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Predominance of Vγ9/Vδ2 T Lymphocytes in the Cerebrospinal Fluid of Children with Tuberculous Meningitis: Reversal after Chemotherapy

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Abstract

Background: We analyzed the γδ T cell composition and responses in the peripheral blood and cerebrospinal fluid (CSF) of children affected by tuberculous meningitis (TBM) and in control children.

Materials and Methods: Peripheral blood and CSF samples were stimulated with different phosphoantigens and IL-2, and expansion of Vγ9/Vδ2 T cells assessed by FACS analysis. Vγ9/Vδ2 lines were obtained by culturing CSF or peripheral blood mononuclear cells (PBMC) in vitro with phosphoantigens and IL-2 for 2 months, and tested for proliferation and cytokine production in response to phosphoantigens. Vδ2(D)Jδ junctional sequence length was assessed by PCR.

Results: The repertoire of γδ T cells from the CSF of TBM patients was characterized by the predominance of Vγ9/Vδ2 T lymphocytes, which accounted for >80% of γδ T cells. Vγ9/Vδ2 cells from the CSF of TBM children responded to different synthetic and natural (mycobacterial) phosphoantigens and produced discrete amounts of IFN-γ and TNF-α. The in vitro expansion of Vγ9/Vδ2 T cells from CSF and peripheral blood of TBM patients prominently decreased following chemotherapy, and similarly, the proportion of ex vivo unstimulated Vγ9/Vδ2 T cells in CSF of TBM patients decreased to levels detected in the CSF of control subjects. Vδ2 CDR3 TCR analysis showed that the remaining Vδ2 cells in the CSF of TBM patients were still polyclonal.

Conclusions: These findings are consistent with an involvement of Vγ9/Vδ2 T cells in TBM.

Introduction

Tuberculosis (TB) remains the leading group of infectious diseases in numerous developing countries and is on the rise in several European nations and in the United States (1). Among individuals infected with *Mycobacterium tuberculosis* (MTB), the ethiologic agent of human TB, only a small number develop post-primary disease in adult age. Development of primary TB and the most severe form represented by TB meningitis (TBM) is inversely related to early childhood age (1). Acquired immunity against

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TB depends on the development of antigen-specific αβ T cells and their interaction with macrophages (2,3). However, circumstantial evidence suggests that γδ T cells may play an important role in the immune response to MTB. γδ T cell–deficient mice suffer a more severe form of TB and fail to control the infection (4; see ref. 5). In humans, γδ T cells contain the highest frequency of MTB-reactive T cells in the peripheral blood (6,7), and the major γδ T cell subset in the periphery (namely the one expressing Vγ9 and Vδ2) proliferates vigorously in vitro to various antigenic preparations of MTB (6,8–10). Several MTB–γδ T cell antigens have been chemically defined and include nonproteic and/or nucleotide molecules containing critical phosphate moieties (11–13).

The precise role played by Vγ9/Vδ2 T cells in human TB is not yet known. Previous studies reported either an increase (14–16) or no change (17,18) in γδ T cells in the peripheral blood of TB patients. Moreover, a dramatic reduction in Vγ9/Vδ2 T cells was found in TB patients, which were refractory to in vitro stimulation by MTB antigens (19). Contrasting data have also been obtained from studying γδ T cells at the site of the disease, showing that γδ cells accumulate in TB lymphoadenitis (20), are not increased in TB granulomas (21), and are strongly decreased in bronchoalveolar lavage of patients affected by pulmonary TB (19). These conflicting results may be a consequence of analyzing γδ T cells from patients affected by different clinical forms of TB or at different stages of disease progression.

In this report, we analyzed the γδ T cell responses in the peripheral blood and in cerebrospinal fluid (CSF) of children affected by TBM, which is a severe form of extrapulmonary TB associated with high mortality. Early diagnosis of TBM in children is life saving but difficult because symptoms are nonspecific. Examination of the CSF may reveal lymphocytosis together with high protein and low glucose concentrations, but these characteristics may also be associated with other diseases such as viral meningitis and purulent meningitis. Delay in diagnosis of TBM is directly related to a poor prognosis, whereas early treatment permits recovery without neurological sequelae. Furthermore, TBM in children is usually due to primary infection (22,23) and represents an important condition for studying γδ cells both in the peripheral blood and at the site of disease soon after infection and following chemotherapy.

Materials and Methods

Patient Population

Peripheral blood and CSF samples were obtained from six children affected by TBM (4 boys, 2 girls; 5.7 ± 4.3 years of age, range 1–12 years) from Children's Hospital G. Di Cristina, Palermo. Diagnosis of TBM (see Table 1) was established by the presence of clinical symptoms, clinical history, computed tomography (CT) scanning, CSF examination, and symptomatic improvement following antituberculous chemotherapy. MTB was detected by polymerase chain reaction (PCR) in CSF from four patients and two of them also had a positive MTB culture. Three out of the six patients included in the study had a positive PPD skin test. Tuberculin (PPD) skin tests were considered positive when the diameter was up to 5 mm at 72 hr of injection of 1 U of PPD (Statens Seruminstitut, Copenhagen, Denmark). Seven children (4 boys, 3 girls; 6 ± 3.5 years of age, range 2–12 years) affected by other, noninflammatory neurological diseases (Table 1) were used as controls, and were also recruited from Children's Hospital G. Di Cristina. Informed consent was obtained for each patient and control subject. None of the TB patients or controls had evidence of HIV infection, nor was any patient being treated with steroids or antitubercular drugs at the time of first sampling. The basic characteristics of subjects used in this study are shown in Table 1.

