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Autoimmune lymphoproliferative syndrome mimicking chronic active Epstein–Barr virus infection

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Abstract Chronic active Epstein–Barr virus infection (CAEBV) is defined as a systemic EBV-associated lymphoproliferative disease characterized by fever, lymphadenopathy, and splenomegaly in apparently immunocompetent persons. Recent studies have revealed that EBV infects T or natural killer cells in most patients with CAEBV; the etiology of CAEBV, however, remains unknown. Autoimmune lymphoproliferative disorder (ALPS) is an inherited disorder associated with defects in apoptosis, and clinically characterized by lymphadenopathy, splenomegaly, hypergammaglobulinemia, and autoimmune disease. ALPS is most often associated with mutations in the FAS gene, which is an apoptosis-signaling receptor important for homeostasis of the immune system. Based on the clinical similarity between ALPS and CAEBV with respect to lymphoproliferation, we have examined the possibility of the co-occurrence of ALPS in patients with a diagnosis of CAEBV. In this study, we have identified FAS gene mutations in three Japanese patients with lymphadenopathy, hepatosplenomegaly, and unusual EBV infection, who were diagnosed with CAEBV. These observations, which indicate that the clinical development of ALPS may be associated with EBV infection, alert us to a potential diagnostic pitfall of CAEBV.

Keywords Autoimmune lymphoproliferative syndrome · Chronic active Epstein–Barr virus · FAS · IL-10

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1 Introduction

Autoimmune lymphoproliferative syndrome (ALPS, MIM 601859) is a disorder characterized by nonmalignant lymphoproliferation, autoimmunity, and an increase in TCR-α/β^+ CD4$^-$ CD8$^-$ double negative T (DNT) cells [1, 2]. Most patients with ALPS display inherited heterozygous mutations in the FAS gene, which encodes a cell surface molecule involved in programmed cell death (apoptosis). Several patients with ALPS display mutations in the FASLG or CASP10 genes [3, 4], and the remaining patients likely display genetic defects that have not been defined. Genetic and immunologic studies have demonstrated that some unaffected family members show elevations of DNT cells and carry the same mutations in the FAS gene, suggesting that the occurrence of ALPS may require not only FAS deficiency but also other genetic or environmental factors.

Chronic active Epstein–Barr virus (EBV) infection (CAEBV) is characterized by chronic or recurrent infectious mononucleosis-like symptoms and unusual patterns of anti-EBV antibodies [5]. The etiology of CAEBV remains to be elucidated. However, recent studies have demonstrated that patients with CAEBV have a high viral

load in their peripheral blood and have a clonal expansion of EBV-infected T cells and natural killer (NK) cells [6]. Lymphadenopathy, hepatosplenomegaly, thrombocytopenia, and hypergammaglobulinemia, which are usually present in patients with CAEBV, are also found in patients with ALPS. We have identified germline *FAS* mutations in three patients diagnosed with CAEBV.

2 Patients and methods

2.1 Patients

Patient 1 was born to nonconsanguineous healthy parents. He had hepatosplenomegaly since the age of 3 months and was admitted to the hospital at the age of 9 months. At admission, he demonstrated systemic lymphadenopathy and mild thrombocytopenia (platelets of 110×10^{-9}/L). The EBV serology included an anti-viral capsid antigen (VCA) IgG antibody titer of 1:640 and an anti-early antigen (EA) IgG antibody titer of 1:80. Anti-VCA IgM antibody and anti-EBV nuclear antigen (EBNA) antibody were negative. The presence of persistent hepatosplenomegaly, lymphadenopathy, and a persistent positive anti-EA IgG antibody suggested that the patient might have CAEBV. He received anti-viral drugs and interferon without improvement. He suffered from aseptic meningitis twice at the age of 13 years. However, he displayed stable hepatosplenomegaly, thrombocytopenia, and elevated titer of EA antibody at the age of 15 years.

