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Tumor Necrosis Factor-α-induced Mononuclear Cell Death May Contribute to Polymorphonuclear Cell Predominance in the Cerebrospinal Fluid of Patients with Bacterial Meningitis

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Abstract

Background: Bacterial meningitis is characterized by a marked predominance of polymorphonuclear leukocytes (PMNs: segmented granulocytes or neutrophils) in the cerebrospinal fluid (CSF), whereas aseptic meningitis is characterized by a predominance of mononuclear leukocytes (MNs: lymphocytes or monocytes). However, the pathophysiology of this predominance of PMNs in the CSF of patients with bacterial meningitis has never, to our knowledge, been clearly described.

Methods: To investigate the predominant cell components of CSF from pediatric patients with bacterial meningitis, we investigated cell death in association with levels of tumor necrosis factor-alpha (TNF-α) in the CSF, using the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay and flow cytometry.

Results: The MTT assay of the CSF revealed that the PMNs had survived, while the MNs rapidly had undergone cell death. Although PMNs survived in CSF with high levels of TNF-α, PMN apoptosis was demonstrated with flow cytometry.

Conclusions: The present study suggests that the pathophysiology of PMN predominance in the CSF of patients in the acute phase of bacterial meningitis is related to the rapid cell death of MNs and the survival of PMNs brought about by high levels of TNF-α.
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Key words: bacterial meningitis, polymorphonuclear leukocytes, mononuclear leukocytes, cerebrospinal fluid, tumor necrosis factor-alpha

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Introduction

Bacterial meningitis is characterized by a marked predominance of polymorphonuclear cells (PMNs: segmented granulocytes or neutrophils) in the cerebrospinal fluid (CSF), whereas aseptic meningitis is characterized by a predominance of mononuclear cells (MNs: lymphocytes or monocytes)[1]. However, although some cytokines or chemokines are reportedly associated with the active transport of leukocytes from the peripheral vessels into the central nervous system[2], the pathophysiology of this marked predominance of PMNs in the CSF of patients with bacterial meningitis has never, to our knowledge, been fully elucidated.

Because apoptosis has been established as the mechanism of leukocyte clearance from the subarachnoid space in patients with meningitis[3], we decided to investigate the predominant cell components of CSF from pediatric patients with meningitis, focusing on cell death in association with levels of tumor necrosis factor-alpha (TNF-α) in the CSF.

Patients and Methods

CSF Samples (Table 1)

Samples of CSF were collected admission via lumbar puncture from 10 patients when clinically indicated. Six of the patients had bacterial meningitis, 2 had mumps meningitis (diagnosed on the basis of bilateral parotic gland swelling and significant elevation of viral antibodies in the serum), 1 had aseptic meningitis (of undetermined origin), and 1 had prolonged febrile seizures (**Table 1**). Although CSF samples were collected from the patients with bacterial meningitis both on the day of admission day and on the following day, samples from the patients with mumps meningitis or febrile seizures were collected only on the day of admission for reasons of clinical convenience. Levels of TNF-α were determined with an enzyme-linked immunosorbent assay kit (Invitrogen, Carlsbad, CA, USA).

Use of CSF from the study subjects was approved by the ethics committee of our institution, and written informed consent was obtained from the guardian(s) of each patient before examination and treatment.

Cell Preparation and Cell Death Analysis

Peripheral blood leukocytes were donated by a healthy volunteer. They were separated into 2 fractions, MNs and PMNs, with a mixture of Ficoll and metrizoate (Mono-Poly Resolving Medium, Dainippon Sumitomo Pharma, Osaka, Japan); 1.0×10^6 cells of each fraction were suspended in 1 mL of the patient's CSF in a 96-well plate and incubated for 10 hours at 37℃. The cell death rate was evaluated with both the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) assay and flow cytometry.

MTT assay: Briefly, 100 μL of MTT (Sigma-Aldrich Japan, Tokyo, Japan) was added to each well and incubated for 10 hours, by which time the samples had turned purple. The absorbance at 540 nm was measured with a microplate reader. The results are expressed as percentages of the control values (control cells suspended in RPMI 1640 medium (Wako Pure Chemical Industries, Osaka, Japan)). To compare the patients' CSF samples, RPMI 1640 media containing 3 different levels (100, 1,000 and 10,000 pg/mL) of TNF-α (human, recombinant) (Wako Pure Chemical Industries) were used as positive controls. Each experiment was performed in triplicate, and the data are presented as means and standard deviations.

