

Differences between T Cell-Type and Natural Killer Cell-Type Chronic Active Epstein-Barr Virus Infection

Hiroshi Kimura,¹ Yo Hoshino,¹ Shinya Hara,¹ Naomi Sugaya,¹ Jun-ichi Kawada,¹ Yukiko Shibata,¹ Seiji Kojima,¹ Tetsuro Nagasaka,² Kiyotaka Kuzushima,³ and Tsuneo Morishima^{4,a}

Departments of ¹Pediatrics and ²Clinical Pathology, Nagoya University Graduate School of Medicine, ³Division of Immunology, Aichi Cancer Center Research Institute, and ⁴Department of Nursing, Nagoya University School of Health Sciences, Nagoya, Japan

Infections of T cells and natural killer (NK) cells play a central role in the pathogenesis of chronic active Epstein-Barr virus (CAEBV) infection. To characterize the virologic and cytokine profiles of T cell-type and NK cell-type infection, 39 patients with CAEBV infection were analyzed. Patients with T cell-type infection had higher titers of immunoglobulin G against early and late EBV antigens, suggesting lytic cycle infection. However, the pattern of EBV gene expression was latency type II; *BZLF1*, which is a hallmark of lytic cycle infection, could not be detected in any patients, regardless of infection type. Patients with CAEBV infection had high concentrations of proinflammatory, T helper cell type 1, and anti-inflammatory cytokines. The cytokine profile in patients with NK cell-type infection was similar to that in patients with T cell-type infection, but the concentration of IL-13 was high in patients with NK cell-type infection. These findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

Epstein-Barr virus (EBV) is a ubiquitous virus that infects most individuals by early adulthood. Primary EBV infection is usually asymptomatic but sometimes progresses to infectious mononucleosis, which resolves spontaneously after the emergence of EBV-specific immunity [1, 2]. EBV infection can be chronic in apparently immunocompetent hosts [3, 4]. Chronic active EBV (CAEBV) infection is characterized by chronic or recurrent mononucleosis-like infectious symptoms, such as fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, and hypersensitivity to mosquito bites [3–5]. Patients with

CAEBV infection have an unusual pattern of EBV-related antibodies and high viral loads in peripheral blood [3–7]. CAEBV infection is associated with high mortality and morbidity.

Recent studies have indicated that the clonal expansion of EBV-infected T cells and natural killer (NK) cells plays a central role in the pathogenesis of CAEBV infection [5, 8–11]. In a previous study, we found that patients with CAEBV infection fall into 2 clinically distinct groups, on the basis of whether the infected cells in their peripheral blood were mainly T cells or NK cells [5]. T cell-type infection is characterized by fever and high titers of EBV-related antibodies, whereas NK cell-type infection is characterized by hypersensitivity to mosquito bites and high titers of IgE. Furthermore, patients with T cell-type infection have significantly poorer outcomes [5, 12]. EBV-infected T cells might become activated and release inflammatory cytokines, such as interferon (IFN)- γ or tumor necrosis factor (TNF)- α , resulting in severe inflammation and fever [13, 14]. However, it is still not known why these 2 manifestations of the disease have different symptoms and courses.

The purpose of the present study was to gain a better

Received 4 May 2004; accepted 23 August 2004; electronically published 6 January 2005.

Financial support: Japan Society for the Promotion of Science (grant JSPS-RFTF97L00703); Japan Ministry of Education, Culture, Sports, Science and Technology (grant 13670793).

^a Present affiliation: Department of Pediatrics, Graduate School of Medicine and Dentistry, Okayama University, Okayama, Japan.

Reprints or correspondence: Dr. Hiroshi Kimura, Dept. of Pediatrics, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan (hkimura@med.nagoya-u.ac.jp).

The Journal of Infectious Diseases 2005;191:531–9

© 2005 by the Infectious Diseases Society of America. All rights reserved.
0022-1899/2005/19104-0008\$15.00

understanding the pathogenesis of CAEBV infection by characterizing the virologic profiles of T cell-type and NK cell-type infection and identifying the differences between them. We analyzed 20 patients with T cell-type infection and 19 patients with NK cell-type infection. The 2 types of CAEBV infection were compared in both virologic and immunologic analyses, including an analysis of cytokine profiles.

PATIENTS, MATERIALS, AND METHODS

Patients. Thirty-nine patients with CAEBV infection were enrolled in the present study. Informed consent was obtained from all of the patients or their parents. Of the 39 patients, 24 had been included in our previous study of the clinical characteristics of CAEBV infection [5]. All of the patients met the following diagnostic criteria [5]: (1) they had EBV-related illness or symptoms for >6 months (including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, uveitis, interstitial pneumonia, hydroa vacciniforme, or hypersensitivity to mosquito bites); (2) they had increased quantities of EBV in either affected tissue or peripheral blood (the quantity of EBV was defined as increased when ≥ 1 of the following criteria was met: EBV DNA was detected in either affected tissue or peripheral blood by Southern-blot hybridization, EBV-encoded small RNA 1 [EBER-1]-positive cells were detected in either affected tissue or peripheral blood [15], or $>10^{2.5}$ copies/ μg DNA was detected in peripheral-blood mononuclear cells [PBMCs] [15]); and (3) they did not manifest evidence of any previous immunologic abnormalities or of any other recent infection that might explain the condition (all of the patients examined were negative for antibody against HIV).

Cells. For the EBV gene-expression experiment, a lymphoblastoid cell line (LCL) that was transformed with B95-8 virus was used as a positive control, and BJAB, an EBV-negative B cell line, was used as a negative control.

Samples. Samples were collected at the time of diagnosis or before the administration of immunosuppressive therapy, such as chemotherapy or hematopoietic stem-cell transplantation. EDTA-treated peripheral blood collected from patients was centrifuged and separated into plasma and cell fractions; the cell fractions were separated into PBMCs on Ficoll-Paque density gradients (Pharmacia Biotech).

Titers of anti-EBV nuclear antigen (EBNA) antibodies were measured by use of an anticomplement immunofluorescence method. Titers of anti-viral capsid antigen (VCA) and anti-early antigen-diffuse restricted (EA-DR) IgG were measured by use of an immunofluorescence method. These titers were measured in all patients.

