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Cytokine mRNA Profiles in Mononuclear Cells in Acute Aseptic Meningoencephalitis

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Cytokines are important modulators of inflammation and immune responses. Using in situ hybridization with radiolabelled cDNA oligonucleotide probes, we studied the expression of mRNA encoding the cytokines gamma interferon (IFN-γ), interleukin 4 (IL-4), IL-6, IL-10, transforming growth factor β (TGF-β), tumor necrosis factor alpha (TNF-α), lymphotoxin, and perforin in mononuclear cells (MNC) from blood and cerebrospinal fluid (CSF) of patients with acute aseptic meningoencephalitis (AM) and from blood of healthy controls. Patients in the acute phase of AM had elevated numbers of IFN-γ mRNA-expressing cells in the blood compared with that of controls and higher numbers of IFN-γ mRNA-expressing cells in their CSF compared with that of convalescent-phase patients, which is in accordance with the antiviral effects of this cytokine. Upregulation of IL-4, IL-6, and IL-10 was found in convalescent-phase patients, which is consistent with the longstanding B-cell response found in AM. TGF-β and perforin were upregulated in both stages of AM, while the numbers of blood and CSF MNC expressing cytokine mRNA of the TNF family (TNF-α and lymphotoxin) did not differ between patients with AM and controls. An even higher elevation in CSF was noticed for MNC expressing most of the cytokines, particularly IL-4 and TGF-β, reflecting the autonomy of the immune response in the CSF. The definition of cytokine profiles in AM, a self-limiting and benign disease, provides a foundation for future comparisons with other infectious and inflammatory nervous system diseases.

Acute aseptic meningoencephalitis (AM) is a benign, self-limiting illness. Characteristic immunological findings include an accumulation of mononuclear cells (MNC) at the sites of inflammation within the central nervous system and reflected in the cerebrospinal fluid (CSF) by elevated immunoglobulin concentrations and oligoclonal bands (18). The viral etiology and involvement of immunological mechanisms suggest a role for immunoregulatory cytokines in AM. Cytokines are low-molecular-weight proteins which control the response of the immune system to infectious agents and trauma (37). Most cytokines have multiple cellular sources and targets and share functions to modulate the levels and effects of each other. Although cytokines can be produced by a variety of cells, blood MNC are considered a major source of these effector molecules. For example, macrophages are known to release large amounts of interleukin 1 (IL-1), IL-6, IL-8, and tumor necrosis factor alpha (TNF-α). Evidence suggesting the existence of polarized human T-cell responses, reminiscent of T helper 1 (Th1) and Th2 subsets described for mice (31), is accumulating. Cells of the Th1 subset produce gamma interferon (IFN-γ), IL-2, and lymphotoxin (LT), while those of the Th2 subset produce IL-4, IL-5, IL-6, IL-10, and IL-13. Th1-like cells enhance cellular immunity, while Th2-like cells help B cells become antibody-producing cells (for a review, see reference 25).

Altered concentrations of cytokines in body fluids have been reported for various diseases, suggesting that these molecules might play a role in the modulation of inflammation and outcome of the disease. Bacterial meningitis and sepsis were studied extensively in this context, and high levels of TNF-α, IL-1β, and IL-6 were reported and correlated with the severity of the disease (2, 9, 15, 22, 29, 33, 36). Studies concerning the involvement and possible role of cytokines in AM are, however, limited or based on determinations of cytokines in body fluids (8, 11, 14, 22, 23, 39), which because of the short half-lives and capture of cytokines by receptors on nearby cells, yield often low or undetectable levels.

Here we report the application of in situ hybridization (ISH) with cDNA probes to examine the expression of mRNA of IFN-γ, transforming growth factor β (TGF-β), IL-4, IL-6, IL-10, TNF-α, LT, and perforin in MNC from patients with AM and healthy controls.