Flow cytometry: Uptake of propidium iodide (PI) was determined with flow cytometry. Cells (1.0×10^6 cells/mL) were incubated with 2.5 mg/mL of PI for 5 minutes at 37℃. Because PI is a fluorochrome and cannot pass through viable cell membranes, PI-positive cells have damaged membranes and are regarded as apoptotic[4]. After incubation, 10,000 cells from each sample were analyzed with a flow cytometer with laser excitation at 488 nm, and the propidium fluorescence was measured. The forward scatter distribution versus FL2-A dot plots was analyzed, and the results were converted to histogram plots with the WinMDI, version 2.9, software program (Joseph Trotter, The Scripps

Table 1 Clinical data of patients

Patient no.	Detected origin of infection			
			Admission day	The next day
1 (F, 0 y 5 m)	*Str. Pneumoniae*	CSF cell count [/3]	740	4,896
		Polymorphonuclear cells [%]	96	89
		Mononuclear cells [%]	4	11
		Peripheral white blood cells [/μL]	2,600	5,300
		Segmented neutrophils [%]	30	50
		Metamyelocytes [%]	3	0
		Stab neutrophils [%]	16	13
		Lymphocytes [%]	49	31
		Monocytes [%]	2	6
		TNF-α [pg/mL]	8,141	87
			Admission day	The next day
2 (M, 0 y 5 m)	*H. Influenzae (type b)*	CSF cell count [/3]	8,800	16,352
		Polymorphonuclear cells [%]	95	97
		Mononuclear cells [%]	5	3
		Peripheral white blood cells [/μL]	7,300	12,400
		Segmented neutrophils [%]	28	68
		Metamyelocytes [%]	6	0
		Stab neutrophils [%]	37	9
		Lymphocytes [%]	24	20
		Monocytes [%]	5	3
		TNF-α [pg/mL]	5,558	44
			Admission day	The next day
3 (M, 0 y 8 m)	*Str. Pneumoniae*	CSF cell count [/3]	11,136	19,856
		Polymorphonuclear cells [%]	97	96
		Mononuclear cells [%]	3	4
		Peripheral white blood cells [/μL]	10,700	14,700
		Segmented neutrophils [%]	28	28
		Metamyelocytes [%]	17	10
		Stab neutrophils [%]	20	28
		Lymphocytes [%]	31	31
		Monocytes [%]	4	3
		TNF-α [pg/mL]	1,477	23
			Admission day	The next day
4 (F, 1 y 0 m)	*H. Influenzae (type b)*	CSF cell count [/3]	28,160	40,192
		Polymorphonuclear cells [%]	98	98
		Mononuclear cells [%]	2	2
		Peripheral white blood cells [/μL]	9,200	22,000
		Segmented neutrophils [%]	57	80
		Metamyelocytes [%]	5	0
		Stab neutrophils [%]	18	5
		Lymphocytes [%]	18	11
		Monocytes [%]	2	4
		TNF-α [pg/mL]	1,040	24.5
			Admission day	The next day
5 (F, 1 y 2 m)	*H. Influenzae (BLNAR)*	CSF cell count [/3]	20,736	30,464
		Polymorphonuclear cells [%]	99	98
		Mononuclear cells [%]	1	2
		Peripheral white blood cells [/μL]	10,400	20,100
		Segmented neutrophils [%]	29	46
		Metamyelocytes [%]	28	0
		Stab neutrophils [%]	26	27
		Lymphocytes [%]	12	23
		Monocytes [%]	5	4
		TNF-α [pg/mL]	956.5	22.5
			Admission day	The next day
6 (M, 2 y 9 m)	*H. Influenzae (BLNAR)*	CSF cell count [/3]	1,664	3,480
		Polymorphonuclear cells [%]	96	95
		Mononuclear cells [%]	4	5
		Peripheral white blood cells [/μL]	9,200	9,300
		Segmented neutrophils [%]	49	69
		Metamyelocytes [%]	10	0
		Stab neutrophils [%]	10	8
		Lymphocytes [%]	26	20
		Monocytes [%]	5	3
		TNF-α [pg/mL]	422	20.5