Purification of Mycobacterial TUBAg1

TUBAg1 was obtained from *Mycobacterium fortuitum*–secreted antigens. Briefly, 2.5 liters of *M. fortuitum* biovar *fortuitum* was grown for 6 weeks as velum on Sauton's medium. Culture medium was collected, filtered, and partitioned twice between chloroform and water. The water phase was concentrated, loaded onto DEAE, and eluted with increasing concentrations of ammonium acetate. The 0.2 M salt fraction was collected, dried three times, and separated on high-performance liquid chromatography (HPLC) C18 in 0.1 M ammonium acetate. The fractions were collected and tested for bioactivity for the G12 γδ T cell clone as described elsewhere (24). The first active peak was eluted after 1.3 Vo was collected, dried, and reseparated by mobile-phase ion pair HPLC (MPIC-HPLC), using a modification of Di Pierro's method, as already described (25). TUBAg1 was further characterized by its Rt upon high-performance anion-exchange chromatog-

Table 1. Baseline characteristics of TBM patients and control children

Patient	PPD Status	MTB detected by PCR	MTB detected by CSF Culture	MTB detected by CSF AFB Smear	Symptomatic Improvement following Therapy
1	Negative	+	+	+	+
2	Negative	+	+	+	+
3	Negative	+	−	−	+
4	Positive	+	−	−	+
5	Positive	−	−	−	+
6	Positive	−	−	−	+

Controls	Diagnosis
7	Congenital hydrocephalus
8	Congenital hydrocephalus
9	Glioma
10	Idiopathic epilepsy
11	No neurological disease
12	Kawasaki disease with meningitis
13	No neurological disease

raphy (HPAEC) and its sensitivity to treatment by alkaline phosphatase (data not shown).

Purified TUBAg1 stock concentration was estimated at around 1 μM from HPAEC conductimetric quantification using an already described method (25). Bioactivity of purified TUBAg1 was titrated as usual (24) and this material was used at a 1:1000 dilution, giving 1 nM final concentration in assays.

Primary Expansion of Vγ9/Vδ2 T Cells

Peripheral blood mononuclear cells (PBMC) or CSF cells were isolated by centrifugation on Ficoll-Hypaque and cultured at 5×10^5 cells/0.5 ml in RPMI 1640 supplemented with 10% heat-inactivated human antibody (AB) serum, 2 mM L-glutamine, 20 mM Hepes, and 10 U/ml penicillin/streptomycin. Cells were cultured with the following phosphoantigens: TUBAg1 (1:1000 v/v final concentration, which corresponds to 1 nM final concentration), ribose-1-P (Rib-1-P; Sigma, St. Louis, MO; 0.5 mM final concentration), xylose-1-P (Xyl-1-P; Sigma; 0.5 mM final concentration), dimethylallylpyrophosphate (DMAPP; Sigma; 0.5 mM final concentration), and isopentenylpyrophosphate (IPP; Sigma; 0.5 mM final concentration). Phosphoantigen concentrations were determined according to Burk et al. (26). After 72 hr, cultures were supplemented with 0.5 ml medium containing 20 U/ml rhIL-2 and every 72 hr, 0.5 ml medium was replaced with 0.5 ml fresh medium containing 20 U/ml rhIL-2 (27). After 14 days, cells were washed three times in medium and expansion of Vγ9/Vδ2 T cells was assessed by FACS as described above. The absolute number of Vδ2 T cells in each culture was calculated according to the following formula:

$$\frac{\%\ V\delta2^+ \text{ cells in each culture} \times \text{total cell count}}{100}$$

The Vδ2 expansion factor (EF) was then calculated by dividing the absolute number of Vδ2 cells in specifically stimulated cultures by the absolute number of Vδ2 cells cultured in the absence of any antigen.

Preparation of Vγ9/Vδ2 T Cell Lines

PBMC or CSF cells were cultured with TUBAg1 as indicated above. After 14 days, cells were washed three times in medium and restimulated

at 10^6/ml with TUBAg1 and irradiated (3000 rads from a Caesium source) allogeneic PBMC (10^6/ml). Every 72 hr, 20 U/ml rhIL-2 was added to the cultures (28). Cultures were harvested 2 months after the initial stimulation and analyzed for cellular composition by FACS analysis. The cell lines used in these studies were 92–98% Vγ9/Vδ2 positive.