MATERIALS AND METHODS

Patients. Twenty-six patients (12 females and 14 males) had AM. A benign clinical course was noted for all patients. Serological tests revealed the following etiologies of AM in the patients: tick bite encephalitis in six patients, herpes simplex type II in three patients, and enterovirus in four patients. The remaining patients had AM of unknown etiology. The age of the patients ranged between 20 and 72 years (mean, 37 years). The interval between first symptoms of AM and sampling of blood and CSF ranged between 1 and 78 days (mean, 14 days). Ten of the patients were examined within 1 week after onset of AM. The MNC count in CSF varied between 10×10^6 and 970×10^6/liter (median, 90×10^6). Fifteen of 23 examined patients had elevated CSF/plasma albumin ratios, reflecting damage to the blood-CSF barrier, and 2 of 23 patients had an increased (>0.7) CSF immunoglobulin G index, indicating intrathecal immunoglobulin G synthesis (19). Three of nine examined patients had oligoclonal immunoglobulin G bands in CSF upon examination of CSF and corresponding plasma by agarose isoelectric focusing combined with immunoblotting and avidin-biotin-amplified double-antibody peroxidase staining (24).

Twenty-three healthy subjects (five females) were examined as controls. The age of these subjects ranged from 21 to 60 years (mean, 35 years).

The study protocol was approved by the Ethics Committee of the Karolinska Institute at Huddinge Hospital, and informed consent was obtained from all patients.

Preparation of blood and CSF cells. Peripheral blood MNC were obtained by density gradient centrifugation on Lymphoprep (Nyegaard, Oslo, Norway). The cells from the interphase were collected, washed three times with Dulbecco's medium (Flow Laboratories, Irvine, United Kingdom) containing antibiotics, washed with phosphate-buffered saline (PBS), and counted.

CSF was centrifuged for 10 min at $200 \times g$. After removal of the supernatant, the cells were washed with PBS and counted.

Cell viability measured by trypan blue exclusion always exceeded 95%.

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TABLE 1. Oligonucleotide probes used for ISH

Probe	GenBank accession no.	Complementary to bases	Reference
IFN-γ	J00129	507–556, 4682–4729, 4660–4707, 4641–4688	13
TGF-β	X02812	1363–1410, 1457–1504, 1766–1813, 1953–2000	4
IL-4	M13982	66–113, 235–282, 286–333, 406–453	41
IL-6	M54894, M38669	53–100, 132–179, 559–606, 634–681	40
IL-10	M57627	31–78, 97–144, 340–387, 376–423	38
TNF-α	X02159, X01394	913–960, 2233–2280, 2383–2430, 2479–2526	26
LT	X01393	82–129, 86–138, 374–421, 540–587	12
Perforin	M28393	376–414, 437–484, 787–834, 1349–1396	34

Detection of cytokine mRNA in MNC by ISH. ISH was performed as described previously by Dagerlind et al. (3) for tissue sections and modified for MNC (21). Aliquots containing 5×10^4 to 10×10^4 MNC were dried onto restricted areas of microscope slides (ProbeOn slides; Fisher Scientific, Pittsburgh, Pa.). The cells were dried at 55°C for about 5 min, fixed for 1 min in 1% formaldehyde, rinsed in PBS, and dehydrated with a sequence of 70, 95, and 99% alcohol. Slides were stored with silica in sealed boxes at −20°C until ISH was performed. Synthetic oligonucleotide probes (Scandinavian Gene Synthesis AB, Köping, Sweden) were labelled with ^{35}S-deoxyadenosine-5′-α-(thio)-triphosphate (Dupont Scandinavia, Stockholm, Sweden) with terminal deoxynucleotidyl transferase (Amersham, Little Chalfont, United Kingdom). For each cytokine, a mixture of four different approximately 48-base-long oligonucleotide probes was used to increase the sensitivity of the method. The oligonucleotide sequences were obtained from GenBank by the MacVector System (Table 1). A constant guanine/cytosine ratio of approximately 60% was used. The oligonucleotide probes were checked for the absence of palindromes and long sequences of homology within the species against available GenBank data. The cells were hybridized for 16 to 18 h at 42°C in a humidified chamber with 10^6 cpm of labelled probe per 100 μl of hybridization mixture consisting of 50% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution (0.02% each of polyvinylpyrrolidone, bovine serum albumin, and Ficoll), 1% sarcosyl (Sigma), 0.02 M phosphate buffer (pH 7.0), 10% dextran sulfate, 500 μg of heat-denatured salmon sperm DNA per ml, and 200 mM dithiotreithol. Following ISH, the slides were rinsed five times for 15 min each time at 55°C in 1× SSC, allowed to come to room temperature, dipped in distilled water, dehydrated through an ethanol gradient (60, 70, and 95%), air dried, dipped in Kodak NTB2 emulsion diluted 1:1 in distilled water, and exposed at 4°C for 15 to 20 days depending on the age of the probe. After developing in Kodak D19, the slides were stained in cresyl violet and mounted with Entellan (Merck, Darmstadt, Germany). Coded slides were examined by dark-field microscopy for positive cells containing >15 grains per cell in a star-like distribution. The intracellular distribution of the grains was always checked by light microscopy at a higher magnification. Labelled cells were expressed as numbers per 10^5 MNC.

