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Short Communication

Cerebrospinal fluid T cells from multiple sclerosis patients recognize autologous Epstein-Barr virus–transformed B cells

Trygve Holmøy and Frode Vartdal

Institute of Immunology, Rikshospitalet University Hospital, Oslo, Norway

The association between multiple sclerosis and Epstein-Barr virus infection could involve Epstein-Barr virus–specific T cells, provided that these T cells get access to the intrathecal compartment. We report that CD4$^+$ T cells from the cerebrospinal fluid of six out of six multiple sclerosis patients, and four out of six patients with other neurological diseases, recognized autologous B cells transformed with Epstein-Barr virus. The cerebrospinal fluid T-cell responses were predominantly HLA-DR restricted. These T cells did not recognize B cells activated through stimulation of CD40 or the inducible autoantigen αB crystalline. These findings support that the immunological response to Epstein-Barr virus could contribute to the pathogenesis of multiple sclerosis. *Journal of NeuroVirology* (2004) **10,** 52–56.

Keywords: cerebrospinal fluid; Epstein-Barr virus; multiple sclerosis; T cells

Several lines of evidence suggest an etiological link between multiple sclerosis (MS) and Epstein-Barr virus (EBV) infection. Almost 100% of MS patients are EBV seropositive (Ascherio and Munch, 2000), and MS risk is related to increased titers of antibodies against EBV (Levin *et al*, 2003). Moreover, 85% of MS patients compared to 13% of control patients display antibody reactivity to the Epstein-Barr nuclear antigen (EBNA) in the cerebrospinal fluid (CSF) (Bray *et al*, 1992). An increased risk of MS is associated with a higher age at childhood infection (Casetta and Granieri, 2000). Further, a single subtype of EBV was found in members of MS clusters (Munch *et al*, 1998). This is compatible with the migration studies, which suggest that a putative environmental factor in MS pathogenesis could be a delayed exposure to a common infection (Kurtzke, 2000).

Although several studies have focused on the antibody response to EBV, little is known about T-cell immunity to EBV in MS. Because CD4$^+$ T lymphocytes orchestrate the immune attack, it is important to chart the CD4$^+$ T-cell response to EBV. Although EBV DNA has not been found in MS brains or CSF, T cells activated by EBV outside the central nervous system (CNS) might contribute to MS pathogenesis by cross-reacting with epitopes on CNS proteins (Wucherpfennig and Strominger, 1995). In line with this, a T-cell clone from an MS patient was recently demonstrated to recognize both a DRB1*1501-restricted myelin basic protein (MBP) peptide and a DRB5*0101-restricted EBV peptide (Lang *et al*, 2002).

Because T cells have to be activated to penetrate the blood-brain barrier (Hickey *et al*, 1991) and induce encephalitis in experimental autoimmune encephalomyelitis (EAE) (Zamvil *et al*, 1985), one would expect that activated EBV-specific T cells must be present within the CNS to be relevant in MS pathogenesis. Because T cells express high-affinity interleukin-2 (IL-2) receptors after antigen stimulation (Waldmann, 1986), activated T-cells can be selectively expanded with IL-2. We therefore generated T-cell lines from CSF and peripheral blood mononuclear cells (PBMCs) from five MS patients (MS1 to MS5 in this study), and single patients with amyotrophic lateral sclerosis (ALS), viral meningitis (ME), encephalitis (EN), Guillain-Barré syndrome (GB), tuberculous meningitis (TB), and cerebral vasculitis associated with rheumatoid arthritis (RA) by primary expansion with IL-2 (Holmøy *et al*, 2003) (Table 1). At the end of the cultivation, the cells

Address correspondence to Trygve Holmøy, Institute of Immunology, Rikshospitalet University Hospital, 0027 Oslo, Norway. E-mail: trygveho@labmed.uio.no

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Table 1 HLA-DR and -DQ genotypes of the MS and control patients

	HLA genotype	
Patients	*DRB1**	*DQB1**
MS1	1501	0602
MS2	0101, 13021	0501, 0604
MS3	0401, 0401	0302, 0303
MS4	1301, 1501	0602, 0603
MS5	0801, 1501	0402, 0602
MS6	13021, 1501	0604, 0602
Amyotrophic lateral sclerosis	0301, 0404	0201, 0301
Tuberculous meningitis	0101, 1301	0501, 0603
Guillain-Barré syndrome	1101, 1501	0301, 0602
Meningitis	0301, 0401	0201, 0302
Rheumatoid arthritis/vasculitis	090102	0303
Encephalitis	0301	0303

were phenotyped in a rosetting assay, using immunomagnetic beads coated with anti-CD4 and anti-CD8 (Dynal ASA, Oslo, Norway). The proportion of $CD4^+$ T cells in the IL-2–expanded T-cell lines exceeded 95%.