DNA was extracted from either 2×10^6 PBMCs or 200 μL of plasma by use of a QIAamp blood kit (Qiagen). To differentiate between free EBV DNA molecules and virions or nucleocapsids, selected plasma samples were examined for EBV content by di-

gestion with deoxyribonuclease (RQ1 RNase-free DNase; Promega) for 30 min at 37°C [16, 17]. As controls, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatants of LCL and BJAB cultures were used. LCL was treated with *n*-butyrate and phorbol 12-myristate 13-acetate, to induce lytic cycle infection, and the culture supernatants were used as a control that contained enveloped virions.

For patients from whom fresh samples were available ($n = 19$), RNA was extracted from 2×10^6 PBMCs by use of an RNA extraction kit (QIAamp RNA Blood Mini Kit; Qiagen). cDNA was synthesized by use of Superscript reverse transcriptase II (Gibco Life Technology), as described elsewhere [18].

Determination of EBV-infected cells. To determine which cells were infected with EBV, PBMCs were fractionated into CD3⁺, CD4⁺, CD8⁺, CD16⁺, CD19⁺, and CD56⁺ cells by use of an immunobead method (DynaBeads; Dynal A/S) [5]. For some patients with T cell-type infection from whom a sufficient quantity of PBMCs was obtained, fractionation into CD4⁺ and CD8⁺ cells was also performed. The fractionated cells were analyzed by either quantitative polymerase chain reaction (PCR) or in situ hybridization with the EBER-1 probe [15]. Patients were defined as having T cell-type infection when CD3⁺ cells either were the main cells giving a positive hybridization signal with EBER-1 or contained more EBV DNA than other cells in the blood sample [5]. Patients were defined as having NK cell-type infection when CD16⁺ or CD56⁺ cells were the main ones containing EBV [5]. Repeated tests were performed for some patients, and similar results were obtained; in the present study, representative results are shown.

For some patients, infected cells were identified in biopsied or autopsied tissues, such as lymph nodes, liver, and spleen. Double labeling by use of in situ hybridization with the EBER-1 probe and immunostaining with surface marker antibody were performed as described elsewhere [19].

Clonality of EBV. The clonality of EBV was determined by Southern blotting with a terminal-repeat probe, as described elsewhere [20, 21]. PBMC-extracted genomic DNA was digested with *Bam*HI, subjected to gel electrophoresis, transferred to a nylon membrane, hybridized with a ³²P-labeled *Xho*I fragment from the terminal region of EBV, and visualized by autoradiography.

Quantification of EBV DNA. Both PBMCs and plasma from all of the patients were assayed for viral load. A real-time quantitative PCR assay with a fluorogenic probe was performed as described elsewhere [15, 22]. As a positive standard for quantification, pGEM-BALF5 was used [15]. The quantity of EBV DNA was calculated as the number of copies per microgram or per milliliter of plasma.

Amplification of EBV-specific RNA transcripts by PCR. To detect latent gene expression (*EBNA1*, *EBNA2*, and *LMP1* [latent member protein]), nested PCR was performed essentially as described elsewhere [23]. For *EBNA1*, 3 different primer

sets were used, to determine promoter usage. Approximately 50 ng of total RNA (converted to cDNA) was used as template. The amplified products were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized by UV light. To detect *BZLF1*, which encodes a transactivator protein and is expressed in lytic cycle infection, nested PCR was performed with the primers described by Prang et al. [24]. To detect the gene for gp350/220, which is also expressed in lytic cycle infection, nested PCR was performed with the outer primers described by Kelleher et al. [25] and newly selected inner primers (5'-CATCACCGGTGACACCAAGT-3' and 5'-TGCTGGCGAACTGGTGGACA-3'). To detect a housekeeping gene, the human glyceraldehyde-3-phosphate dehydrogenase gene was amplified by single PCR; the sequences of the primer pair were 5'-GAAGGTGAAGGTCGGAGT-3' and 5'-GAAGATGGTGATGGGATTTC-3'. All of the primer pairs used in the present study were designed to span introns, to avoid amplification of genomic DNA.

Quantification of cytokine gene transcription by use of a real-time PCR assay. Cytokine gene transcription was quantified by use of a real-time PCR assay, as described elsewhere [18]. Transcription of the genes for IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p35, IL15, IFN- γ , and TNF- α was assessed by use of a TaqMan Cytokine Gene Expression Plate 1 (Applied Biosystems). A predeveloped primer/probe (Assays-On-Demand Gene Expression Products; Applied Biosystems) was used to measure IL-13 separately. Each well contained TaqMan primers and probes for assaying human cytokine mRNA and rRNA as an endogenous control. Approximately 25 ng of total RNA (converted to cDNA) was used to quantify the expression of each cytokine gene. All of the assays were conducted in duplicate. To calculate the relative expression of a cytokine gene in cells, the value for the expression of a cytokine gene was divided by that for the internal control rRNA, by use of the comparative threshold cycle method described by the manufacturer (P/N 4306744; Applied Biosystems) [18, 26].

Determination of plasma cytokine concentration by use of ELISA. Plasma cytokine concentrations were determined in all of the patients. Plasma concentrations of IL-1 β , IL-4, IL-10, IL-12, IL-13, and IFN- γ were determined by use of sandwich-type ELISA kits (R&D Systems); these assays were conducted in accordance with the manufacturer's instructions [18, 27]. Sample values were determined from a standard curve. The minimum detectable concentrations of IL-1 β , IL-4, IL-10, IL-12, IL-13, and IFN- γ were 1, 10, 3.9, 5, 32, and 8 pg/mL, respectively.

Statistical analyses. Statistical analyses were conducted by use of StatView software (version 5.0; SAS Institute). Fisher's exact test or the χ^2 test was used to compare differences in clinical measurements. The Mann-Whitney *U* test was used for statistical comparisons of laboratory data, viral load, cytokine

gene expression, and plasma cytokine concentrations. $P < .05$ was considered to be statistically significant.