Proliferation Assay

This assay was performed according to the protocol of Sireci et al. (28), using 2.5×10^4 responder cell lines per well and 10^5 irradiated allogeneic PBMC. The following antigens were used to assess proliferation, at the stated final concentrations: Rib-1-P (0.5 mM), Xyl-1-P (0.5 mM), DMAPP (0.5 mM), IPP (0.5 mM), TUBAg (1:1000 v/v), *M. tuberculosis* strain H37Ra (10 μg/ml), phytohemagglutinen (PHA) (1 μg/ml), and irradiated (12,000 rads) Daudi Burkitt lymphoma cells (10^5/well). After 48 hr, 1 μCi/well of ^{3}H-thymidine (Amersham) was added and the cultures were harvested after an additional 18–24 hr. Results are expressed as mean counts per minute (cpm) ± standard deviation (SD). The SD was always <10% of the mean.

Lymphokine ELISA Assay

Cell lines were cultured with PHA or TUBAg for 24 hr, as described previously. Culture supernatants were harvested and tested for interleukin-4 (IL-4), interferon γ (IFN-γ), or tumor necrosis factor α (TNF-α) by two–monoclonal antibody sandwich ELISA with commercially available kits (R&D, Minneapolis, MN), according to the manufacturer's recommendations.

Monoclonal Antibodies and Flow Cytometry

The following monoclonal antibodies (MAbs) specific for human surface antigens were used: anti-CD3-Quantum Red (UCHT-1, Sigma), anti-TCR γδ-PE (B1.1, Pharmingen), anti-TCR Vδ2-FITC (IMMU389), and anti-Vγ9 (IMMU360), both from Immunotech (Marseille, France, through Delta Biologicals, Rome, Italy). PBMC or CSF cells [10^6 in 100 μl phosphate-buffered saline (PBS) with 1% heat-inactivated fetal calf serum (FCS) and 0.02% Na-azide] were incubated at 4°C for 30 min with the anti-Vγ9 MAb, followed by phycoerythrin (PE)-conjugated secondary MAb, and then Quantum Red–conjugated anti-CD3 and FITC-conjugated anti-Vδ2 MAbs were added simultaneously. Alternatively, the cells were simultaneously labeled with Quantum Red–conjugated anti-CD3, PE-conjugated anti-TCRγδ, and FITC-conjugated anti-Vδ2 MAbs. Fluorochrome-conjugated, isotype-matched MAbs were used as negative controls. After washing, the cells were suspended in PBS with 1% FCS and analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), using forward scatter/side scatter gating to select the lymphocyte population for analysis.

PCR Analysis of Vδ2(D)Jδ Junctional Sequences

This analysis was carried out according to the protocol of Poccia et al. (29). Total RNA from Ficoll-Hypaque-purified CSF cells was prepared by guanidinium thiocyanate-phenol-chloroform extraction (30) with 10 μg of P815 RNA carrier. Single-stranded DNA was synthesized by means of a cDNA synthesis kit (Perkin Elmer, Rome, Italy) and an aliquot of the reaction, corresponding to an equivalent of $4–5 \times 10^3$ $\gamma\delta^+$ $CD3^+$ T cells, was subjected to PCR amplification in a total volume of 50 μl, using the Vδ2 5′ primer (5′-AACCTGGCTGTACTTAAGATAC-3′) and the Cδ antisense primer (5′-CGGATGGTTTGGTATGAGGC-3′) in saturating conditions as described in Poccia et al. (29). Two microliters of the Vδ2-Cδ PCR reaction was subjected to a cycle of elongation (runoff) in 10 μl of reaction buffer containing 3 mM $MgCl_2$ (final concentration) and 0.1 μM of one of the fluorescent Jδ-specific antisense primers (Jδ1 = 5′-XTTCCACAGTCACACGGGTTC-3′; Jδ2 = 5′-XTTCCACGATGAGTTGTGTTCC-3′; Jδ3 = 5′-XCACGAAGAGTTTGATGCCAG-3′, in which X designates the dye bound to the primer through the amino-2 group). Separation and analysis of the runoff products were performed as described previously (31) using an Applied Biosystem 373A DNA sequencer. Dye-labeled size standards were included in each electrophoresis run. This allows for precise determination of the size of each Vδ2/Jδ runoff DNA fragment. CDR3, defined in T cell receptors (TCRs) from amino acid residue 95 (located in the V region gene) to amino acid residue 106 (located in the J gene) can thus be easily determined. For the Vδ2 gene segment–specific oligonucleotide, this corresponds to a distance of 66 nt upstream from residue 95, and for the Jδ gene segment–specific oligonucleotides to a distance of 30 nt (for Jδ1 and Jδ2) or 27 nt (for Jδ3) downstream from residue 106. The fluorescence intensity is expressed in arbitrary units

Table 2. Comparison of absolute numbers of CD3, γδ, and Vγ9/Vδ2 cells in peripheral blood and CSF of children affected by TBM and control subjects

Source	Absolute Number/μl ± S.E.		
	$CD3^+$ Cells	Total $\gamma\delta^+$ Cells	$V\delta2^+$ Cells
TBM patients			
CSF	167 ± 24*	6.7 ± 1.9* (4)[a]	5.9 ± 0.9* (88)[b]
PBMC	1580 ± 268	78 ± 19 (4.9)[a]	56 ± 14 (72)[b]
Controls			
CSF	20 ± 5*	0.7 ± 0.3* (3.4)[a]	0.2 ± 0.1* (29)[b]
PBMC	1625 ± 418	84 ± 26 (5.2)[a]	60 ± 14 (71)[b]

PBMC and CSF samples were analyzed using two- and three-color flow cytometry as described in Materials and Methods. The differences in CSF values between TBM patients and control children were all significant (*$p < 0.001$).