To validate the estimation of cytokine mRNA-positive cells, slides representing three patients were chosen, and the numbers of grains in randomly selected cells judged positive and cells judged negative were counted. The numbers of grains on cells judged positive for IFN-γ, TGF-β, IL-4, IL-10, TNF-α, and LT were 61 ± 15, 53 ± 13, 47 ± 9, 52 ± 11, 71 ± 16, and 38 ± 9, respectively. In many positive cells, the grains were so heavily accumulated within and around the cells that it was not possible to count every single grain. In cells judged negative, the number of grains was 2 ± 1 per cell. In cells negative by this standard, the grains were also scattered randomly over the cells and not distributed in a star-like fashion. The results suggest a bimodal distribution of cells: cytokine mRNA-positive cells with many grains and cytokine mRNA-negative cells with background grains. Variation between duplicates was <10%. As control probes, the sense sequences for the exons 4 of IFN-γ, TGF-β, IL-4, IL-6, IL-10, TNF-α, LT, and perforin, respectively, were always used in parallel on the cells from each subject, without revealing any positive cells. The number of cells used in ISH was not necessarily equal to the number of cells that was ultimately detected on the slide. Cell losses ranged between 10 and 50% (mean value, around 30%) from cell application to cell counting. The preferential loss of certain cell types is not ruled out. To compensate for cell losses, the total number of cells on the slides was regularly counted. With the help of a microscope grid used as a measuring unit, the radius (r) of the surface area (A) covered by cells was determined. The area A was calculated by the formula $A = r^2 \times \pi$. Cells were usually counted in two grids at the periphery and one grid at the center of the surface covered by cells. In the case of uneven cell distribution, cells in additional grids were counted. The mean value of the number of cells per grid was determined and multiplied by A.

Statistical analyses. The nonparametric Mann-Whitney test was used for group comparisons, and the Wilcoxon signed rank test for pairs was used to evaluate the differences between blood and CSF values. Spearman's rank correlation test was used for correlation analysis. Reported P values are two tailed, and a P of <0.05 was considered statistically significant.

RESULTS

The 26 AM patients were subgrouped into 10 patients who were examined during the acute phase of AM, i.e., within 7 days after onset of clinical symptoms, and 16 patients who were examined in the convalescent phase, i.e., more than 7 days after onset.

The patients examined during the acute phase of AM had elevated numbers of IFN-γ, IL-6, TGF-β, and perforin mRNA-expressing MNC in their peripheral blood compared with that of the healthy controls (Table 2 and Fig. 1). The numbers of IL-4, IL-10, TNF-α, and LT mRNA-positive MNC did not differ between patients with AM and controls. The numbers of IFN-γ, IL-4, and TGF-β mRNA-expressing cells were further elevated in the CSF of the patients compared with those in their blood on examination during the acute phase of AM ($P < 0.05$ for all comparisons). There were also tendencies for the numbers of some of the remaining cytokines to be elevated in the CSF (Fig. 2A), but the small number of patients examined precludes conclusions.

