64	—	67	57	64	61	1	—	—	—	—	—	2	15
8	0	8	4	9	4	97	0	91	95	91	88	91	90	<1	—	—	—	77	—	0	<1
9	—	—	—	39	—	—	—	4	58	53	52	55	55	1	55	—	—	—	—	—	—
10†	0	98†	87†	99†	11	5	—	0	67	<1	0	0	<1	<1	—	—	—	<1	—	<1	<1
11	—	74	61	98	10	—	—	—	—	23	21	—	2	20	—	—	—	—	—	—	1
12	—	70	61	94	9	—	—	—	27	28	26	—	1	26	—	—	—	—	—	<1	5
13	—	12	7	96	4	2	—	0	72	85	85	51	<1	49	85	<1	87	—	—	0	<1
14	0	<1	<1	<1	<1	89	—	0	0	0	0	0	0	0	0	0	54	96	89	<1	<1
15	0	13	12	15	3	—	0	<1	72	87	82	84	86	1	>80	>80	0	—	1	<1	<1
16	0	1	<1	2	<1	>95	>95	>95	>95	28	>95	0	<1	<1	—	—	—	—	88	<1	<1
17	0	—	11	15	7	3	0	74	50	52	—	88	83	<1	>80	>80	<1	22	—	<1	<1
18	0	74	56	80	23	20‡	0	1	23	18‡	13‡	9‡§	9‡§	6‡§	10‡§	9‡§	11‡§	19	—	—	3
19	0	—	79	97	16	—	0	0	—	—	<1	—	<1	<1	—	—	—	—	—	—	1
20	0	83	58	87	26	24	0	<1	27	5	—	1§	3§	3§	—	—	—	—	—	—	1
21	0	74	—	77	—	—	0	<1	—	—	—	3	—	—	—	—	—	—	—	2	10
22	0	61	41	62	20	26	—	2	17	10	13	9	—	—	—	5	2	8	9‖	10	14
23a	48	53	53	96	21	63¶	45	49	22¶	1	1	—	<1	<1	—	—	—	16	—	4	<1
23b	6#	—	48	80	25	—	1#	10	6¶	—	<1	—	—	—	—	—	—	—	—	1	12
24	0	25	16	26	9	0	0	27	5	61	24	62	61	0	57	59	1	37	46	0	12

Abbreviations: s, surface; c, cytoplasmic; TfR, transferrin receptor; Non-Ly, nonlymphoid cells (monocytes, granulocytes, histiocytes/ependymal cells).

Figures in bold type comprise or include malignant cells among others.

*Partial denaturation of glutaraldehyde-sensitive CD3 and CD4 by excess fixation at the start of this study.

†In blood, tested to exclude underlying T-CLL,[35,36] there were 75% B-CLL cells and 21% T helper cells. The latter cells included 17% Ia$^+$ cells, thus reflecting the activated T-helper cell pleocytosis in CSF.

‡Differences in values reflect loss of CD24, CD20, and surface immunoglobulin, and acquisition of cytoplasmic immunoglobulin and CD38 at different stages of development of B cells into plasma cells, as identified by morphology (Fig 2).

§Difference between μ heavy chain and the sum of light chains presumably reflects the switch from IgM to other immunoglobulin classes.

‖Morphologically, positive cells mainly comprised cells of the plasma cell series.

¶According to combined-antibody incubations, Ia$^+$ cells were activated CD3$^+$/CD1$^-$ T cells, whereas CD38$^+$ cells comprised both activated and malignant T cells.

#Loss of TdT and in part of CD1 in the course of disease was confirmed by reevaluation of malignant cells at time of death.

Table 3. Immunocytochemical Diagnosis and Clinical Outcome in 26 Cases of Nondiagnostic Morphologic CSF Cell Assessment