Patient 2 was born to healthy, unrelated parents and was admitted to the hospital at the age of 2 years because of hepatosplenomegaly for 6 months. At admission, he had cervical lymphadenopathy. He showed an unusual pattern of anti-EBV antibodies: an anti-VCA IgG antibody titer of 1:640, an anti-EA antibody titer of 1:80, and an anti-EBNA antibody titer of 1:160. At the age of 3 years, he complained of abdominal pain and had severe anemia (hemoglobin level of 5.8 g/dL) and jaundice. Laboratory data showed elevated levels of indirect bilirubin and reticulocytes, and decreased levels of haptoglobin, a pattern which is indicative of hemolytic anemia. The Coombs test, however, was negative. The anemia and hepatosplenomegaly improved gradually without treatment, but the EBV serology persistently showed an unusual pattern: an anti-VCA IgG antibody titer of 1:2,560 and an anti-EA antibody titer of 1:2,560. The patient had thrombocytopenia (platelets of 9×10^{-9}/L), and responded to pulse steroid therapy at the age of 13 years. He had been followed as a patient with CAEBV associated with hemolytic anemia until the age of 14 years. EBV had mainly infected his CD19$^+$ B cells (1,210 copies/μg DNA).

Patient 3 was born to healthy, unrelated parents. He had presented with hepatosplenomegaly and thrombocytopenia since early infancy. At the age of 2 years, the patient contracted a primary EBV infection and has had cervical lymphadenopathy since then. He showed high titer of anti-VCA IgG (1:1,280) and increased copies of EBV-DNA (3,000 copies/μg DNA) in the peripheral blood. His sister also developed lymphadenopathy and an urticarial rash since a primary EBV infection.

Clinical symptoms, including fever, persistent hepatitis, and fatigue, were not observed in these patients. Patients 1 and 3 had persistent thrombocytopenia, but anti-platelet antibodies were not measured.

2.2 Analysis of lymphocyte subpopulations and Fas-mediated apoptosis

Heparinized venous blood samples were obtained from patients and healthy donors after informed consent was obtained. Peripheral blood mononuclear cells (PBMC) were separated by Ficoll-Hypaque gradient centrifugation. Lymphocyte subpopulations were analyzed by flow cytometry (EPICS XL-MCL; Beckman Coulter KK, Tokyo, Japan).

Peripheral blood mononuclear cells were stimulated with 0.1% phytohemagglutinin (Sigma-Aldrich, Inc., St. Louis, MO, USA) in the presence of 100 U/mL recombinant human IL-2 (Takeda, Osaka, Japan) in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol, 200 U/mL penicillin G, 10 μg/mL gentamicin, and 25 mM HEPES. After 4 days, the culture medium was supplemented with 50 U/mL of IL-2 for 7–10 days. Activated T cells were treated with different concentrations of anti-Fas mAb (CH11, MBL, Nagoya, Japan) for 12 h. Apoptosis was evaluated by a flow cytometric method previously described [7].

2.3 Mutational analysis of the *FAS* gene

Total RNA was extracted from the patients' PBMC by the Trizol reagent (Invitrogen), and single-stranded cDNA was synthesized using Superscript II transcriptase (Invitrogen). Oligonucleotide primer sets were used for PCR amplification of the four overlapping regions, which included all nine exons of *FAS* mRNA previously described [7]. PCR was performed by conventional methods. The nucleotide sequence was determined using each amplified PCR product with the BigDye Terminator Cycle Sequence Kit (Applied Biosystems, Foster City, CA, USA) with an automated ABI PRISM 310 DNA sequencer (Applied Biosystems). Genomic DNA was isolated from whole blood leukocytes by Qiagen Blood Mini Kit (Qiagen, Hilden, Germany). The primer pairs, 5′-GCTTAGTTTCTGGCAAGGCCG-3′ and

5′-GCTTAGTTTCTGGCAAGGCCG-3′ were used for amplifying the exon 8 boundaries of the *FAS* gene.

2.4 Measurement of IL-10

Serum IL-10 concentrations were evaluated by a commercial ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's recommendation.