RESULTS

Characteristics of T cell-type and NK cell-type CAEBV infection. Of the 39 patients with CAEBV infection, 20 had infections that were defined as T cell type, and 19 had infections that were defined as NK cell type. The differences in clinical and laboratory measurements between the 2 types of CAEBV infections are shown in table 1. The patients with T cell-type infection had a poorer prognosis (death rate, 60%); 12 of them died, with the causes of death including hepatic failure ($n = 4$), malignant lymphoma ($n = 2$), and cardiac complications ($n = 2$) (for death by other causes, $n = 4$). Three patients received hematopoietic stem-cell transplantation; 2 are still alive, and 1 relapsed and died shortly afterward. T cell-type infection was strongly characterized by high fever and anemia; other characteristics of T cell-type infection were hepatomegaly and lymphadenopathy, although the statistical significance was marginal. In contrast, the patients with NK cell-type infection had a better prognosis (death rate, 26%); 5 of them died, with the causes of death including complications related to hematopoietic stem-cell transplantation ($n = 3$), sepsis ($n = 1$), and interstitial pneumonia ($n = 1$). Seven patients received hematopoietic stem-cell transplantation; 4 are still alive, and 3 died of transplantation-related complications. NK cell-type infection was characterized by large granular lymphocytosis, hypersensitivity to mosquito bites, and a high IgE concentration. These observed differences between the 2 types of CAEBV infection are in agreement with the results of our previous study [5].

Determination of EBV-infected cells. For 28 patients, fractionation of PBMCs followed by quantitative PCR was used to determine the cell types that were infected (table 2). Using this method, we determined that 15 patients had T cell-type infection, because CD3 $^+$ cells contained more EBV DNA than did the other cell populations. In 3 of these patients, mainly CD4 $^+$ T cells were infected; in 2 of these patients, mainly CD8 $^+$ T cells were infected. In contrast, mainly CD16 $^+$ cells (not CD3 $^+$ cells) were infected in 13 patients, who were therefore determined to have NK cell-type infection (table 2). In some patients, both CD16 $^+$ and CD19 $^+$ cells contained more EBV DNA than did unfractionated cells, suggesting that both NK and B cells were infected with EBV.

For the remaining 11 patients, in situ hybridization was used to determine which cell types were infected. For 3 patients with NK cell-type infection, infected cells were identified by fractionating PBMCs and then performing EBER-1 in situ hybridization. EBER-1 was detected in 15%, 25%, and 60% of the CD56 $^+$ cells in these 3 patients, indicating that most of the infected cells were NK cells (as described elsewhere [28]). For the other 8 patients, tissue samples were used to identify in-

Table 1. Differences in clinical and laboratory measurements between T cell-type and NK cell-type chronic active Epstein-Barr virus (EBV) infection.

	T cell-type infection (n = 20)	NK cell-type infection (n = 19)	P
Sex, M:F, no.	10:10	11:8	.43
Age at onset, mean \pm SD, years	12.6 \pm 9.5	8.0 \pm 4.9	.19
Death rate, %	60	26	.03
Time to death from onset, mean \pm SD, years	2.9 \pm 1.3	5.4 \pm 3.8	.27
Symptoms			
Fever >1 day/week, %	81	38	.01
Hepatomegaly, %	79	53	.09
Lymphadenopathy, %	58	32	.096
Large granular lymphocytosis, %	11	58	.003
Hypersensitivity to mosquito bites, %	11	68	.0002
Laboratory data			
WBC count, mean \pm SD, cells/ μ L	5200 \pm 5500	6100 \pm 3200	.11
Hemoglobin concentration, mean \pm SD, g/dL	10.7 \pm 1.2	12.1 \pm 1.9	.03
Platelet count, mean \pm SD, 10 ⁴ cells/ μ L	21.4 \pm 7.6	19.2 \pm 8.5	.52
IgG level, mean \pm SD, mg/dL	1990 \pm 1440	1590 \pm 450	.36
IgE level, mean \pm SD, mg/dL	190 \pm 220	5650 \pm 6470	.002
EBV-related antibody			
Anti-VCA IgG level, GMT	2010	310	.001
Anti-EA-DR IgG level, GMT	610	70	.007
Anti-EBNA level, GMT	27	45	.12
Viral load			
PBMCs, mean \pm SD, copies/ μ g	10 ^{3.9 \pm 0.7}	10^{4.4 \pm 0.6}	.03
Plasma, mean \pm SD, copies/mL	10 ^{2.8 \pm 1.1}	10 ^{2.5 \pm 2.0}	.87

NOTE. Values in boldface indicate statistically significant results. Either Fisher's exact test or the χ^2 test was used to compare symptoms; the Mann-Whitney *U* test was used to compare laboratory data and viral load. EA-DR, early antigen-diffuse restricted; EBNA, EBV nuclear antigens; F, female; GMT, geometric mean titer; M, male; PBMCs, peripheral-blood mononuclear cells; VCA, viral capsid antigen; WBC, white blood cell.

fects cells. Double labeling with EBER-1 and surface markers showed that, in 5 patients, most EBER-1-positive cells were CD3⁺, indicating that they had T cell-type infection. In 3 patients, most EBER-1-positive cells were CD16⁺ (not CD3⁺), indicating that they had NK cell-type infection. Some of these results have been described elsewhere [29].

Virologic analyses. EBV-related antibody titers were compared between the 2 types of CAEBV infection. The patients with T cell-type infection had significantly higher titers of anti-VCA and anti-EA-DR IgG (table 1). The anti-EBNA antibody titers were comparable between the 2 types of CAEBV infection. The higher titers of antibody against early and late EBV antigens (i.e., anti-EA-DR IgG and anti-VCA IgG) but not against latent antigen (i.e., anti-EBNA antibody) suggested the possibility of lytic cycle infection in T cells.

To examine whether lytic cycle infection existed in EBV-infected T or NK cells, for 19 patients from whom fresh samples were available (for T cell-type infection, *n* = 11; for NK cell-type infection, *n* = 8), reverse-transcription PCR was used to examine EBV gene expression in PBMCs. *BZLF1*, which encodes a transactivator protein and is a hallmark of lytic cycle infection [1], was not detected in any of the 19 patients (table 3); another

lytic gene, for gp350/220, was also not detected. *EBNA1*, *LMP1*, and *LMP2A* were detected in PBMCs from nearly one-half of the 19 patients, indicating that they had the latency type II pattern. The Qp promoter, but not the Cp/Wp promoter, was used for *EBNA1* transcription. Representative results are shown in figure 1. Because it was possible that lytic cycle infection was present at sites other than PBMCs, EBV gene expression was examined in autopsy or biopsy samples from 2 patients with T cell-type infection. The *BZLF1* and gp350/220 genes were not detected in the livers, lymph nodes, or spleens of these patients (data not shown). The pattern of EBV gene expression in these tissue samples was also latency type II.