[a]The percentage of total $\gamma\delta^+$ cells, within $CD3^+$ cells, is shown in parentheses.

[b]The percentage of $V\delta2^+$ T cells, within total $\gamma\delta^+$ cells, is shown in parentheses.

through Immunoscope software. Thus, for a given Vδ PCR reaction, it is possible to quantitate the percentage of representation of each DNA fragment size.

Statistics

The Mann-Whitney *U* test was used for statistical analysis. Values of $p < 0.05$ were chosen for rejection of the null hypothesis.

Results

Distribution of Vγ9/Vδ2 T Lymphocytes in Peripheral Blood and CSF of Children with TBM

Two- and three-color FACS analysis was carried out to assess the percentage of total γδ and Vγ9/Vδ2 T lymphocytes in the peripheral blood and CSF of children with TBM, as well as in children affected by noninflammatory neurological diseases (Table 2). In control subjects the percentage of total γδ cells was greater in PBMC than in CSF (4.1 ± 1.6% in PBMC and 3.4 ± 0.9% in CSF), whereas in TBM patients the percentage of total γδ cells was greater in CSF than in PBMC (3.6 ± 1.2% in PBMC and 4 ± 1.1% in CSF). Vδ2 T lymphocytes represented less than 30 of the γδ T cells in the CSF of control children but accounted for >80% of γδ T cells in the CSF of TBM children.

Because of the increased number of T lymphocytes found in the CSF of TBM children (Table 2), the absolute number of total γδ and Vδ2 T lymphocytes increased 9- and 30-fold, respectively, in the CSF of TBM patients compared to the number detected in the CSF of control children (Table 2). Further analysis confirmed that all the Vδ2 cells co-expressed the Vγ9 chain (data not shown). This means that the CSF of children affected by TBM is characterized by accumulation of Vγ9/Vδ2 T lymphocytes.

Vγ9/Vδ2 T Lymphocyte Response to Mycobacterial and Synthetic Antigens

To evaluate the response of Vγ9/Vδ2 T lymphocytes to stimulation by mycobacterial (TUBAg) and synthetic phosphoantigens, PBMC and CSF cells from children affected by TBM and from control subjects were cultured with different molecules known to selectively stimulate Vγ9/Vδ2 T cells (Table 3). Although there was great individual variation in the expansion of Vγ9/Vδ2 cells, a pronounced expansion of Vγ9/Vδ2 cells was observed in PBMC and CSF cells from TB patients, with CSF cells usually showing more expansion than PBMC. Furthermore, a certain hierarchy was found in the expansion of Vγ9/Vδ2 cells toward the different antigens, with the highest E.F. obtained with TUBAg, the lowest E.F. with Rib-1-P and Xyl-1-P, and intermediate E.F. with IPP and DMAPP. In contrast, in control subjects the Vγ9/Vδ2 population expanded at a

Table 3. Vγ9/Vδ2 expansion factor to several different phosphoantigens in vitro

Group	Source	Vγ9/Vδ2 EF after In Vitro Culture with				
		Rib-1-P	Xyl-1-P	IPP	DMAPP	TUBAg
TBM patients						
1	CSF	14	13	24	28	35
	PBMC	7	2	5	6	8
2	CSF	38	30	61	54	68
	PBMC	22	12	30	28	40
3	CSF	15	10	40	30	64
	PBMC	10	8	32	26	44
4	CSF	17	21	44	49	79
	PBMC	12	20	45	53	72
5	CSF	35	27	60	48	90
	PBMC	20	18	34	40	54
6	CSF	15	17	78	80	143
	PBMC	7	12	12	18	32
Controls						
7	CSF	1	3	1	7	5
	PBMC	1	1	2	4	5
8	CSF	3	1	2	10	5
	PBMC	1	1	6	4	7
9	CSF	5	2	1	6	8
	PBMC	6	7	ND	ND	5
10	CSF	3	7	7	4	2
	PBMC	1	4	5	5	12
11	CSF	2	3	1	9	11
	PBMC	2	4	4	7	7
12	CSF	5	6	8	8	11
	PBMC	6	4	7	9	9
13	CSF	1	4	5	3	5
	PBMC	3	7	7	9	14

Paired samples of PBMC and CSF cells from children affected by TBM were stimulated in vitro with the indicated phosphoantigens, and the Vγ9/Vδ2 expansion factor (EF) was determined as described in Materials and Methods. ND, not determined.

very low frequency in both CSF cells and PBMC, compared to the rate in TBM children.