The patients examined during convalescence after AM also had elevated numbers of IL-6, TGF-β, and perforin mRNA-expressing cells in their blood compared with that of the healthy controls (Table 2 and Fig. 1). In addition, the numbers of IL-4 and IL-10 mRNA-expressing MNC were elevated in comparison with the numbers encountered in the controls. In contrast, the elevation of IFN-γ mRNA-expressing cells noticed in the blood of patients examined during the acute phase of AM compared with that of healthy controls was not detectable in the convalescent phase. In the CSF of convalescent-phase AM patients, the numbers of IL-4 and TGF-β mRNA-expressing cells were significantly higher than those in the blood of the AM patients ($P < 0.05$), as was the case during the acute phase of AM, while the difference encountered during the acute phase of AM for IFN-γ mRNA-expressing cells was not demonstrable in the convalescent phase. In addition, the numbers of perforin mRNA-expressing cells were higher ($P < 0.05$) in the CSF than in the blood in the convalescent phase after AM. For the remaining four cytokines, there were tendencies for elevated values to be present in CSF compared with those in the blood (Fig. 2B).

When comparing the data obtained from examination during the acute and convalescent phases in the AM patients, the numbers of IFN-γ mRNA-expressing MNC in CSF were higher during the acute phase ($P < 0.05$). There was also a trend of elevation of IL-4 and TGF-β mRNA-expressing cells in CSF during the convalescent phases, but these differences did not reach statistical significance (Table 2). In the AM patients, we found no differences between acute- and convalescent-phase numbers of blood MNC expressing mRNA of any of eight cytokines under study.

Evaluations of the cytokines of the TNF family, i.e., TNF-α and LT, did not reveal any differences between the AM patients and the healthy controls or between the AM patients examined during the acute phase and those examined during convalescence.

When comparing patients with different etiologies of AM, we observed that two of three patients with confirmed herpes

TABLE 2. Numbers of IFN-γ, TGF-β, IL-4, IL-6, IL-10, TNF-α, LT, and perforin mRNA-positive cells per 10^5 MNC from peripheral blood (PBL) and CSF of patients with AM and from PBL of healthy subjects[a]

Cytokine	Measured in:	Acute-phase AM patients[b]				Convalescent-phase AM patients[b]				Healthy subjects		
		No. of MNC (10^5)		No. examined	P^c	No. of MNC (10^5)		No. examined	P^c	No. of MNC (10^5)		No. examined
		Range	Mean ± SD			Range	Mean ± SD			Range	Mean ± SD	
IFN-γ	CSF	0–80	18 ± 23	10	<0.01	0–20	7 ± 8	16	NS[d]	ND[e]	ND	ND
	PBL	0–10	4 ± 3	10		0–10	3 ± 3	16		0–6	1.6 ± 1.7	23
TGF-β	CSF	0–20	9 ± 7	10	<0.05	0–192	29 ± 51	14	<0.05	ND	ND	ND
	PBL	0–6	3 ± 2.3	10		1–12	5 ± 3	14		0–5	1.6 ± 1.6	23
IL-4	CSF	0–20	8 ± 7	8	<0.05	0–290	38 ± 77	16	<0.01	ND	ND	ND
	PBL	0–8	1.5 ± 2.8	8		0–8	2.6 ± 2.4	16		0–4	1.1 ± 1.3	23
IL-6	CSF	0–10	2 ± 4	9	NS	0–13	5 ± 5	9	NS	ND	ND	ND
	PBL	0–13	5 ± 4	9		1–5	2.3 ± 1.4	9		0–3	0.7 ± 1	21
IL-10	CSF	0–15	7 ± 7	6	NS	0–60	15 ± 30	9	NS	ND	ND	ND
	PBL	0–8	3 ± 2	6		1–12	4 ± 4	9		0–5	1.7 ± 1.6	22
TNF-α	CSF	0–33	7 ± 11	8	NS	0–12	5 ± 5	11	NS	ND	ND	ND
	PBL	0–7	2.8 ± 2.6	8		0–20	1.2 ± 0.8	11		0–5	1.5 ± 1.6	22
LT	CSF	0–15	3 ± 5	7	NS	0–10	1.9 ± 4	8	NS	ND	ND	ND
	PBL	0–4	1.7 ± 1.8	7		0–6	1.3 ± 2	8		0–6	1.1 ± 1.7	20
Perforin	CSF	3–25	11 ± 11	6	NS	3–20	10 ± 6	10	<0.01	ND	ND	ND
	PBL	0–8	5 ± 3	6		1–6	3 ± 4	10		0–5	1.5 ± 1.7	22