Case No.	Immunocytochemical Diagnosis	Outcome (Time of Observation)
1	No LM: mature/activated T-Plc	Alive with no CNS disease (39 mo)*
2	No LM: mature/activated T-Plc	Alive with no CNS disease (41 mo)
3	No LM: mature T-Plc	Alive with no CNS disease (46 mo)
4	No LM: normal CSF	Alive with no CNS disease (7 mo)
5	ALL LM	CNS-T, died of systemic disease (4 mo)
6a	C-ALL LM + T-Plc	CNS-T, see 6b
6b	Persisting LM, low proliferation rate† + T-Plc	Chronic LM, died of CNS disease (40 mo)
7	Lb-B-NHL LM	CNS-T, died of CNS disease (4 wk)
8	Lb-B-NHL LM + mature/activated T-Plc	CNS-T, residual CNS disease (4 mo)
9	B-NHL LM + T-Plc	CNS-T, died of CNS disease (2 wk)
10	No LM: mature/activated T-Plc	Symptoms and signs resolved (4 wk)
11	B-CLL LM + T-Plc	No progression despite no CNS-T (4 mo)
12	B-CLL LM + T-Plc	CNS-T, alive with no CNS disease (25 mo)
13	B-CLL LM + T-Plc	CNS-T, died of systemic disease (2 mo)
14	Plasmablastic MM LM, high proliferation rate	CNS-T, died of systemic disease (3 mo)
15	Lymphoplasmacytoid NHL‡ LM, low proliferation rate + T-Plc	CNS-T, residual spinal cord disease (30 mo)
16	C-ALL LM, high proliferation rate + T-Plc	CNS-T, died of systemic disease (5 mo)
17	Lb-B-NHL LM + T-Plc	CNS-T, died of CNS disease (5 wk)
18	No LM: mature T + polyclonal mature/activated B-Plc	Alive with no CNS/systemic disease (24 mo)
19	No LM: normal CSF	Died of undefined CNS tumor (3 mo)
20	No LM: mature/activated T-Plc	Died of presumed encephalitis (3 wk)
21	No LM: mature T-Plc	Sarcoidosis, residual CNS disease (20 mo)
22	No LM: mature/activated T + B-Plc	Died of tuberculous meningitis (1 wk)
23a	Lb-T-NHL primary LM§ + T-Plc	CNS-T, see 23b
23b	Residual LM + T-Plc	Died of combined CNS/systemic‖ disease (16 mo)
24	Lb-B-NHL primary LM§, high proliferation rate + T-Plc	CNS-T, died of CNS disease (8 mo)

Abbreviations as in Table 1.

*No treatment was given to the CNS unless otherwise indicated.

†Proliferation rate was assessed by Ki-67 MoAb.

‡Diagnosis was made on the basis of negativity of CD5 and the morphologic finding of a mixed population of monoclonal small B lymphocytes and plasmacytoid cells.

§A detailed workup, including BM and PB cytology, BM histology, chest radiograph and CT scan, ultrasonic examination and CT scan of the abdomen, as well as liver biopsy had revealed no evidence of concurrent systemic disease.

‖Leukemic dissemination occurred only in the terminal stage of his disease, although no systemic treatment had been given.

with acetone or even methanol. Moreover, in contrast to the latter fixatives, glutaraldehyde allowed selective staining of surface antigens unless detergent was used to permeabilize the cell membranes. Morphologic details were equally well preserved in both methods, but the three-dimensional shape of nondehydrated cells permitted better distinction of surface from intracellular staining, including staining of endogenous peroxidase, at different focal planes. Glutaraldehyde-fixed cells could be stored in a humidified chamber at 4°C for several days or cryopreserved at −80°C with dimethyl sulfoxide (DMSO).

Findings in CSF With Nondiagnostic Cytomorphology

Immunocytochemical data obtained in 26 CSF samples are shown in Table 2; the definitive diagnoses and details of patient follow-up are shown in Table 3. By combining morphologic, cytochemical (myeloperoxidase), and immunologic multimarker analysis, we were able to identify >85% of cells in all specimens and almost 100% in most of them (Table 2). The various types of cell could be defined by their composite immunophenotype rather than by single antigens only. By evaluating large numbers of cells, closely corresponding values were obtained with corresponding antigens, best exemplified by cases 11 through 13, in which CD5-positive cells equaled the sum of T cells, coexpressing CD3 and CD4 or CD8, and B-CLL cells, coexpressing monotypic immunoglobulin, Ia (HLA-DR), CD20, and CD24 (Table 2).