Patient no.	Detected origin of infection		
			Admission day
7 (M, 5 y 6 m)	mumps	CSF cell count [/3]	263
		Polymorphonuclear cells [%]	46
		Mononuclear cells [%]	54
		Peripheral white blood cells [/μL]	4,800
		Segmented neutrophils [%]	48
		Stab neutrophils [%]	7
		Lymphocytes [%]	40
		Monocytes [%]	4
		Eosinophils [%]	1
		TNF-α [pg/mL]	28.5
8 (F, 2 y 3 m)	mumps	CSF cell count [/3]	1,212
		Polymorphonuclear cells [%]	28
		Mononuclear cells [%]	72
		Peripheral white blood cells [/μL]	9,000
		Segmented neutrophils [%]	49
		Stab neutrophils [%]	11
		Lymphocytes [%]	35
		Monocytes [%]	4
		Eosinophils [%]	1
		TNF-α [pg/mL]	20.5
			Admission day
9 (M, 5 y 9 m)	aseptic (unknown)	CSF cell count [/3]	930
		Polymorphonuclear cells [%]	10
		Mononuclear cells [%]	90
		Peripheral white blood cells [/μL]	17,700
		Segmented neutrophils [%]	65
		Stab neutrophils [%]	17
		Lymphocytes [%]	16
		Monocytes [%]	2
		TNF-α [pg/mL]	15.5
			Admission day
10 (F, 3 y 4 m)	Febrile Seizures (unknown)	CSF cell count [/3]	5
		Peripheral white blood cells [/μL]	10,400
		Segmented neutrophils [%]	51
		Stab neutrophils [%]	9
		Lymphocytes [%]	35
		Monocytes [%]	5
		TNF-α [pg/mL]	12.5

Institute, La Jolla, CA, USA). The percentage of PI-positive cells was evaluated. Cell populations in the R6 square (out of 10,000 cells) showed a high uptake of PI in this experiment. Flow cytometry was performed with cells from 5 representative patients (3 with bacterial meningitis, 1 with mumps meningitis, and 1 with febrile seizures).

Data are presented as means ± standard deviations; the statistical significance of differences was determined with one-way analysis of variance.

Results

Clinical Data of Patients (Table 1)

In comparison with the peripheral blood white cell population, each CSF sample taken from the patients with bacterial meningitis on admission showed high TNF-α levels and marked pleocytosis comprised almost entirely of PMNs. However, the TNF-α levels decreased rapidly in as little as 1 day. In contrast, each CSF sample obtained on admission from the patients with aseptic meningitis (including mumps meningitis) showed pleocytosis, but not with a particularly high level of PMNs; the samples from the patients with febrile seizures did not show pleocytosis.

MTT Assay (Table 2)

In the patients with bacterial meningitis, the higher the CSF TNF-α level was, the higher the MN mortality rate tended to be; however, the PMNs survived in the CSF for 10 hours despite high levels of TNF-α. In contrast, in the patients with aseptic meningitis (including mumps meningitis) or febrile

Table 2 MTT assay

Patient no.	Clinical Diagnosis	TNF-α [pg/mL]	MNs survival rate [%]		PMNs survival rate [%]	
1 (F, 0 y 5 m)	Bacterial meningitis	8,141	37.8		92.4	
2 (M, 0 y 5 m)	Bacterial meningitis	5,588	54.9		93.8	
3 (M, 0 y 8 m)	Bacterial meningitis	1,477	58.8		100	
4 (F, 1 y 0 m)	Bacterial meningitis	1,040	68.2		100	
5 (F, 1 y 2 m)	Bacterial meningitis	956.5	65.4		100	
6 (M, 2 y 9 m)	Bacterial meningitis	422	75.5		100	
n=6	(Bacterial meningitis)	2,937 ± 3,165	60.1 ± 13.1			
7 (M, 5 y 6 m)	Mumps meningitis	28.5	88.5		100	
8 (F, 2 y 3 m)	Mumps meningitis	20	91.5		100	
9 (M, 5 y 9 m)	Aseptic meningitis	15.5	100		100	
n=3	(Aseptic meningitis)	21.3 ± 6.6	93.3 ± 6.0			
10 (F, 3 y 4 m)	Prolonged febrile seizures	12.5	100		100	
	Positive control #1	10,000	39.2 ± 7.5	P<0.01	79.2 ± 8.4	P<0.05
	Positive control #2	1,000	88.2 ± 5.5		100	
	Positive control #3	100	96.2 ± 6.6	P<0.05	100	