EBV-transformed lymphoblastoid B-cell lines (EBV-LCLs) elicit proliferation of EBV-specific $CD4^+$ T cells (Savoldo *et al*, 2002), and are therefore relevant when probing EBV-specific T cells. Thus, the proliferative response of T cells against an autologous EBV-LCL generated by infecting PBMCs with a supernatant from a B95.8 EBV-infected marmoset cell line were tested by incubation of 1.0×10^5 T cells with a 0.2×10^5 irradiated (80 Gy) EBV-LCL in triplicate cultures for 72 h, adding 1 μCi of [methyl-^{3}H]thymidine for the last 16 h. Responses were considered positive when the difference between counts per minute (cpm) of stimulated cells and cpm of T cells and the EBV-LCLs cultivated separately (Δcpm) was greater than 1000, and their ratio, named stimulatory index (SI), exceeded 3. Except for the ALS and GB patients, all IL-2–expanded T-cell lines from both blood and CSF responded to autologous EBV-LCL. The experiments were performed at least twice, and representative results are shown in Figure 1. In addition, 10 CSF T-cell lines from another MS patient (MS6) and 1 T-cell line from patient MS3 were expanded with the T-cell mitogen phytohemagglutinin (PHA) and IL-2. All but one of the PHA-expanded CSF T-cell lines from MS1 responded to autologous EBV-LCL. The median thymidine incorporation of the responding PHA-expanded CSF T-cell lines (Δcpm) were 22359 (range 3781 to 52547), and the median SI 42.0 (range 8.8 to 97.2).

To our knowledge, this is the first demonstration of EBV-LCL–reactive T-cells in the CSF. These CSF T-cell lines were derived from a limited number of precursor cells. Given that the frequency of $CD4^+$ T-cells among CSF cells in MS is about 70% (Cepok *et al*, 2001), each CSF T-cell line was derived from approximately 1400 to 5000 $CD4^+$ T cells. All but one of the

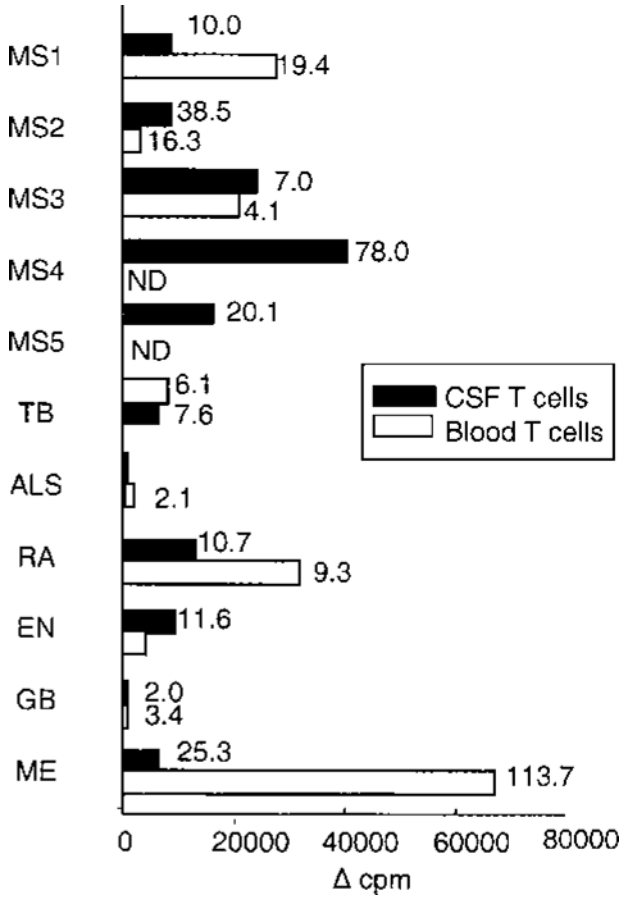

Figure 1 Proliferative responses of IL-2–expanded T-cell lines from CSF and peripheral blood stimulated with autologous EBV-LCLs. Results are given as Δcpm (cpm of stimulated wells after subtraction of cpm of T cells and an irradiated EBV-LCL cultivated separately), and stimulatory index are given at the end of each bar where the Δcpm exceeded 1000.

CSF T-cell lines from the MS patients turned out to respond to EBV-LCL. It can therefore be assumed that EBV-LCL–responsive T-cells occur quite frequently in the CSF, and probably outnumbers the frequency of MBP-specific T-cells found in the spinal cord of rats with MBP-induced EAE, which is calculated to be 1/8000 (Ishigami *et al*, 1998).