[a] Comparing data for acute-phase patients with those of convalescent-phase patients, the P value was <0.05 for IFN-γ and not significant for all other cytokines. Comparing blood data for acute-phase patients with those of healthy subjects, P values were <0.05 for IFN-γ, TGF-β, and perforin, <0.005 for IL-6, and not significant for all other cytokines. Comparing blood data for convalescent-phase patients with those of healthy subjects, P values were <0.05 for IL-4, IL-10, and perforin, <0.005 for IL-6, <0.0005 for TGF-β, and not significant for the other cytokines.

[b] Acute-phase patients were patients 1 to 7 days from onset of disease. Convalescent-phase patients were patients >7 days from onset of disease.

[c] P for CSF value compared with PBL value.

[d] NS, not significant.

[e] ND, not done.

simplex type II etiology had the highest numbers of TGF-β and IL-4 mRNA-expressing cells in the convalescent phase. The groups were too small for statistical comparisons. No association was found between any cytokine under study and clinical signs (pure meningitis or meningoencephalitis) or CSF findings.

There were no correlations between the numbers of cytokine mRNA-expressing cells when we compared one cytokine with the others or blood with CSF data. The only exceptions were the observations of a positive correlation between IL-4 and TGF-β in CSF ($r = 0.5$, $P < 0.02$) and a negative correlation for perforin mRNA-expressing cells in blood versus those in CSF ($r = -0.6$, $P < 0.03$), but the marginal significance of these correlations could occur by chance, considering the multitude of comparisons performed.

DISCUSSION

Recent studies have emphasized the importance of cytokines in modulating immune responses and controlling the intensity

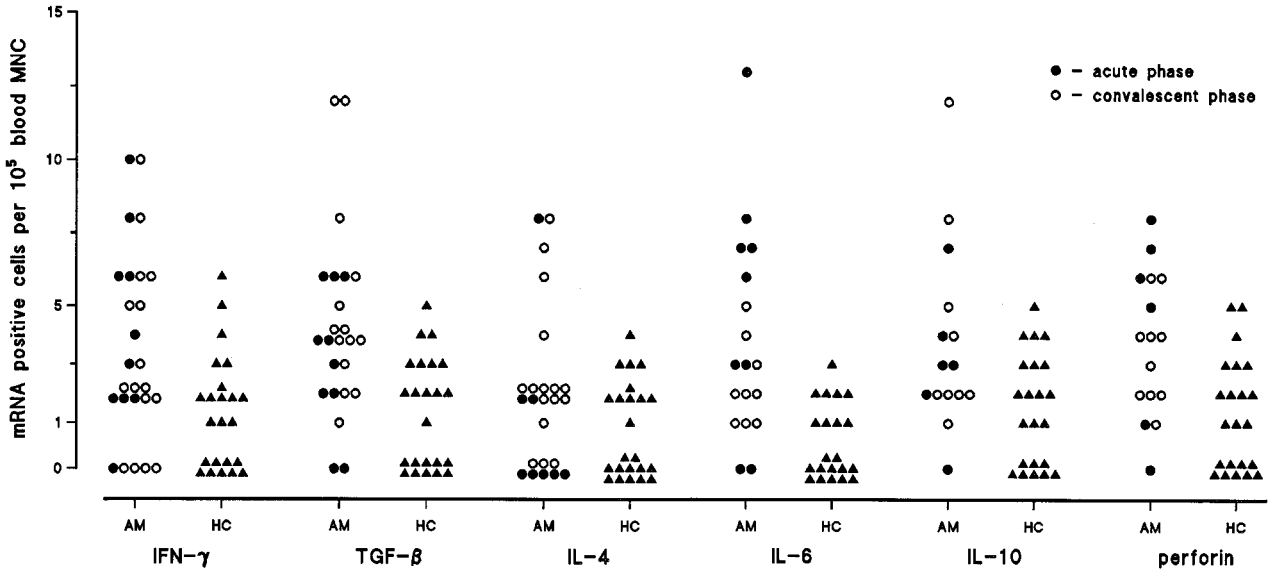

FIG. 1. Numbers of IFN-γ, TGF-β, IL-4, IL-6, IL-10, and perforin mRNA-expressing MNC in peripheral blood of AM patients and healthy controls (HC).