Table 3 PI-positive rate on flow cytometry

Patient no.	Clinical Diagnosis	TNF-α [pg/mL]	MNs PI-positive rate [%]	PNMs PI-positive rate [%]
2 (M, 0 y 5 m)	Bacterial meningitis	5,588	3.54	4.99
3 (M, 0 y 8 m)	Bacterial meningitis	1,477	2.56	2.08
5 (F, 1 y 2 m)	Bacterial meningitis	956.5	1.19	1.35
7 (M, 5 y 6 m)	Mumps meningitis	28.5	0.73	0.37
10 (F, 3 y 4 m)	Prolonged febrile seizures	12.5	0.23	0.32

seizures, most MNs and all PMNs survived. The MN survival rate in the patients with bacterial meningitis (60.1% ± 13.1%, n=6) was significantly lower ($p<0.01$) than that in the patients with aseptic meningitis (93.3% ± 6.0%, n=3).

Flow Cytometry (Table 3 and Fig. 1)

The PI-positive rate indicated that both MNs and PMNs incubated in CSF from the patients with bacterial meningitis had undergone apoptosis. However, leukocytes (MNs and PMNs) incubated in CSF from the patient with mumps meningitis or the patient with febrile seizures largely failed to absorb PI (raw flow cytometric data for 1 representative patient with bacterial meningitis [patient 2]) and 1 patient with mumps meningitis [patient 7]) are presented in **Fig. 1**).

Discussion

The inflammatory cells in the CSF of patients with meningitis are MNs and PMNs, and it is generally thought that a marked pleocytosis of white blood cells comprised almost entirely of PMNs is present in the CSF of patients with bacterial meningitis[1]. However, the mechanism by which PMNs overwhelmingly predominate in the CSF during the acute phase of bacterial meningitis is unclear. Some cytokines or chemokines reportedly play a role in the active transport of leukocytes into the central nervous system compartment[2], and TNF-related apoptosis-inducing ligand (TRAIL) has been hypothesized to regulate the influx of the infiltrating leukocyte population[5]. According to this hypothesis, TRAIL regulates the acute inflammatory response