EBV-responsive T cells in the CSF is not exclusive for MS patients, as these specificities were also detected in the CSF T-cell repertoire of the TB, ME, RA, and EN patients. The absence of EBV-LCL–responsive T cells in the CSF of the ALS patient could be due to the low number of CSF cells obtained from this patient (total 600), or the lack of EBV-LCL–responsive T cells in the blood, which were probably the case in the GB patient. However, the finding of EBV-LCL–responsive T cells in the CSF of non-MS patients does not contradict T-cell responses to EBV as a pathogenetic factor in MS, because the environmental contribution to MS etiology is more likely to be a common infectious agent than a specific "MS-microbe" (Ebers *et al*, 1995).

The HLA restriction of T-cell responses to EBV-LCL were analyzed by incubating T cells and irradiated EBV-LCLs in the presence and absence of 20 μg/ml monoclonal antibodies to human leukocyte antigen (HLA) class I (cloneW6/32; IgG2a; American Tissue Culture Collection [ATCC], Manassas, VA, USA), HLA-DR (clone L243; IgG2a; ATCC), HLA-DQ (clone SPVL-3; IgG2a; gift from Dr. Hergen Spits, Netherlands Cancer Institute, Amsterdam, The Netherlands), HLA-DP (clone B7/21; IgG3; gift from I. Trowbridge, The Salk Institute, La Jolla, CA, USA); and a monoclonal IgG2a and IgG3 isotype control (Diatec, Oslo, Norway). In MS1, the proliferative response to the autologous EBV-LCL elicited in the CSF T-cell lines was blocked by antibodies to HLA-DR, whereas antibodies to other class II molecules or class I or isotype controls did not block the T-cell response (Figure 2A). Similar blocking results were obtained when CSF T-cells from MS1 were stimulated with EBV-LCLs of identical HLA-DR and -DQ (data not shown). The PBMC T-cell responses to autologous EBV-LCL were suppressed to a lesser extent than the corresponding CSF T-cell responses (Figure 2B). In MS4, MS5, and the TB patient, the anti-DR antibody suppressed the CSF T-cell responses to autologous EBV-LCL by approximately 40% to 75%.

As a positive control of their proliferative capacity, the T cells were stimulated with allogeneic EBV-LCLs. Interestingly, CSF T cells from MS1 did not recognize allogeneic EBV-LCLs, but proliferated vigorously upon stimulation with autologous or HLA-matched EBV-LCLs. This was confirmed in several experiments. In contrast, the remaining CSF and peripheral blood lymphocytic (PBL) T-cell lines recognized allogeneic EBV-LCLs (SI 8.5 to 156). The lack of alloreactive T cells in the CSF T-cell lines from MS1 enabled us to determine their HLA restriction by testing against a panel of HLA-matched and -mismatched EBV-LCLs. Because MS1 is homozygous for the MS associated DRB1*1501, DQB1*0602 haplotype, we generated an EBV-LCL from an individual (KS) with a rare DRB1*0301 haplotype carrying the DQA1*0201 and DQB1*0602. (Rønningen *et al*, 1991). Both the KS donor and the MS1 patient share the DQ(α1*0102, β1*0602) molecule, but express different DR molecules. This EBV-LCL therefore enabled us to determine if the restriction element is DR or DQ. In contrast to T cells from the blood, the CSF T cells recognized HLA-DR–matched EBV-LCL exclusively (Table 2), suggesting that the T cells in the CSF constitute a compartmentalized population. This is in line with studies on EAE (Weissert *et al*, 2001) and coeliak disease (Molberg *et al*, 1998), and underscores the differences between T cells from the diseased organ compared to the blood and peripheral lymphoid tissue. The HLA-DR restriction also indicate that the CSF T-cell responses in MS1 are elicited by an antigen presented on HLA-DR molecules rather than a superantigen or other polyclonal activators, which has been proposed as mechanism of T-cell stimulation by EBV-LCLs (Boylston and Anderson, 1979; Sutkowski *et al*, 2001).

Because the observed T-cell responses to EBV-LCL could be elicited by either epitopes from EBV proteins or endogenous activation–induced proteins, we stimulated CSF and blood T cells from MS1 and the TB patient with autologous B cells, which had been activated through stimulation of CD40 by biweekly stimulation with irradiated (96 Gy) murine fibroblasts transfected with the human CD40