Immunocytochemistry disproving malignant meningitis. In ten CSF samples, malignant meningitis could be ruled out not only by excluding malignant but also by positively identifying benign mature cells (samples 1 through 4, 10, and 18 through 22; Table 2). Normal CSF and nonmalignant pleocytoses contained mainly T lymphocytes, on the average accounting for 84% of total CSF cells. In all cases, helper cells predominated, accounting for up to 88% of T lymphocytes (case 10). Activated T cells, which amounted to up to 67% of total T lymphocytes (case 10), represented a major source of ambiguity in morphologic cell assessment (Fig 1). Their lack of expression of immature antigens and coexpression of Ia helped to distinguish them from malignant lymphoblasts. In contrast, only a few mature B cells (≤3% in five of nine nonmalignant specimens tested), and, except in cases 18 (Fig 2) and 22, no cells of the plasma cell series were found. In the former patient, multiple myeloma had been erroneously presumed on the basis of a monoclonal hypergammaglobulinemia, an excessive BM plasmacytosis (30%), and CSF cytomorphology. Follow-up in this group of patients (Table 3) was in keeping with a diagnosis of reactive (intrathecal treatment) or infectious meningitis/meningoencephalitis except in case 19, with a parenchymal tumor of unknown type not exfoliating cells into the CSF, and in case 21, with sarcoidosis subsequently diagnosed by mediastinal lymph node histology.

Strongly Fc receptor-bearing cells of presumed ependymal origin constituted a potential diagnostic pitfall in immunophenotyping CSF cells, especially in paucicellular specimens. They range in diameter from 10 to >25 μm, show a characteristic finely villous surface in their three-dimen-

sional appearance on the PLL-coated slide, sometimes contain phagocytosed material, are invariably negative for myeloperoxidase, and express Ia, CD11b, and CD11c, features shared with pleural histiocytes (unpublished observations). In CSF with normal to slightly elevated cell count, they amounted up to 3% of cells, whereas in reactive conditions their percentage decreased with increasing cell counts. Due to their strongly binding antibodies such as anti-CD1a, these cells may be mistaken for a small residual population of malignant cells unless Fc receptors are blocked by serum or heat-aggregated IgG.

Immunocytochemistry proving and subtyping malignant cells in CSF. Malignant cells, expressing immature antigens (CD1a, CD10, and/or TdT) and/or monotypic immunoglobulin, could be definitively identified in 16 specimens obtained from 14 patients (samples 5 through 9, 11 through 17, 23, and 24; Table 2), none of which had been contaminated by blood or BM. In most cases, the malignant cell population was accompanied by mature T cells, amounting up to 110 cells/μL (case 24) and contributing to the difficulties in morphologic diagnosis.

In nine patients, the composite immunophenotype of malignant cells in their CSF corresponded to the type of systemic malignancy diagnosed before, including c-ALL in cases 5 and 6, high-grade B-cell NHL in cases 7 through 9, B-CLL in cases 11 through 13 and multiple myeloma in case 14. Morphologic CSF cell assessment had been equivocal as to whether malignant cells were present in six specimens and as to the type of malignant cell in one specimen (case 14). In the latter case, a shift in morphology from mature cell-type to plasmablastic multiple myeloma[37] together with an excessively elevated percentage of mitotic figures (1.8%) and of cells expressing the proliferation-associated nuclear antigen Ki-67[38] (Fig 3) as well as transferrin receptors in high density suggested transformation from low to high malignancy. This assumption was corroborated clinically by fulminant development of widely disseminated multiple large tumors leading to death soon after. In the remaining three specimens, false-negative diagnoses had been made on the basis of cytomorphology, including two specimens containing a normal number of cells (samples 5 and 7) and one specimen obtained from a patient with AIDS and Burkitt-type NHL (sample 8), whose CSF cells had been morphologically misinterpreted as activated lymphocytes on repeated occasions during a 3-week period of CNS disease.

In five patients with no known or with ill-defined underlying malignancy, multimarker analysis of CSF cells not only proved the presence of malignant cells but also accurately identified, for the first time, the type of malignancy, specifying a previous histologic diagnosis of "low-grade NHL" in case 15 (lymphoplasmacytoid NHL, later substantiated by development of a monoclonal IgM paraproteinemia), correcting a previous histologic diagnosis of "reticulum cell sarcoma" in case 16 (c-ALL) and one of "undifferentiated, probably epithelial neoplasia" in case 17 (lymphoblastic B-NHL, Fig 4), and classifying rare primary meningeal lymphoid malignancy[39] in cases 23 (lymphoblastic T-NHL) and 24 (lymphoblastic B-NHL). In contrast to typical primary CNS lymphoma, no parenchymal CNS mass lesions were detectable in the two latter patients by computed tomographic (CT) scans and magnetic resonance imaging (MRI). Patients 16 and 17 had not received prophylactic CNS treatment, based on their previous incorrect histopathologic diagnoses, and patient 24 had been treated erroneously for tuberculous meningitis, presumed on the basis of low CSF sugar values and a morphology mistaken for activated lymphocytes.