3 Results

Three patients with hepatosplenomegaly, lymphadenopathy, cytopenia, hypergammaglobulinemia and an unusual pattern of EBV infection received a tentative diagnosis of

CAEBV (Table 1). Monoclonal or oligoclonal EBV infection of T or NK cells is one of the characteristics of CAEBV [6]. Because the clinical findings of hepatosplenomegaly, lymphadenopathy, cytopenia, and hypergammaglobulinemia are also found in patients with ALPS, we suspected the diagnosis of ALPS. The percentage of DNT cells was increased (Table 1; Fig. 1). Fas-mediated apoptosis assays demonstrated that the cells of these patients displayed no apoptosis after stimulation with an anti-Fas monoclonal antibody (Fig. 2). The patients were therefore tentatively diagnosed with ALPS. *FAS* gene analysis was performed in these patients. Patient 1 had a mutation in intron 8 characterized by a splicing donor site (IVS8+5G>T) resulting in no transcription of exon 8. Patients 2 and 3 had the same nonsense mutation, Q226X,

Table 1 Clinical and laboratory features of patients at the time of diagnosis of CAEBV

	Patient 1	Patient 2	Patient 3
Present age	27 years	27 years	12 years
Age at onset	3 months	1 year	2 years
Sex	Male	Male	Male
Splenomegaly	+	+	+
Hepatomegaly	+	+	+
Lymphadenopathy	+	+	+
Anemia	−	+	−
Thrombocytopenia	+	−	+
Hypergammaglobulinemia	+	+	+
VCA-IgG	1:640	1:640	1:1,280
VCA-IgM	<1:10	<1:10	<1:10
EA-IgG	1:80	1:80	<1:10
EBNA	<1:10	1:160	1:10
EBV-DNA	+	+	+
TCRα/β^+ CD4$^-$ CD8$^-$ (%)	10.4	20.3	12.5
Serum IL-10 (pg/mL)	173	NE	296

NE not examined

Fig. 1 Analysis of DNT cells. PBMC from Patient 1 and from a normal control were analyzed by flow cytometry with a monoclonal antibody specific for TCR-α/β, CD4, and CD8. The percentages of DNT cells are indicated in the *upper left* quadrants
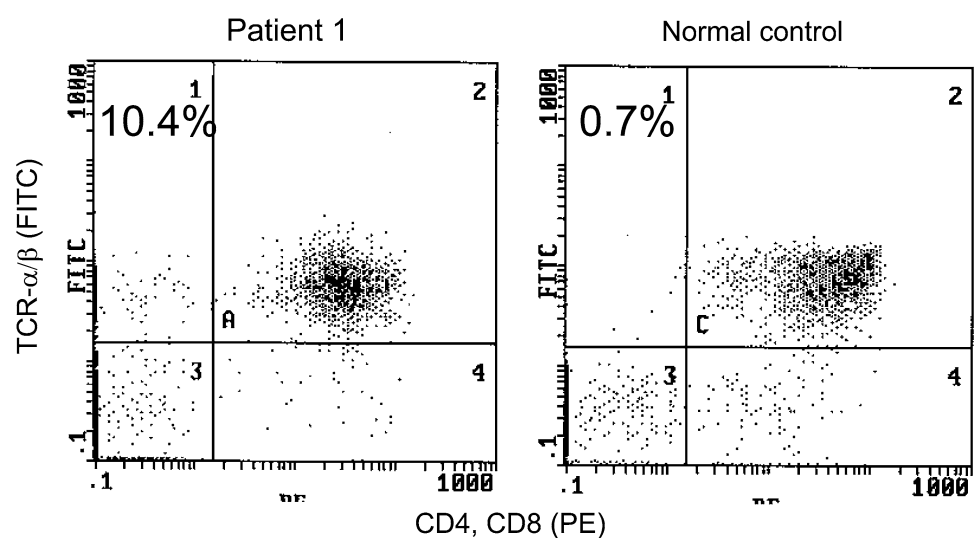
caused by a single base substitution (1020C>T). The mutations of Patients 1 and 2 were previously reported [8]. All the mutations produced a truncated protein of a defective intracellular domain, causing a defect in Fas-mediated apoptosis. Patients with ALPS frequently have markedly elevated levels of IL-10, vitamin B12 and soluble Fas ligand [9]. In this study, serum IL-10 levels were markedly elevated (normal value <15 pg/mL) in Patients 1 and 3.
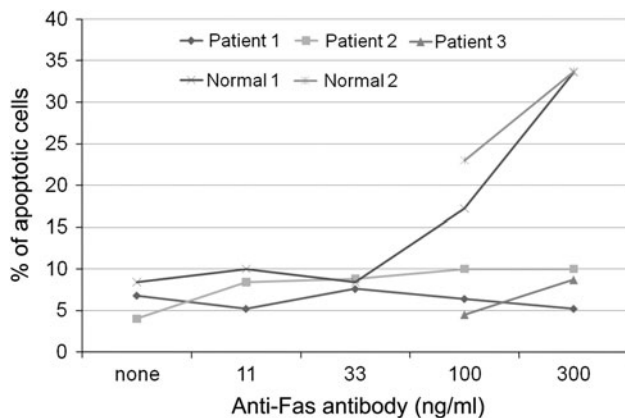
4 Discussion