Next, the viral load in peripheral blood was investigated by use of real-time PCR. The viral load in PBMCs was higher in patients with NK cell-type infection (table 1). Interestingly, the viral load in plasma was similar between the patients with each type. To examine the viral load in plasma, plasma samples from selected patients were digested with deoxyribonuclease before extraction of DNA. As a preliminary experiment, pGEM-BALF5, a control plasmid DNA containing an EBV target gene, and the supernatant of an LCL culture containing enveloped virions were tested. The control plasmid DNA was sensitive to deoxy-

Table 2. Determination of Epstein-Barr virus (EBV)-infected cells in peripheral-blood mononuclear cells.

Type of chronic active EBV infection, patient	EBV DNA, copies/ μ g					Unfractionated cells	Mainly infected cells	Clonality of cells
	Fractionated cells							
	CD3 ⁺	CD4 ⁺	CD8 ⁺	CD16 ⁺	CD19 ⁺			
T cell								
1	220,000	760	230,000	100,000	760	210,000	T (CD8 ⁺)	Monoclonal
2	90,000	ND	ND	17,000	15,000	24,000	T	ND
3	43,000	21,000	1500	12,500	2400	8600	T (CD4 ⁺)	Monoclonal
4	23,000	ND	ND	1100	1600	14,000	T	Monoclonal
5	18,000	ND	ND	6200	0	7300	T	ND
6	17,000	ND	ND	4300	5200	16,000	T	ND
7	12,000	ND	ND	5700	2700	11,000	T	ND
8 ^a	12,000	4500	2500	6500	5000	51,000	T	Monoclonal
9	11,600	ND	ND	2700	2000	6100	T	Monoclonal
10	10,000	27,000	4900	1900	1200	42,000	T (CD4 ⁺)	Oligoclonal
11	7600	ND	ND	80	260	6600	T	ND
12	6600	ND	ND	0	900	3900	T	Polyclonal
13	3600	ND	ND	840	950	3200	T	ND
14	400	720	90	250	20	340	T (CD4 ⁺)	Monoclonal
15	160	10	220	0	20	200	T (CD8 ⁺)	Polyclonal
NK cell								
1	120,000	ND	ND	400,000	470,000	210,000	NK, B	Monoclonal
2	21,000	ND	ND	170,000	3200	110,000	NK	Monoclonal
3	7400	ND	ND	89,000	17,000	78,000	NK	ND
4	11,000	ND	ND	86,000	18,000	75,000	NK	Monoclonal
5	10,000	ND	ND	54,000	23,000	36,000	NK	Monoclonal
6	3300	ND	ND	35,000	1900	20,000	NK	Monoclonal
7	7600	ND	ND	25,000	1700	28,000	NK	ND
8 ^a	1800	ND	ND	16,000	9200	7000	NK, B	Oligoclonal
9	300	ND	ND	15,000	0	2000	NK	ND
10	200	ND	ND	15,000	1800	31,000	NK	ND
11	1600	ND	ND	4500	570	8100	NK	Monoclonal
12	50	ND	ND	4300	110	1600	NK	Oligoclonal
13	0	ND	ND	2700	1300	820	NK, B	ND

NOTE. Values in boldface indicate that EBV DNA was concentrated after fractionation. ND, not done.

^a Infected cells were confirmed by double labeling of tissue samples.

ribonuclease (percentage of reduction of EBV DNA after digestion, 99.9%), but the LCL supernatant was resistant to the enzyme (percentage of reduction of EBV DNA after digestion, 55.7%). Five plasma samples from each group of patients were tested. After deoxyribonuclease digestion, the percentages of reduction of EBV DNA were 100%, 92.8%, 96.8%, 97.3%, and 100% in the samples from the patients with T cell-type infection and 99.8%, 98.3%, 100%, 100%, and 100% in the samples from the patients with NK cell-type infection. Thus, plasma from both groups of patients was sensitive to deoxyribonuclease, indicating that most of the EBV DNA in plasma, rather than consisting of enveloped virions, consisted of free EBV DNA molecules, which were likely derived from dead or damaged cells.

The clonality of EBV was analyzed by use of Southern blotting. The majority of both T cell-type and NK cell-type infections were monoclonal (table 2). There was a trend in that

those patients with polyclonal or oligoclonal proliferation had lower viral loads, although there was no difference between the 2 types of CAEBV infection.

Cytokine profiles. Differences in the symptoms or immunologic responses between the 2 types of CAEBV infection might be due to the differences in the cytokine production profiles of either EBV-infected cells themselves or inflammatory cells. The plasma concentrations of cytokines (IL-1 β , IL-4, IL-10, IL-12, IL-13, and IFN- γ) were estimated and compared between the 2 types of CAEBV infection. IL-1 β , IL-10, and IFN- γ , none of which are detected in healthy individuals, were detected in plasma from many patients with CAEBV infection (figure 2); there were no significant differences between the T cell-type and NK cell-type infections. IL-13, which also is not detected in healthy individuals, was frequently detected in the patients with NK cell-type infection, and the concentration was higher than that in the patients with T cell-type infection. The patients who developed

Table 3. Summary of Epstein-Barr virus (EBV) gene expression in peripheral-blood mononuclear cells.

Type of chronic active EBV infection	EBNA1 ^a			EBNA2	LMP1	LMP2A	BZLF1	gp350/220	GAPDH
	Common	Qp	Cp/Wp						
T cell (<i>n</i> = 11)	4	1	0	0	4	3	0	0	11
NK cell (<i>n</i> = 8)	4	3	0	0	4	4	0	0	8

NOTE. Data are no. of samples positive for the indicated gene. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

^a For EBNA1, 3 different primer sets were used to determine promoter usage.

hemophagocytic syndrome had high concentrations of inflammatory cytokines, such as IL-1 β and IFN- γ .

Next, the transcription of cytokine genes in PBMCs was investigated in the 19 patients from whom fresh samples were available. Assays were performed for IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-10, IL-12p35, IL-13, IL-15, IFN- γ , and TNF- α . Transcription of the genes for IL-1 α , IL-1 β , IL-10, IL-13, IL-12p35, IL-15, TNF- α , and IFN- γ was high in the patients with CAEBV infection, whereas transcription of the genes for IL-4 and IL-5 was undetectable in most patients. There were no statistical differences in the transcription of these cytokine genes between the 2 types of CAEBV infection, including for IL-13.