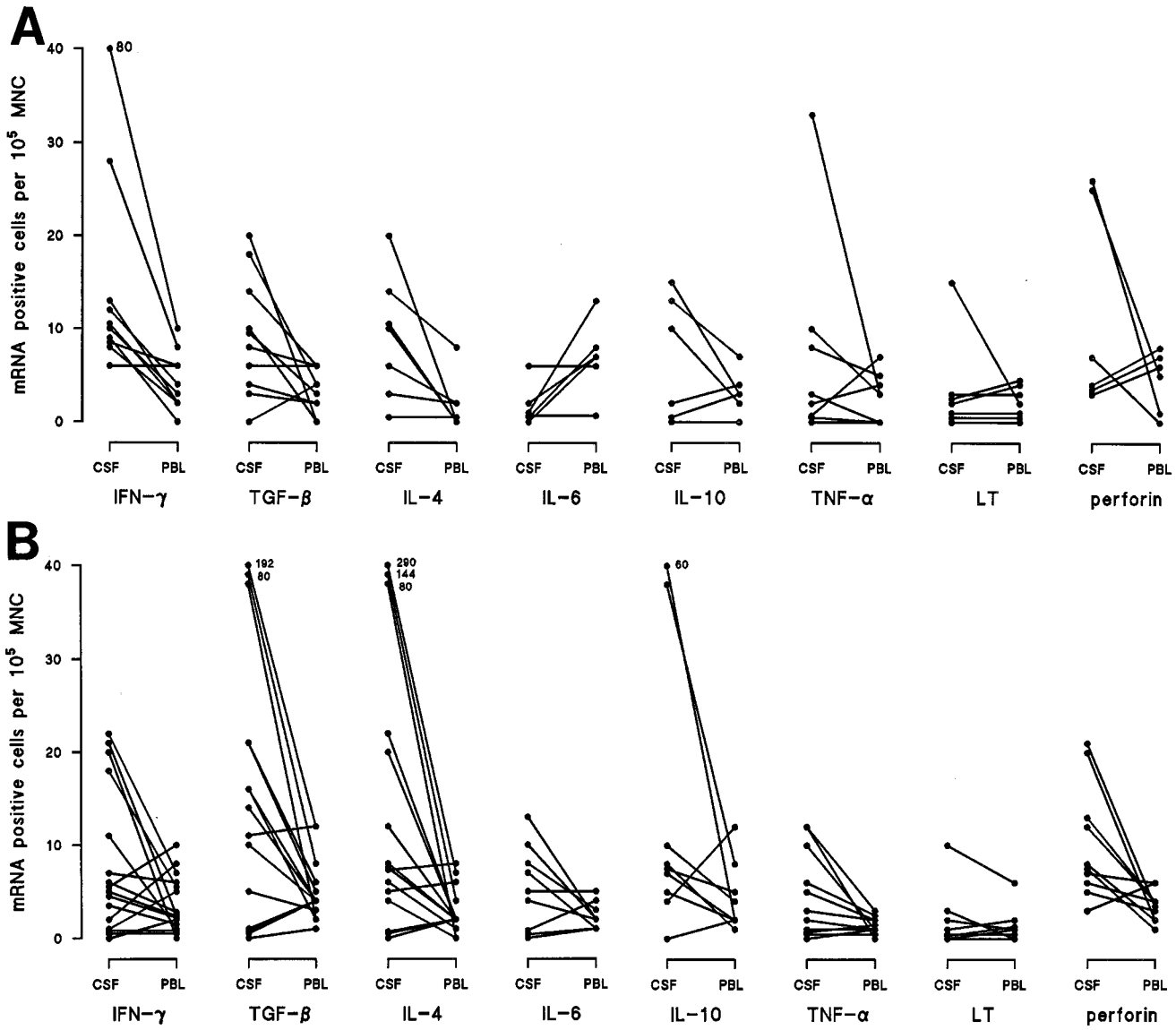

FIG. 2. Numbers of IFN-γ, TGF-β, IL-4, IL-6, IL-10, TNF-α, LT, and perforin mRNA-expressing MNC in CSF and blood (PBL) of AM patients examined during the acute (A) and convalescent (B) phases.