DISCUSSION

Our study underscores the serious potential for misinterpretation of CSF cells with morphologic assessment only. Activated lymphocytes, mimicking features of malignant blast cells, represent an important diagnostic pitfall.[22] The overall larger size of cells and the more prominent nucleoli in cytospin preparations contribute to the difficulties in interpretation. The activated lymphocytes in the CSF were most often T cells, but activated B cells may also cause confusion, as documented by case 18. Conversely, malignant cells may be mistaken for activated cells, as exemplified by cases 8 and 24, or missed in small volume disease, as in cases 5 through 7, especially if no symptoms or signs indicate CNS involvement.[7,13,25,29,40,41] As has also been stressed by other researchers,[22,41] distinction between normal and malignant cells is virtually impossible in mature-type lymphoid malignancies, as in cases 10 through 13 and case 15. Finally, the type of neoplasm from which the malignant cells originate often cannot be identified reliably, as emphasized here by cases 15 through 17 as well as 23 and 24. Since patients with immune deficiencies are not only prone to opportunistic infection but also have an increased risk of developing primary CNS lymphoma,[42] differentiation between these two conditions and subtyping of the latter may gain further importance with the increasing prevalence of AIDS.

Previous attempts at increasing diagnostic accuracy in CSF by immunophenotyping cells were often compromised by low, and moreover variable, cell recoveries,[24-29] which is in keeping with detailed data of a recent methodologic study[43] as well as our own experience when using multiple washing centrifugations and/or cytospin preparations. Immunophenotyping was thus applicable to paucicellular CSF only in cases of a known, immunologically subclassified disease with analysis tailored to one antigen in question and still entailed the risk of selective cell loss and of technical pitfalls not detected because additional positive and negative controls were lacking. Moreover, the number of cells counted (as few as 10 to 50 cells only[24,25,29]) was often too small to provide reliable information. In contrast, the higher cell recovery achieved by binding cells to slides optimally coated with PLL and performing all washing procedures on the slide allowed appropriate numbers of cells to be evaluated and multiple antigens to be tested even with small volumes of paucicellular CSF. This enabled us not only to prove or disprove malignant meningitis more reliably, but also to identify the type of malignant cell in cases with no known or with ill-defined systemic disease. The achievement in cell recovery was combined with an excellent cell morphology and a high sensitivity in the detection of antigens.

Except for the less common, phenotypically mature so-

called peripheral T cell leukemias/lymphomas not encountered in this study, the various other types of malignant lymphoid cell can be recognized by their expression of immature antigens (CD1a, CD10, TdT) and/or monotypic immunoglobulin. Since normal CSF and CSF in reactive conditions contain no phenotypically immature cells, according to our experience and that of other investigators,[25,26,29] and few immunoglobulin-bearing cells only, even minute populations of such types of malignant cell can be accurately identified, as we documented by cases 5 through 7,11,12, and 23b. In most cases, therapeutic conclusions can easily be drawn from such findings, provided that the specimen was not contaminated by peripheral blood or, conceivably, BM. In our study, the sole questions remaining concerned the interpretation of the small numbers of malignant cells found in the CSF in two of four cases with B-CLL (cases 11 and 12). Clinically overt CNS disease has very seldom been described in CLL,[15-17,35,36,44,45] at least in the common B-cell type. In contrast to clinical experience, however, postmortem examination showed that ~50% of previously asymptomatic patients with CLL had CNS involvement.[40,46] This raises the intriguing possibility that the few B-CLL cells in our two cases merely represented incidental findings in patients whose symptoms and signs were due to some other complication such as infection or small parenchymal bleeding. Furthermore, the recent history of bacterial meningitis in patient 12 even raises the question of whether B-CLL cells, like their normal counterparts, may enter the CSF compartment in the course of infectious CNS disease. Therefore, care should be taken not to miss other possible causes of CNS disease and not to deduce cytostatic treatment unreservedly from such findings of minor populations of B-CLL cells in the CSF unless more immunocytologic data have clarified the above considerations.

Our study does not answer questions regarding the frequency of ambiguous or even false diagnoses in morphologic CSF cell assessment. However, it does show that within a broad range of lymphoid malignancies definitive diagnoses can be reached in equivocal clinical settings, virtually irrespective of CSF cell concentration, provided that the methodologic details given are strictly adhered to. The approach may likewise increase diagnostic yield in detecting nonhematologic malignant disease,[47,48] and may help to analyze in more detail changes in CSF subpopulations in some neurologic nonmalignant conditions.

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