DISCUSSION

It has been shown that infections of T and NK cells play a central role in the pathogenesis of CAEBV infection. A recent national

survey in Japan showed that the majority of patients with CAEBV infection had infections that belonged to either the T cell type or the NK cell type; only 2 of 82 patients had an infection that mainly involved B cells [12]. EBV-infected T cell or NK cell lines have been established from patients with CAEBV infection [20, 21]. In the present study, we identified 20 patients with CAEBV infection in whom mainly T cells were infected and 19 patients with CAEBV infection in whom mainly NK cells were infected. Recently, a small-scale study reported that, during acute EBV-associated hemophagocytic lymphohistiocytosis, EBV infection was predominant in CD8⁺ cells, but that, in patients with CAEBV infection, EBV infection was predominant in non-CD8⁺ cells [30]. However, the results of the present study indicated that, in some of our patients with CAEBV infection, EBV infection was predominant in CD8⁺ T cells.

To determine which cells were infected with EBV, we used immunomagnetic cell isolation to fractionate PBMCs, followed by quantitative PCR. This method is very rapid and convenient, but its disadvantage is the relatively poor purity of the selected cells. EBV-infected cells can contaminate uninfected cell fractions. Furthermore, the real-time PCR assay is so sensitive that the contaminating EBV genome can be detected; therefore, this method can determine only the cell population that is mainly infected. The low levels of EBV DNA seen in other cell populations do not always mean that they are infected with EBV; however, the results of the present study showed that, in some of our patients, both NK cells and B cells were infected with EBV (table 2). Perhaps >1 cell lineage harbors EBV in some patients with CAEBV infection. Electric cell sorting followed by EBER-1 in situ hybridization is a more accurate method for determining EBV-infected cell lineages. Using this method, Kasahara et al. found that, in some patients with CAEBV infection, different cell lineages were infected [30].

Originally, CAEBV infection was characterized by an unusual pattern of EBV-related antibodies, such as high titers of anti-VCA and anti-EA-DR IgG or the absence of anti-EBNA antibody [3, 4, 6, 31], although recent observations indicate that high titers of these EBV-related antibodies are not necessary for CAEBV infection to be diagnosed [5]. In patients with CAEBV infection—and especially in patients with T cell-type infection who have high titers of EBV-related antibody—high

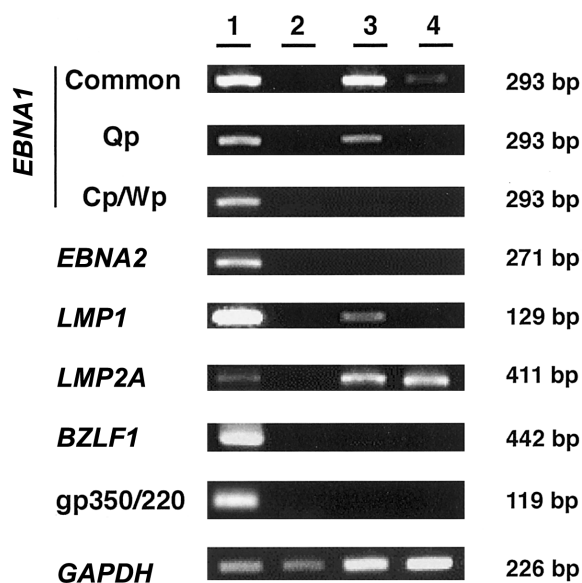


Figure 1. Expression of Epstein-Barr virus (EBV) genes in peripheral blood. Lane 1: EBV-positive lymphoblastoid cell line. Lane 2: BJAB, an EBV-negative B cell line. Lane 3: Peripheral blood from a patient with T cell-type chronic active EBV (CAEBV) infection. Lane 4: Peripheral blood from a patient with NK cell-type CAEBV infection. EBNA, EBV nuclear antigens; GAPDH, human glyceraldehyde-3-phosphate dehydrogenase; LMP, latent membrane protein.

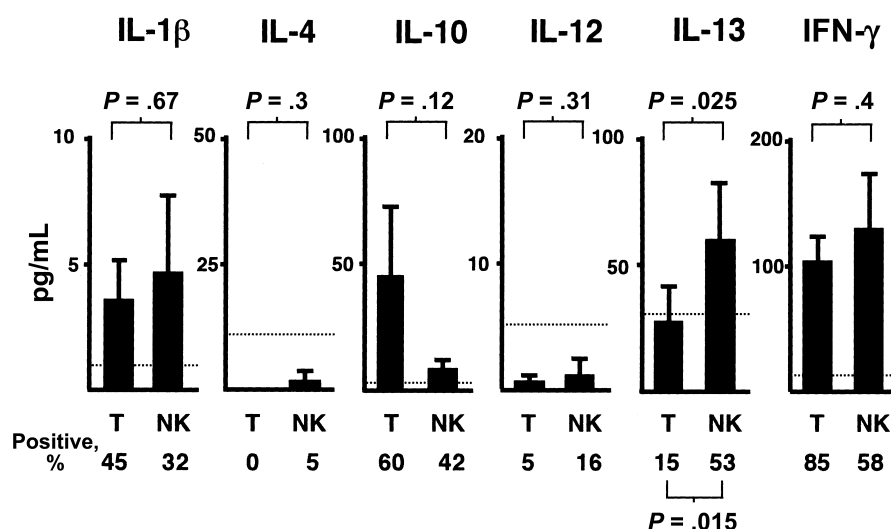


Figure 2. Comparison of plasma cytokine concentrations. Concentrations were estimated by use of an immunoassay and compared between patients with T cell-type ($n = 20$) and NK cell-type ($n = 19$) chronic active Epstein-Barr virus infection. Boxes and error bars indicate means and SEs, respectively; the dotted lines indicate the upper limits of healthy individuals. The Mann-Whitney U test was used to compare plasma cytokine concentrations, and Fisher's exact test was used to compare positivity rates. IFN, interferon; IL, interleukin.

titers of antibodies against the early and late EBV antigens and the existence of cell-free EBV DNA in plasma suggest the possibility of lytic cycle infection [6, 32, 33]. In the present study, a deoxyribonuclease-digestion experiment showed that the presence of EBV DNA in plasma was attributable to free nucleic acids that were likely released from dead or damaged cells. Furthermore, the pattern of EBV gene expression in PBMCs was latency type II, which supports the absence of lytic cycle replication in the PBMCs, at least, of patients with CAEBV infection. Lytic cycle infection may occur in tissue, although our results for tissue samples, while limited, showed no sign of a lytic cycle. It is also possible that we did not detect the occurrence of lytic cycle replication if lytic cycle infection occurred in $<1\%$ of EBV-infected cells. There is no definite proof of lytic cycle replication in tissues from patients with CAEBV infection. Some investigators have reported the expression of early or late EBV gene transcripts, such as those for BZLF1 or viral IL-10 [30, 34, 35], whereas other investigators have reported the absence of expression of these transcripts in tissue samples [36].