Recognition of Phosphoantigens by Vγ9/Vδ2 T Cell Lines Isolated from CSF of TBM Patients

Vγ9/Vδ2 T cell lines were derived from PBMC and CSF cells of each TBM patient after in vitro culture with TUBAg for 2 months in vitro. These lines contained only 1–4% of αβ T lymphocytes and 2–3% of $CD16^+$ cells. Proliferation in vitro occurred in response to several known stimulants of Vγ9/Vδ2 T lymphocytes using lines derived from PBMC and CSF cells of two patients (Fig. 1). TUBAg-derived T cell lines showed a good proliferative response to different phosphoantigens and similar patterns of reactivity were detected with other cell lines derived from PBMC and CSF. Furthermore, all cell lines strongly proliferated in response to stimulants known to activate Vγ9/Vδ2 T cells, such as *M. tuberculosis* H37Ra and Daudi Burkitt lymphoma cells. No proliferation occurred in unstimulated or PPD-stimulated cultures (Fig. 2).

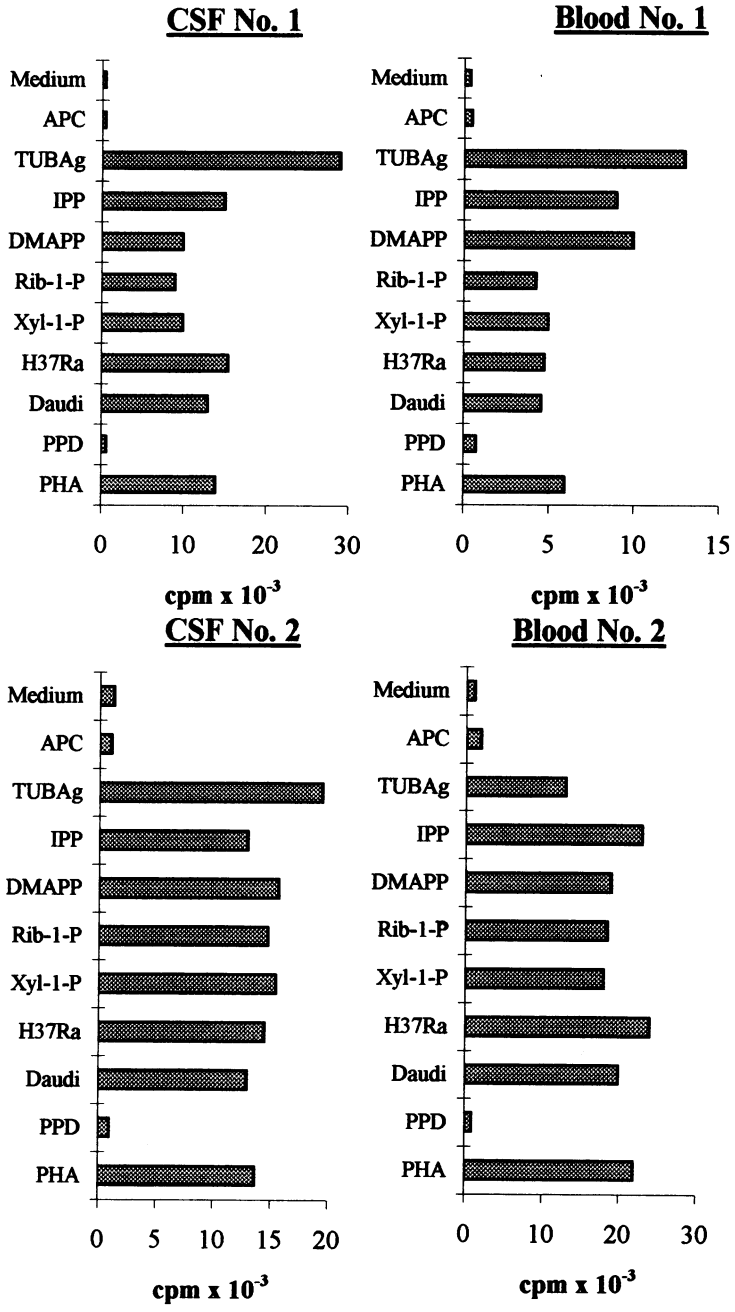

Fig. 1. Broad reactivity of CSF-derived Vγ9/Vδ2 T cell lines to different phosphoantigens. Vγ9/Vδ2 T cell lines were isolated by stimulation of PBMC or CSF cells with TUBAg. The lines (2.5×10^4/well) were cultured with different stimulants in the presence of irradiated allogeneic PBMC (10^5/well) as APC, as described in Materials and Methods. Results are shown with lines derived from paired PBMC and CSF samples of two patients affected by TBM.