Hepatosplenomegaly, lymphadenopathy, cytopenia, hypergammaglobulinemia, and autoimmunity are detected in patients with both CAEBV and ALPS [1, 2, 5, 6]. Hematologic complications, including hemolytic anemia and thrombocytopenia, may be due to autoimmunity. The current results suggest that patients with ALPS displaying unusual patterns of EBV antibodies may be misdiagnosed as having CAEBV. The EBV might mainly infect B cells in these patients, whereas the EBV infects T or NK cells in most patients with CAEBV. The cell type of EBV infection may be a critical point for differentiating ALPS from

Fig. 2 Fas-mediated apoptosis of activated T cells from the patients and normal controls. PBMC were activated by incubation with PHA and IL-2 for 2 weeks and then tested for Fas-mediated apoptosis

CAEBV. Some patients with *FAS* mutations are asymptomatic, and the onset of ALPS may be associated with additional environmental or genetic factors. Arkwright et al. [10] described two boys with ALPS who were infected with cytomegalovirus (CMV) during infancy. CMV infection may trigger the onset of ALPS in these patients. In our patients, EBV infection may have triggered the onset of ALPS. Therefore, we believe that ALPS should be differentiated from EBV-associated lymphoproliferative disorders (LPD) (Table 2). X-linked lymphoproliferative syndrome type 1 (XLP-1), a genetic immunodeficiency caused by mutations in the *SH2D1A* gene, is characterized clinically not only by fulminant infectious mononucleosis or severe EBV-associated hemophagocytic lymphohistiocytosis (HLH), but also by dysgammaglobulinemia and malignant lymphoma [11–13]. Patients with XLP do not present with autoimmunity. Posttransplant LPD (PTLPD) is a form of EBV-LPD. Patients with PTLPD can show hypogammaglobulinemia, but no hypergammaglobulinemia. PTLPD is not associated with HLH or autoimmunity.

IL-10 is a pleiotropic cytokine that is produced mainly by Th2 lymphocytes and monocytes. Serum or plasma IL-10 is increased in patients with ALPS and CAEBV. Cytokine imbalance and Th1 to Th2 shift may play a pivotal role in the pathogenesis of ALPS, and IL-10 may be a key cytokine [14]. In ALPS, IL-10 is mainly produced by DNT cells [8]. More than half of the patients with CAEBV demonstrated elevated levels of plasma IL-10 [15], and IL-10 may be mainly produced by EBV-infected T cells [16]. IL-10 may be associated with lymphoproliferation in CAEBV. Therefore, IL-10 may be a key cytokine in the pathogenesis of EBV-associated LPD.

Clinical and immunological findings of hepatosplenomegaly, lymphadenopathy, cytopenia, and hypergammaglobulinemia overlap in ALPS and CAEBV, and ALPS may be misdiagnosed as CAEBV. In addition, EBV infection may trigger the onset of ALPS. Serum or plasma IL-10 is increased in both diseases, and IL-10 may not only play a key role in the pathogenesis of this disease due to its anti-apoptotic function but may also account for

Table 2 ALPS-FAS and EBV-associated lymphoproliferative diseases

Disease	ALPS-FAS	XLP-1	CAEBV	PTLPD
Inheritance	Autosomal	X-linked	?	?
Sex	Both	Male only	Both	Both
Gene locus	10q25	Xq25	?	?
Gene	*FAS*	*SH2D1A*	?	?
HLH	Rarely	Frequent	Sometimes	Rarely
Malignant lymphoma	Sometimes	Frequent	Sometimes	Sometimes
Immunoglobulin	Hyper	Hypo or Hyper	Hyper	Hypo ∼ normal
Autoimmunity	++	−	+	−

autoimmunity. Although a genetic defect in CAEBV has not been identified, a gene related to the apoptosis pathway may be associated with CAEBV. Further studies should address this question.

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