Our observation here of the absence of lytic cycle infection is particularly important with regard to selection of the treatment strategy for patients with CAEBV infection. Antiviral drugs that suppress viral DNA polymerase and lytic cycle replication may not be suitable for the treatment of CAEBV infection; however, therapies that reduce or eliminate EBV-infected T cells or NK cells may be suitable choices. Chemotherapy or hematopoietic stem-cell transplantation are suitable in this regard—the successful treatment of CAEBV infection by hematopoietic stem-cell transplantation has been reported [37, 38]. Alternatively,

EBV-related antigens expressed in T cells or NK cells may be the targets of treatment. Cytotoxic T cells that were generated from LCL and targeted to latency type III antigens have been administered to patients with CAEBV infection [39, 40]. On the basis of the present result that the pattern of EBV gene expression was latency type II, cytotoxic T cells specific for latency type II antigens, such as LMP1 or LMP2A, would be more favorable for the control and eradication EBV-infected cells if they are inducible [41, 42].

In the present study, patients with CAEBV infection had high concentrations of proinflammatory (IL-1 β), Th1-type (IFN- γ), and anti-inflammatory (IL-10) cytokines. Transcription of the genes for these cytokines was also high in PBMCs. The up-regulation of various cytokine genes has also been reported in patients with CAEBV infection in other studies [43–45]. These cytokines are thought to be produced either by EBV-infected T cells or NK cells or by reacting inflammatory cells. On the one hand, it has been shown that EBV-infected T cells produce proinflammatory (IL-6 and TNF- α), Th1-type (IL-2 and IFN- γ), and anti-inflammatory (transforming growth factor β 1) cytokines [14, 46]. Shen et al. reported that, during EBV-infected nasal NK/T cell lymphoma, human IL-10, an anti-inflammatory cytokine that suppresses cytotoxicity against EBV-infected cells, was expressed [47]. On the other hand, reacting inflammatory cells, such as macrophages, can produce most of the cytokines seen in the present study. Unfortunately, because of our study design, it is impossible to determine whether EBV-infected or reacting cells were the main sources of these cytokines. However, the high concentrations of and the elevated

transcription of genes for various cytokines must contribute to the diverse symptoms seen in patients with CAEBV infection.

One of the purposes of the present study was to find virologic differences between the T cell-type and the NK cell-type infection. One main difference was that we found frequent detection and a high concentration of IL-13 in the patients with NK cell-type infection. IL-13 is a Th2-type cytokine that induces the differentiation of B cells, the production of antigen-specific antibody, and a class switch to IgE and that also suppresses the cytotoxic functions of monocytes and macrophages [48]. IL-13 is primarily produced by activated T cells and is not usually detected in plasma from healthy individuals [49]. The cytokine is produced by Reed-Sternberg cells during Hodgkin disease, which is associated with EBV infection [50]. Although the reason why IL-13 was produced in the patients with NK cell-type infection is unclear, the high concentration of IL-13 may explain the high serum IgE levels and the hypersensitivity to mosquito bites, both of which are frequently seen in patients with NK cell-type infection [5].

The other difference between the 2 types of CAEBV infection is that the patients with NK cell-type infection had a higher viral load in PBMCs. This is particularly interesting, because NK cell-type infection is usually milder and progresses slowly [5, 12]. In contrast, the viral load in plasma was similar between the 2 types of CAEBV infection. These results suggest that sources of EBV DNA other than PBMCs exist in patients with T cell-type disease, the more severe, rapid type of CAEBV infection. In patients with T cell-type infection, the cell-free EBV DNA may come from tissue, such as lymph nodes or the spleen, where EBV-infected T cells infiltrate and proliferate. Indeed, patients with T cell-type infection have a higher incidence of hepatomegaly and lymphadenopathy, as was shown in the present study. The higher viral load in plasma could also be explained by the naturally high rate of apoptosis in activated T cells. It is still unclear why T cell-type infection is severe and progresses rapidly. The distribution of infected cells, determined by the differences in homing receptors among cells, may determine the symptoms and prognosis. A recent animal model showed that activated T cells are selectively trapped in the liver, primarily by intracellular adhesion molecule 1, which is constitutively expressed on sinusoidal endothelial cells and Kupffer cells [51]. We previously reported a patient with primary EBV infection who had severe hepatitis and whose liver was infiltrated with EBV-infected CD8⁺ cells. In patients with T cell-type CAEBV infection, EBV-infected, presumably activated T cells might accumulate in the liver and cause hepatitis. Although further studies are necessary, our findings should help to clarify the pathogenesis of CAEBV infection and facilitate the development of more-effective treatments.

Acknowledgments

We thank the following individuals for their contributions to the study: Chikako Kanazawa (Yamagata University); Masaki Ito, Mitsuaki Hosoya, and Atushi Kikuta (Fukushima Medical University); Shinichi Toyabe (Niigata University); Yuichi Hasegawa (Tsukuba University); Hidemitsu Kurosawa and Kenichi Sugita (Dokkyo University); Tsutomu Oh-ishi (Saitama Children's Medical Center); Miho Maeda (Nippon Medical School); Hiroko Kurozumi (Yokohama Minami Kyosai Hospital); Hirokazu Kanegane (Toyoama Medical and Pharmaceutical University); Tsuyoshi Ito (Toyohashi City Hospital); Kuniaki Kitamura (Ichinomiya Municipal Hospital); Yoshitoyo Kagami (Aichi Cancer Center); Ikuya Tsuge and Kayoko Matsunaga (Fujita Health University); Takahide Nakano (Kansai Medical University); Masahiro Sako (Osaka City General Hospital); Shiro Oshima (Osaka University); Takayuki Okamura and Keisei Kawa (Osaka Medical Center and Research Institute for Maternal and Child Health); Takanori Teshima (Okayama University); Hiroyuki Moriuchi (Nagasaki University); and Hitoshi Kiyoi, Tomohiro Kinoshita, and Tomoki Naoe (Nagoya University).