Cytokine Production by Vγ9/Vδ2 T Cell Lines Isolated from CSF of TB Patients

Culture supernatants obtained after stimulation with PHA or TUBAg1 showed substantial variation among T cell lines, but discrete amounts of IFN-γ and TNF-α were produced by Vγ9/Vδ2 T cell lines from each TB patient (Table 4). A similar pattern of cytokine production was observed upon stimulation of the cell lines with TUBAg. However, we found no significant correlation between phosphoantigen-induced proliferation and cytokine production; for instance, bulk Vγ9/Vδ2 T cell line from patient 3 produced about half the amount of IFN-γ and TNF-α compared to bulk T cell line from patient 2 (Table 4) after TUBAg stimulation, but Vγ9/Vδ2 T cells from these patients expanded equally upon primary in vitro TUBAg stimulation (Table 3). A similar lack of correlation was found between proliferation and cytokine production by Vγ9/Vδ2 T cell lines. In no case was IL-4 detected in supernatants of Vγ9/Vδ2 T cell lines, thus indicating their Th1-type pattern of cytokine production.

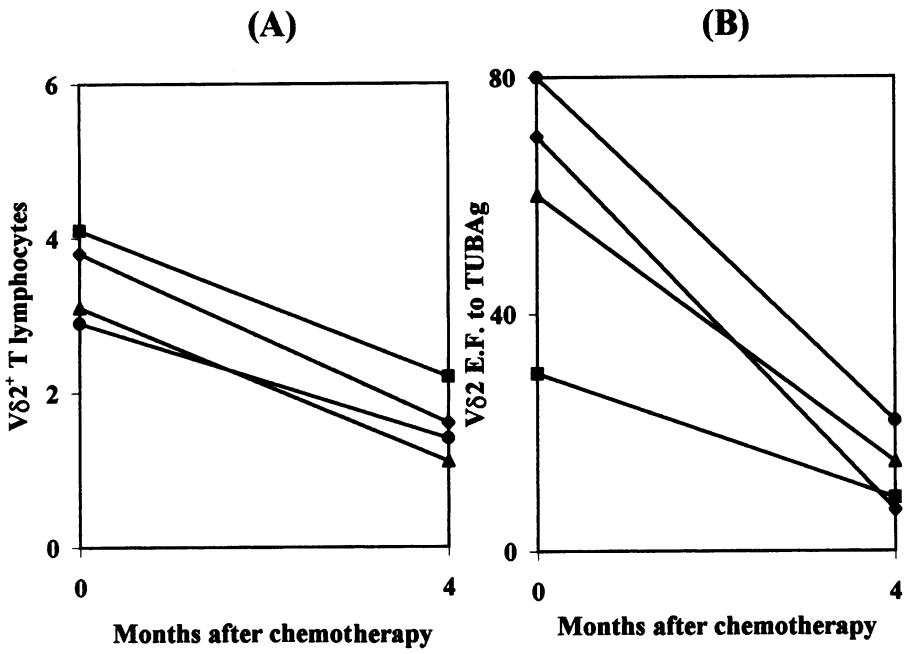

Fig. 2. Comparison of the proportion and expansion of Vγ9/Vδ2 cells in the CSF of children affected by TBM before and during chemotherapy. (A) percentage of Vγ9/Vδ2 cells, within $CD3^+$ cells; (B) Vδ2 E.F. in response to TUBAg.

Table 4. Cytokine production by Vγ9/Vδ2 T cells lines from CSF samples

Line from Patient	Stimulation	TNF-α (pg/ml)	IFN-γ (pg/ml)
1	PHA	210	900
	TUBAg	160	1400
2	PHA	250	1400
	TUBAg	240	1100
3	PHA	140	1100
	TUBAg	120	580
4	PHA	200	970
	TUBAg	210	840
5	PHA	200	950
	TUBAg	170	700
6	PHA	380	1600
	TUBAg	450	1800

Vγ9/Vδ2 T cell lines obtained from CSF cells of patients affected by TBM were stimulated in vitro with PHA or TUBAg and TNF-α and IFN-γ production assessed by ELISA as described in Materials and Methods.

Effect of Chemotherapy on Vγ9/Vδ2 T Cell Responses

The Vγ9/Vδ2 T cell responses were retested in four patients halfway (approximately 4 months) through chemotherapy. Figure 2A shows that chemotherapy caused a modification of the γδ subsets in CSF, and the percentage of $V\gamma9/V\delta2^+$ cells decreased nearly to that detected in CSF of control subjects. Parallel to this finding was the Vγ9/Vδ2 T cell response to TUBAg, which strongly decreased during drug treatment, both in CSF cells (Fig. 2B) and PBMC (data not shown).

Vδ2-Jδ Junctional Diversity in Vγ9/Vδ2 T Cells Isolated from CSF of TBM Patients before and after Chemotherapy

To assess whether the decrease in Vγ9/Vδ2 T cells observed in CSF after chemotherapy concerned some specific clones, we analyzed the CDR3 size distribution of Vδ2-Jδ1, Vδ2-Jδ2, and Vδ2-Jδ3 rearrangements of TUBAg1-stimulated CSF cells from four TBM children before and after chemotherapy. As Vδ2-Jδ2 were rare (data not shown), our analysis focused on Vδ2-Jδ1 and Vδ2-Jδ3 rearrangements. As shown in Figure 3, no significant difference was detected in the mean representation of each CDR3 fragment size among Vδ2-Jδ1 and Vδ2-Jδ3 rearrangements, indicating that the decrease in Vδ2 cells after chemotherapy does not involve either a particular CDR3 size rearrangement or a specific Jδ usage.