of inflammation. However, studies of cytokines have focused mainly on the determination of cytokine levels in body fluids. Because of their autocrine and paracrine actions, short half-lives, and high affinity to nearby receptors, reported levels of circulating cytokines have been mostly low or undetectable, and results from different groups have often been contradictory. The presently used principle for the detection of cytokines by ISH and subsequent enumeration of cytokine mRNA-expressing cells enabled us to study the involvement of IFN-γ, IL-4, IL-6, IL-10, TNF-α, LT, TGF-β, and perforin in the blood and CSF of the AM patients.

Elevated levels of cells encoding for IFN-γ were found in the blood, and these levels were further enriched in the CSF compartment in acute-phase AM. This finding is in agreement with a previous report, where high CSF levels of IFN-γ were detected in the acute but not the convalescent phase in children with AM (23). IFN-γ exhibits potent antiviral activity and might thus play an important role in the development of antiviral immunity and in viral clearance (30).

IL-4, IL-6, and IL-10 mRNA-expressing cells were detected at higher numbers in the CSF of AM patients in the convalescent phase than in those in the acute phase. Furthermore, the numbers of IL-4, IL-6, and IL-10 mRNA-expressing blood MNC were higher in AM patients examined during convalescence than in healthy controls. IL-4, IL-6, and IL-10 are produced by Th2 cells and have important regulatory effects on B-cell responses. Local production of antibodies in the CSF compartment is a characteristic finding in AM and can persist for years after uneventful recovery (10, 18). In this context, our observation of upregulation of Th2-like cytokines during convalescence from AM is not unexpected and suggests that these molecules might be important in supporting persistent B-cell activation in AM.

We found no evidence for upregulation of TNF-α or LT in

the blood or CSF in AM patients. Although TNF-α exerts antiviral activity (30), it has a major impact in the pathogenesis of bacterial meningitis and sepsis (9, 15). TNF-α is thought to induce blood-brain barrier injury and brain edema (33), and elevated levels have been reported in the CSF of bacterial meningitis patients, while viral meningitis was associated with low or undetectable levels (9, 17, 22). Our observation is in accordance with the view that viral infection differs in cytokine profiles involved in immune response from bacterial or parasitic infection (1). Determination of TNF-α production may hold a potential for differential diagnosis between the viral and bacterial etiologies of disease.

Perforin, which is a specific marker of functionally active cytotoxic lymphocytes (7), was also found to be elevated in the blood and CSF in both the acute and convalescent phases of AM. The functions of perforin in AM are not known, but it may play a role in specific cytotoxicity.

Interestingly, we found higher numbers of TGF-β mRNA-expressing MNC in CSF during the convalescent phase of AM than during the acute phase and higher numbers of TGF-β mRNA-expressing cells in blood from AM patients than in that from healthy controls. TGF-β is an immunosuppressive cytokine that downregulates both B- and T-cell immunity and promotes tissue repair (5, 32). TGF-β has also been shown to exert antiviral activity in vitro through its capacity to suppress human immunodeficiency virus replication in monocyte-macrophage cell lines (27). A beneficial role of TGF-β has been reported for mice infected with lymphocytic choriomeningitis virus (6) and for immune-mediated Borna disease virus-induced encephalomyelitis (35). TGF-β was also suggested to have a beneficial role in experimental (16, 28) and human autoimmune diseases (20, 21). Our results with AM similarly point to beneficial effects of TGF-β in modulating inflammation.

In conclusion, we have shown that some of the hematopoietins (IL-4, IL-6, IL-10), one of the interferons (IFN-γ), perforin, and TGF-β are upregulated in patients with AM. In contrast, cytokines of the TNF family (TNF-α and LT) previously found to be associated with bacterial meningitis are unaffected in AM. The upregulation of cytokines in AM was detectable systemically, and a further increment was found in CSF.

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