References

1. Rickinson AB, Kieff E. Epstein-Barr Virus. In: Knipe DM, Howly PM, eds. *Virology*. 4th ed. Vol. 2. Philadelphia: Lippincott Williams and Wilkins, 2001:2575–627.
2. Cohen JI. Epstein-Barr virus infection. *N Engl J Med* 2000; 343:481–92.
3. Rickinson AB. Chronic, symptomatic Epstein-Barr virus infection. *Immunol Today* 1986; 7:13–4.
4. Straus SE. The chronic mononucleosis syndrome. *J Infect Dis* 1988; 157:405–12.
5. Kimura H, Hoshino Y, Kanegane H, et al. Clinical and virologic characteristics of chronic active Epstein-Barr virus infection. *Blood* 2001; 98:280–6.
6. Okano M, Matsumoto S, Osato T, Sakiyama Y, Thiele GM, Purtilo DT. Severe chronic active Epstein-Barr virus infection syndrome. *Clin Microbiol Rev* 1991; 4:129–35.
7. Maeda A, Wakiguchi H, Yokoyama W, Hisakawa H, Tomoda T, Kurashige T. Persistently high Epstein-Barr virus (EBV) loads in peripheral blood lymphocytes from patients with chronic active EBV infection. *J Infect Dis* 1999; 179:1012–5.
8. Jones J, Shurin S, Abramowsky C, et al. T-cell lymphomas containing Epstein-Barr viral DNA in patients with chronic Epstein-Barr virus infections. *N Engl J Med* 1988; 318:733–41.
9. Kikuta H, Taguchi Y, Tomizawa K, et al. Epstein-Barr virus genome-positive T lymphocytes in a boy with chronic active EBV infection associated with Kawasaki-like disease. *Nature* 1988; 333:455–7.
10. Kawa-Ha K, Ishihara S, Ninomiya T, et al. CD3-negative lymphoproliferative disease of granular lymphocytes containing Epstein-Barr viral DNA. *J Clin Invest* 1989; 84:51–5.
11. Quintanilla-Martinez L, Kumar S, Fend F, et al. Fulminant EBV⁺ T-cell lymphoproliferative disorder following acute/chronic EBV infection: a distinct clinicopathologic syndrome. *Blood* 2000; 96:443–51.
12. Kimura H, Morishima T, Kanegane H, et al. Prognostic factors for chronic active Epstein-Barr virus infection. *J Infect Dis* 2003; 187:527–33.
13. Kawaguchi H, Miyashita T, Herbst H, et al. Epstein-Barr virus-infected T lymphocytes in Epstein-Barr virus-associated hemophagocytic syndrome. *J Clin Invest* 1993; 92:1444–50.
14. Lay JD, Tsao CJ, Chen JY, Kadin ME, Su JJ. Upregulation of tumor necrosis factor- α gene by Epstein-Barr virus and activation of macrophages in Epstein-Barr virus-infected T cells in the pathogenesis of hemophagocytic syndrome. *J Clin Invest* 1997; 100:1969–79.
15. Kimura H, Morita M, Yabuta Y, et al. Quantitative analysis of Epstein-Barr virus load by using a real-time PCR assay. *J Clin Microbiol* 1999; 37:132–6.
16. Cabrerizo M, Bartolome J, De Sequera P, Caramelo C, Carreno V. Hepatitis B virus DNA in serum and blood cells of hepatitis B surface