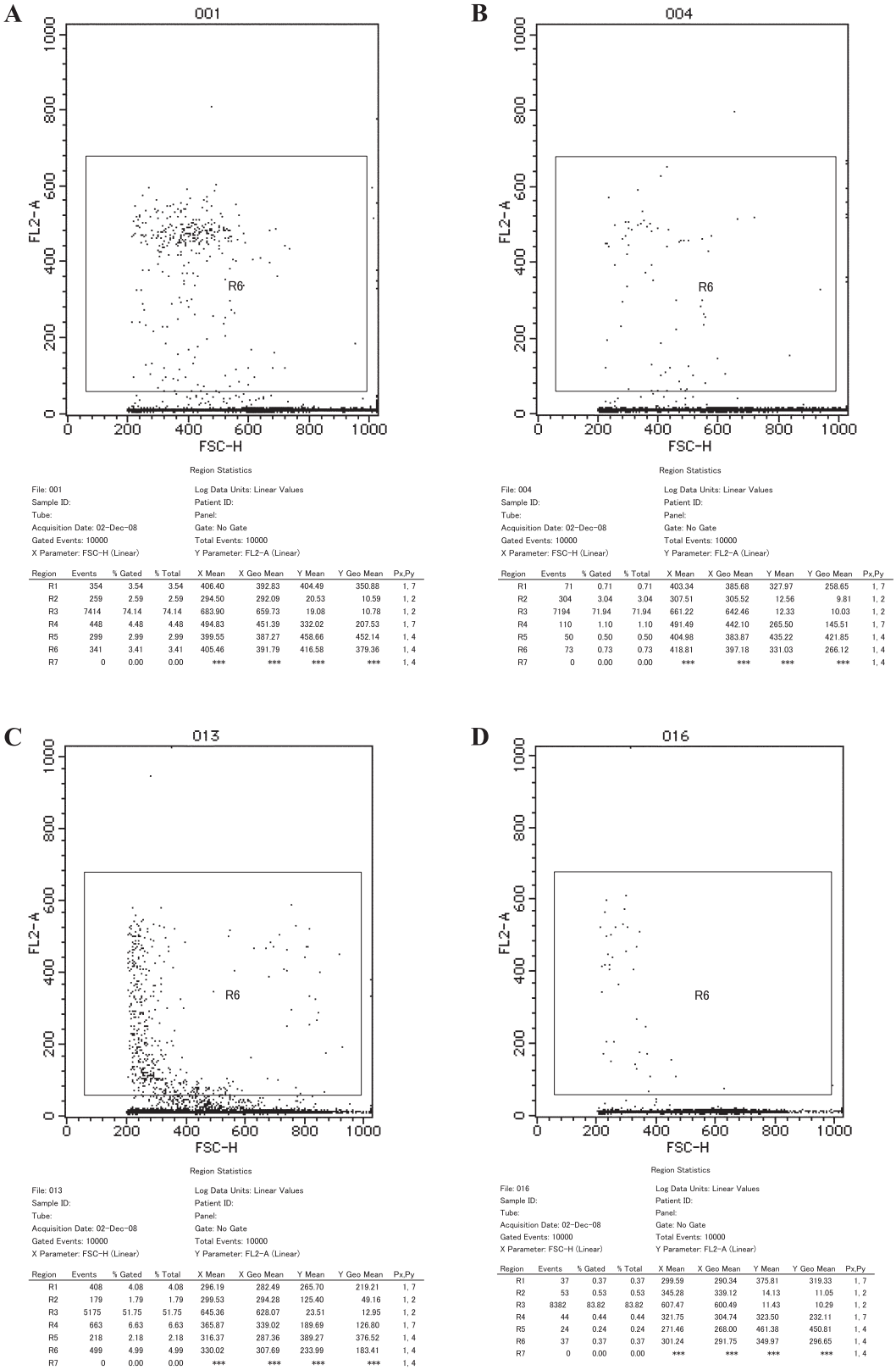

Fig. 1 Cells in each fraction were preincubated with CSF from patients with meningitis before the addition of PI. Cell populations in the R6 square showed high uptake of PI (PI-positive).
(A) MNs in the CSF of a patient with bacterial meningitis (patient 2); (B) MNs in the CSF of a patient with mumps meningitis (patient 7); (C) PMNs in the CSF of patient 2; (D) PMNs in the CSF of patient 7.

characterized by a massive influx of PMNs from the peripheral blood stream into the CSF compartment and by delayed recruitment of MNs; the concentration of TRAIL correlates with the number of leukocytes in the CSF of patients with bacterial meningitis[5]. However, protein array analysis has shown that CSF levels of granulocyte chemotactic protein-2 and interleukin-8 in patients with aseptic meningitis are similar to those in patients with bacterial meningitis and that CSF levels of monocyte chemoattractant protein-1 and 2 in patients with bacterial meningitis are similar to those in patients with aseptic meningitis[6]. Therefore, active transport mediated by some types of chemoattractive factor (adhesion molecules or chemokines) cannot, contrary to common belief, fully explain the pathophysiology of the marked predominance of PMNs in the CSF of patients with bacterial meningitis.

Our study suggests another possibility: TNF-α plays a role in the phenomenon by allowing PMNs to survive while MNs rapidly undergo cell death. Our findings are compatible with those of previous studies, which have shown that inflammatory neutrophil apoptosis (a mode of cell death) is inhibited by various inflammatory mediators[7,8], such as granulocyte-macrophage colony-stimulating factor, lipopolysaccharide, and TNF-α[9].

On the other hand, the mechanism by which leukocytes are finally cleared from sites of tissue inflammation is apoptosis[3,10]. Although the scope of the present study was limited, our flow cytometry findings suggest that apoptosis of not only MNs but also of PMNs starts in the CSF of patients with bacterial meningitis in the acute phase.

Because the levels of TNF-α were markedly decreased on the day of admission in all 6 patients with bacterial meningitis, the potency of TNF-α activity may be limited during the superacute phase of the disease. Moreover, although limiting factors of our present study were the use of peripheral blood leukocytes from a healthy volunteer rather than from a patient with meningitis and the use of the MTT assay, which is only a quantitative assay of cell survival[11] and cannot determine the mode of cell death, we suggest that the rapid cell death of MNs and the survival of PMNs brought about by high levels of TNF-α are factors in the pathophysiology of PMN predominance in the CSF of patients in the acute phase of bacterial meningitis.

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