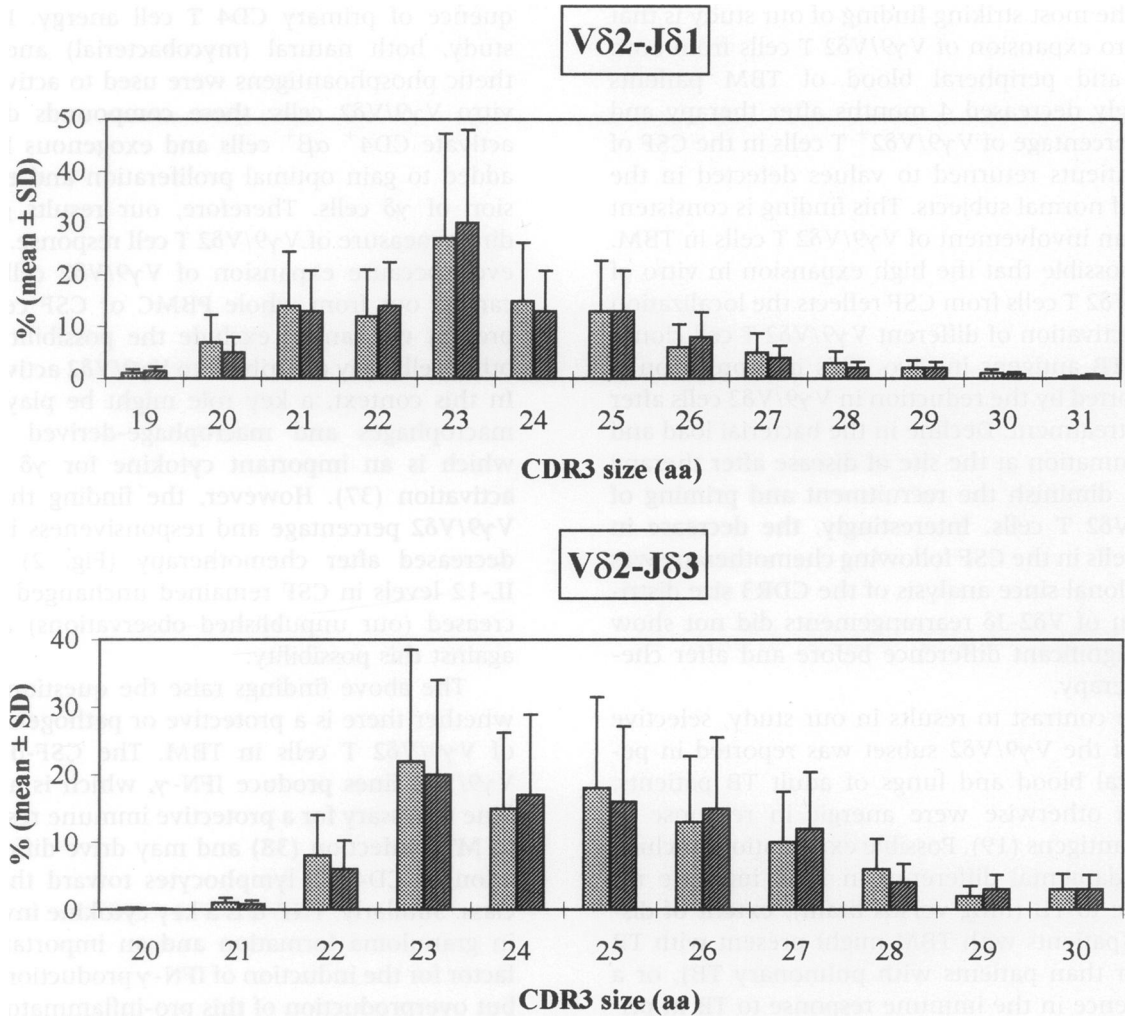

Fig. 3. Distribution of the Vδ2-Jδ1 (upper panel) and Vδ2-Jδ3 (lower panel) CDR3 size frequency in the CSF of TBM children before and after therapy. The percentage of representation of each size fragment of the CDR3 was calculated by the ratio of the intensity of each size fragment to the sum of all of them. CDR3 size is indicated as number of amino acids. Gray columns, before chemotherapy; hatched columns, after chemotherapy. Bars represent SD of the means.

Discussion

In this report we have shown that γδ T cells isolated from the CSF of children affected by TBM are characterized by a predominance of Vγ9/Vδ2 T lymphocytes. The Vγ9/Vδ2 subsets made up <30% of the γδ T cells in the CSF of normal individuals (Table 2 and ref. 32), but >80% of γδ T cells in the CSF of children with TBM. The absolute number of total γδ and Vγ9/Vδ2 T lymphocytes in the CSF of TBM patients increased 9- and 30-fold, respectively. This finding indicates compartmentalization of Vγ9/Vδ2 T lymphocytes at the site of disease.