- antigen-negative hemodialysis patients and staff. *J Am Soc Nephrol* **1997**; 8:1443–7.
17. Shotelersuk K, Khorprasert C, Sakdikul S, Pornthanakasem W, Voravud N, Mutirangura A. Epstein-Barr virus DNA in serum/plasma as a tumor marker for nasopharyngeal cancer. *Clin Cancer Res* **2000**; 6:1046–51.
 18. Kawada J, Kimura H, Ito Y, et al. Systemic cytokine responses in patients with influenza-associated encephalopathy. *J Infect Dis* **2003**; 188: 690–8.
 19. Kimura H, Nagasaka T, Hoshino Y, et al. Severe hepatitis caused by Epstein-Barr virus without infection of hepatocytes. *Hum Pathol* **2001**; 32:757–62.
 20. Imai S, Sugiura M, Oikawa O, et al. Epstein-Barr virus (EBV)-carrying and -expressing T-cell lines established from severe chronic active EBV infection. *Blood* **1996**; 87:1446–57.
 21. Tsuge I, Morishima T, Morita M, Kimura H, Kuzushima K, Matsuoka H. Characterization of Epstein-Barr virus (EBV)-infected natural killer (NK) cell proliferation in patients with severe mosquito allergy: establishment of an IL-2-dependent NK-like cell line. *Clin Exp Immunol* **1999**; 115:385–92.
 22. Hoshino Y, Kimura H, Tanaka N, et al. Prospective monitoring of the Epstein-Barr virus DNA by a real-time quantitative polymerase chain reaction after allogeneic stem cell transplantation. *Br J Haematol* **2001**; 115:105–11.
 23. Chen F, Zou JZ, di Renzo L, et al. A subpopulation of normal B cells latently infected with Epstein-Barr virus resembles Burkitt lymphoma cells in expressing EBNA-1 but not EBNA-2 or LMP1. *J Virol* **1995**; 69:3752–8.
 24. Prang NS, Horneff MW, Jager M, Wagner HJ, Wolf H, Schwarzmann FM. Lytic replication of Epstein-Barr virus in the peripheral blood: analysis of viral gene expression in B lymphocytes during infectious mononucleosis and in the normal carrier state. *Blood* **1997**; 89:1665–77.
 25. Kelleher CA, Paterson RK, Dreyfus DH, et al. Epstein-Barr virus replicative gene transcription during de novo infection of human thymocytes: simultaneous early expression of BZLF-1 and its repressor RAZ. *Virology* **1995**; 208:685–95.
 26. Giulietti A, Overbergh L, Valckx D, Decallonne B, Bouillon R, Mathieu C. An overview of real-time quantitative PCR: applications to quantify cytokine gene expression. *Methods* **2001**; 25:386–401.
 27. Ito Y, Ichiyama T, Kimura H, et al. Detection of influenza virus RNA by reverse transcription-PCR and proinflammatory cytokines in influenza-virus-associated encephalopathy. *J Med Virol* **1999**; 58:420–5.
 28. Morita M, Tsuge I, Matsuoka H, et al. Calcification in the basal ganglia with chronic active Epstein-Barr virus infection. *Neurology* **1998**; 50: 1485–8.
 29. Nakagawa A, Ito M, Saga S. Fatal cytotoxic T-cell proliferation in chronic active Epstein-Barr virus infection in childhood. *Am J Clin Pathol* **2002**; 117:283–90.
 30. Kasahara Y, Yachie A, Takei K, et al. Differential cellular targets of Epstein-Barr virus (EBV) infection between acute EBV-associated hemophagocytic lymphohistiocytosis and chronic active EBV infection. *Blood* **2001**; 98:1882–8.
 31. Tosato G, Straus S, Henle W, Pike SE, Blaese RM. Characteristic T cell dysfunction in patients with chronic active Epstein-Barr virus infection (chronic infectious mononucleosis). *J Immunol* **1985**; 134:3082–8.
 32. Alfieri C, Ghibu F, Joncas JH. Lytic, nontransforming Epstein-Barr virus (EBV) from a patient with chronic active EBV infection. *Can Med Assoc J* **1984**; 131:1249–52.
 33. Kikuta H, Osato T, Matsumoto S. Sites of Epstein-Barr virus replication in acute and chronic active Epstein-Barr virus infections. *Intervirology* **1989**; 30:346–50.
 34. Xu ZG, Iwatsuki K, Oyama N, et al. The latency pattern of Epstein-Barr virus infection and viral IL-10 expression in cutaneous natural killer/T-cell lymphomas. *Br J Cancer* **2001**; 84:920–5.
 35. Kanegane H, Bhatia K, Gutierrez M, et al. A syndrome of peripheral blood T-cell infection with Epstein-Barr virus (EBV) followed by EBV-positive T-cell lymphoma. *Blood* **1998**; 91:2085–91.
 36. Yoshioka M, Ishiguro N, Ishiko H, Ma X, Kikuta H, Kobayashi K. Heterogeneous, restricted patterns of Epstein-Barr virus (EBV) latent gene expression in patients with chronic active EBV infection. *J Gen Virol* **2001**; 82:2385–92.
 37. Okamura T, Hatsukawa Y, Arai H, Inoue M, Kawa K. Blood stem-cell transplantation for chronic active Epstein-Barr virus with lymphoproliferation. *Lancet* **2000**; 356:223–4.
 38. Taketani T, Kikuchi A, Inatomi J, et al. Chronic active Epstein-Barr virus infection (CAEBV) successfully treated with allogeneic peripheral blood stem cell transplantation. *Bone Marrow Transplant* **2002**; 29:531–3.
 39. Kuzushima K, Yamamoto M, Kimura H, et al. Establishment of anti-Epstein-Barr virus (EBV) cellular immunity by adoptive transfer of virus-specific cytotoxic T lymphocytes from an HLA-matched sibling to a patient with severe chronic active EBV infection. *Clin Exp Immunol* **1996**; 103:192–8.
 40. Savoldo B, Huls MH, Liu Z, et al. Autologous Epstein-Barr virus (EBV)-specific cytotoxic T cells for the treatment of persistent active EBV infection. *Blood* **2002**; 100:4059–66.
 41. Straathof KC, Bollard CM, Rooney CM, Heslop HE. Immunotherapy for Epstein-Barr virus-associated cancers in children. *Oncologist* **2003**; 8:83–98.
 42. Gottschalk S, Edwards OL, Sili U, et al. Generating CTLs against the subdominant Epstein-Barr virus LMP1 antigen for the adoptive immunotherapy of EBV-associated malignancies. *Blood* **2003**; 101:1905–12.
 43. Xu J, Ahmad A, Jones JF, et al. Elevated serum transforming growth factor β 1 levels in Epstein-Barr virus-associated diseases and their correlation with virus-specific immunoglobulin A (IgA) and IgM. *J Virol* **2000**; 74:2443–6.
 44. Ohga S, Nomura A, Takada H, et al. Epstein-Barr virus (EBV) load and cytokine gene expression in activated T cells of chronic active EBV infection. *J Infect Dis* **2001**; 183:1–7.
 45. Ohga S, Nomura A, Takada H, Hara T. Immunological aspects of Epstein-Barr virus infection. *Crit Rev Oncol Hematol* **2002**; 44:203–15.
 46. Roncella S, Cutrona G, Truini M, et al. Late Epstein-Barr virus infection of a hepatosplenic $\gamma\delta$ T-cell lymphoma arising in a kidney transplant recipient. *Haematologica* **2000**; 85:256–62.
 47. Shen L, Chiang AK, Liu WP, Li GD, Liang RH, Srivastava G. Expression of HLA class I, β_2 -microglobulin, TAP1 and IL-10 in Epstein-Barr virus-associated nasal NK/T-cell lymphoma: implications for tumor immune escape mechanism. *Int J Cancer* **2001**; 92:692–6.
 48. Larche M, Robinson DS, Kay AB. The role of T lymphocytes in the pathogenesis of asthma. *J Allergy Clin Immunol* **2003**; 111:450–63.
 49. Fiumara P, Cabanillas F, Younes A. Interleukin-13 levels in serum from patients with Hodgkin disease and healthy volunteers. *Blood* **2001**; 98: 2877–8.
 50. Kapp U, Yeh WC, Patterson B, et al. Interleukin 13 is secreted by and stimulates the growth of Hodgkin and Reed-Sternberg cells. *J Exp Med* **1999**; 189:1939–46.
 51. Mehal WZ, Juedes AE, Crispe IN. Selective retention of activated CD8⁺ T cells by the normal liver. *J Immunol* **1999**; 163:3202–10.