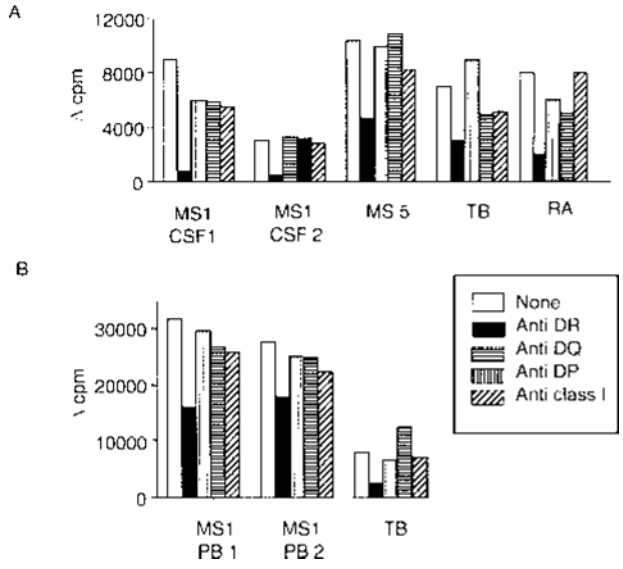

Figure 2 Proliferative responses of CSF T-cell lines (**A**) and peripheral blood T-cell lines (**B**) stimulated with autologous EBV-LCL in the presence and absence of monoclonal antibodies to HLA-DR, -DQ, and -DP and HLA class I molecules. Results are given as Δcpm (cpm of stimulated wells, after subtraction of cpm of T cells and an irradiated EBV-LCL cultivated separately). The mean background was 1331 (range 996 to 1612).

Table 2 Proliferative responses[a] of CSF T cells from patient MS1 when stimulated with autologous, allogeneic, and HLA-matched EBV-LCLs

Stimulating EBV-LCL	*DRB1**	*DQB1**	*Δcpm*	*SI*
Autologous	1501	0602	3088	3.9
	1501	0602		
Healthy (KS)	0301	0602	437	1.8
	1101	0301		
Healthy	1501	0603	7107	25.2
	0401	0301		
IHW[b] cell line 9008	1501	0602	2857	4.0
	1501	0603		
MS	1501	0602	7107	11.5
	1501	0602		
IHW[b] cell line 9023	0301	0201	278	1.0
	0301	0201		
TB	0101	0501	677	2.0
	1301	0603		

[a]Values are thymidine incorporation measured as Δcpm (cpm of stimulated cells minus cpm of T cells and irradiated EBV-LCLs cultivated separately) and stimulatory index (SI).
[b]IHW, International Histocompatibility Workshop.

Table 3 Proliferative responses[a] of cerebrospinal fluid T-cell lines stimulated with autologous Epstein-Barr virus–transformed B-cell lines (EBV-LCLs), αB-crystalline, and myelin basic protein (MBP)

	Stimulating cell or antigen		
Patients	*EBC-LCL*	*αB-Crystalline*	*MBP*
MS1	40079 (59.4)	−295	−210
MS2	8962 (50.9)	−93	206
MS3	13946 (3.5)	101	5045 (1.7)
MS5	3169 (23.7)	588	99
Amyotrophic lateral sclerosis	288	229	413
Tuberculous meningitis	6835 (8.7)	Not determined	21

[a]Results are given as thymidine incorporation measured as Δcpm (cpm of stimulated cells minus cpm of T cells and irradiated EBV-LCLs or PBMCs cultivated separately), and stimulatory index of those with Δcpm exceeding 1000 in parenthesis.

ligand gene (NIH-3T3 cells; kind gift from Dr. Joachim Schultze, Boston, MA, USA) (Schultze *et al*, 1997). The T cells, which responded to EBV-LCLs in the same experiment, did not recognize autologous B cells activated through CD40, suggesting that activation of B cells by itself is not sufficient to stimulate autoreactive T cells.

In order to see if the EBV-LCL–responsive T cells could cross-recognize putative MS autoantigens from the myelin sheath, the CSF T-cell responses to autologous EBV-LCLs were compared to those elcited by αB-crystallin (kind gift from Dr. Johannes van Noort, Division of Immunological and Infectious Diseases, TNO Prevention and Health, Leiden, the Netherlands) and human MBP (Chemicon, Temecula, CA, USA), using 100,000 irradiated (25 Gy) autologous PBMCs as antigen-presenting cells. αB-Crystallin is expressed on myelin from MS patients and on EBV-LCLs (van Sechel *et al*, 1999), and T-cell responses to αB-crystalline are suggested to be a link between MS and viral infections (van Noort *et al*, 2000). None of the EBV-LCL–responsive CSF T-cell lines displayed significant responses to MBP or αB-crystalline (Table 3).

This study does not pinpoint the actual antigens driving the response to autologous EBV-LCL. The lack of responses to CD40-activated B cells and αB-crystallin favors that the responses are directed against a viral epitope, but does not exclude other EBV-induced autoantigens. It is demonstrated that $CD4^+$ T cells from healthy donors can recognize dendritic cells loaded with EBNA1 and synthetic peptides from EBNA1 and EBNA3c, as well as EBNA1 processed endogenously by EBV-LCLs (Munz *et al*, 2000; Leen *et al*, 2001), indicating that EBNA peptides are presented on HLA class II molecules by EBV LCLs, and recognized by EBNA-specific $CD4^+$ T cells. Taken together with our data, this suggests that EBV might be involved in the pathogenesis of MS by activating or expanding autoreactive $CD4^+$ T cells.

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