Our study showed that Vγ9/Vδ2 cells from the CSF of TBM children responded to five different phosphoantigens to a similar or greater extent than those in the peripheral blood. In striking contrast, expansion of Vγ9/Vδ2 cells was very low in the CSF cells and PMBC from control children. To study this Vγ9/Vδ2 population in more detail, we evaluated the antigen reactivity of bulk T cell lines. The CSF-derived Vγ9/Vδ2 T cell lines proliferated not only in response to the natural (mycobacterial) phosphoantigen TUBAg but also to four synthetic phosphoantigens, *M. tuberculosis* H37Ra strain, and Daudi Burkitt lymphoma cells. Furthermore, after in vitro stimulation with PHA or TUBAg, the Vγ9/Vδ2 T cell lines produced discrete amounts of IFN-γ and TNF-α, but not IL-4.

The most striking finding of our study is that in vitro expansion of Vγ9/Vδ2 T cells from both CSF and peripheral blood of TBM patients strongly decreased 4 months after therapy and the percentage of Vγ9/Vδ2$^+$ T cells in the CSF of TB patients returned to values detected in the CSF of normal subjects. This finding is consistent with an involvement of Vγ9/Vδ2 T cells in TBM. It is possible that the high expansion in vitro of Vγ9/Vδ2 T cells from CSF reflects the localization and activation of different Vγ9/Vδ2 T cell clones by MTB antigens in vivo. This interpretation is supported by the reduction in Vγ9/Vδ2 cells after drug treatment. Decline in the bacterial load and inflammation at the site of disease after therapy could diminish the recruitment and priming of Vγ9/Vδ2 T cells. Interestingly, the decrease in Vδ2 cells in the CSF following chemotherapy was polyclonal since analysis of the CDR3 size distribution of Vδ2-Jδ rearrangements did not show any significant difference before and after chemotherapy.

In contrast to results in our study, selective loss of the Vγ9/Vδ2 subset was reported in peripheral blood and lungs of adult TB patients, which otherwise were anergic in response to MTB antigens (19). Possible explanations include a fundamental difference in local immune response to TB (lung versus brain), extent of disease (patients with TBM might present with TB earlier than patients with pulmonary TB), or a difference in the immune response to TB in primary disease versus reactivation. In fact, our study has been carried out using children affected by TBM and analysis has been performed before and after drug treatment. Furthermore, in the above-reported studies, the whole MTB has been used as antigen to stimulate γδ cells. This makes it difficult to dissect selective activation of γδ cells. In fact, it is known that for MTB to activate γδ cells, it also needs to activate CD4$^+$ αβ$^+$ cells to release IL-2, which is absolutely required for expansion of γδ cells (33). Therefore, increased or decreased expansion of γδ cells upon in vitro culture with MTB might reflect increased or decreased activation of αβ cells and IL-2 production. For instance, the apparent anergy of Vγ9 cells in HIV-infected individuals appears to be primarily due to deficiency of antigen-specific CD4 Th1 cells and is completely restored by addition of exogenous IL-2 (27). Because CD4 T cell anergy is a rather common phenomenon in patients with active pulmonary TB (34–36), the possibility remains that the observed Vγ9/Vδ2 anergy might be the consequence of primary CD4 T cell anergy. In our study, both natural (mycobacterial) and synthetic phosphoantigens were used to activate in vitro Vγ9/Vδ2 cells: these compounds do not activate CD4$^+$ αβ$^+$ cells and exogenous IL-2 is added to gain optimal proliferation and expansion of γδ cells. Therefore, our results give a direct measure of Vγ9/Vδ2 T cell response. However, because expansion of Vγ9/Vδ2 cells was carried out from whole PBMC or CSF cells, at present we cannot exclude the possibility that other cells may contribute to Vγ9/Vδ2 activation. In this context, a key role might be played by macrophages and macrophage-derived IL-12, which is an important cytokine for γδ T cell activation (37). However, the finding that the Vγ9/Vδ2 percentage and responsiveness in CSF decreased after chemotherapy (Fig. 2) while IL-12 levels in CSF remained unchanged or increased (our unpublished observations) argues against this possibility.

The above findings raise the question as to whether there is a protective or pathogenic role of Vγ9/Vδ2 T cells in TBM. The CSF-derived Vγ9/Vδ2 lines produce IFN-γ, which is a cytokine necessary for a protective immune response to MTB infection (38) and may drive differentiation of CD4$^+$ T lymphocytes toward the Th1 class. Similarly, TNF-α is a key cytokine involved in granuloma formation and an important cofactor for the induction of IFN-γ production (39), but overproduction of this pro-inflammatory cytokine might be crucial for the pathogenesis of meningitis.

The selective increase in Vγ9/Vδ2 cells in the CSF of TBM children indicates that they contribute to the immune response. While the decrease in Vγ9/Vδ2 cells during recovery could be interpreted as contributing to pathology, their clear type 1 phenotype also suggests a protective role.

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