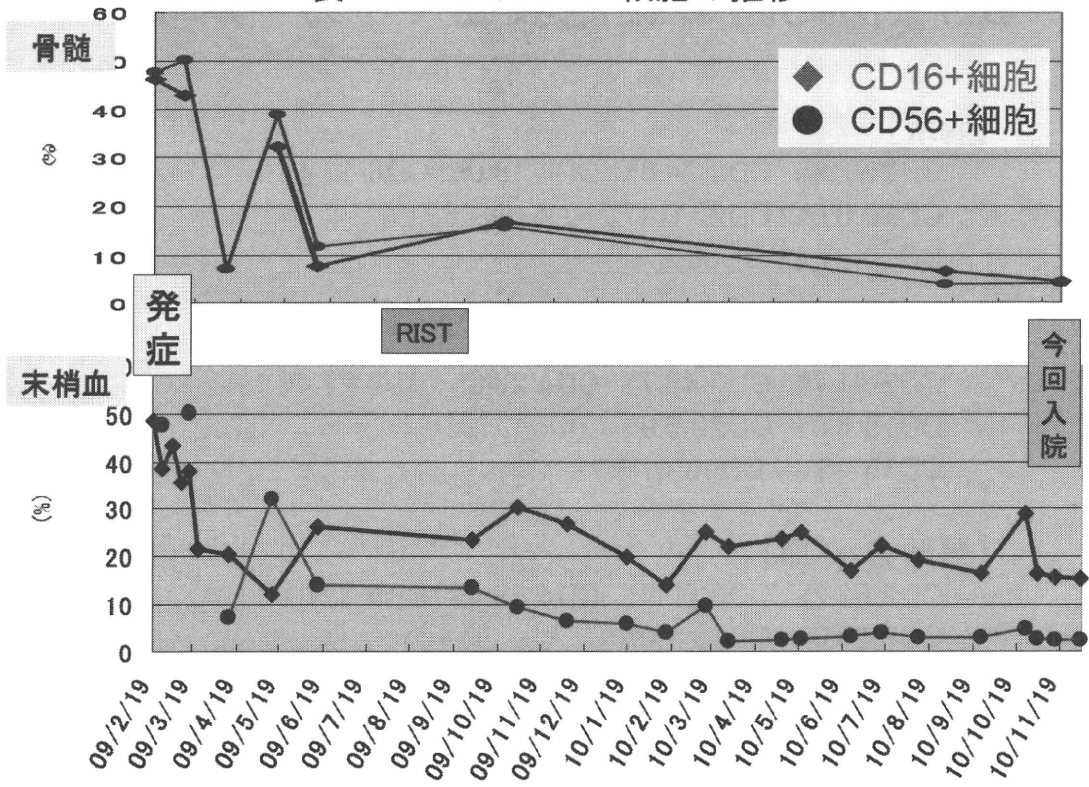表3. CD16+/CD56+細胞の推移

CAEBV モデルマウスの開発と応用

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研究要旨　慢性活動性 EB ウイルス（EBV）感染症（CAEBV）患者末梢血単核細胞（PBMC）を NOD/Shi-scid/IL-2R γ c^{null}（NOG マウス）に移植すると、CD4, CD8, γδT, NK の 4 タイプの CAEBV 症例全てにおいて、EBV 感染 T 或いは NK 細胞の生着が認められた。しかし、それぞれのタイプの患者 PBMC から EBV 感染細胞を含む CD4$^+$, CD8$^+$, γδT, NK 細胞分画を単離して移植すると、CD4$^+$細胞の場合を除いて生着しなかった。また、PBMC から CD4$^+$細胞を除いて移植すると全てのタイプで生着しなかった。単離した CD8$^+$, γδT, NK 細胞分画に CD4$^+$細胞を加えて移植すると生着した。PBMC 移植後のマウスに OKT-4 抗体を投与して CD4$^+$細胞を除くと生着が妨げられた。以上の結果から CAEBV 由来の EBV 感染 T 及び NK 細胞の NOG マウスへの生着には CD4$^+$細胞が重要な役割を果たすことが示唆された。CD4$^+$細胞を標的とする新規治療法の可能性が考えられる。

A. 研究目的

EB ウイルス（EBV）には優れた感染モデル動物が存在せず、特に薬剤や治療法の開発に有用な小動物モデルが不十分であった。我々は、以前免疫不全マウスの一系統 NOD/Shi-scid/IL-2R γ c^{null}（NOG マウス）にヒト造血幹細胞を移植して作成したヒト化マウスを用いて EBV 感染モデルを作成し、移植後リンパ増殖性疾患などの病態を再現することに成功した。しかし、慢性活動性 EBV 感染症（CAEBV）のように EBV が T 或いは NK 細胞に感染する病態は、これまでのところこのヒト化マウスを用いては再現されていない。そこで、ヒト化していない NOG マウスに患者末梢血単核細胞（PBMC）を異種移植する方法によりモデル作成を試み、EBV 感染 T あるいは NK 細胞の増殖と全身臓器への浸潤、latency II 型 EBV 遺伝子発現、高サイトカイン血症など、CAEBV に特徴的な病態を再現することに成功した。今年度は、このモデルを用いて CAEBV の発症機構を探るために、EBV 感染細胞が NOG マウスに生着し増殖するための条件を解析することを目的として研究を進めた。

B. 研究方法

1. NOD/Shi-scid/IL-2R γ c^{null}（NOG マウス）

NOG マウスは NOD/Shi-*scid* マウスと、IL-2 受容体コモンγ鎖ノックアウトマウスを掛け合わせたもので、造血幹細胞を含めたヒト造血系細胞の生着に最も適した免疫不全マウスの一つである。NOG マウスは実験動物中央研究所より購入し、国立感染症研究所無菌飼育室で飼育した。

2. CAEBV 患者末梢血単核細胞の移植

末梢血より Ficoll 比重遠心法により単核細胞を分離し、$1～5×10^6$ 細胞を尾静脈より注射した。移植後定期的に、末梢血中の EBV DNA 量およびヒト $CD45^+$ 細胞数を測定し、EBV 感染細胞生着の指標とした。

3. リンパ球分画の調製とマーカー発現解析

$CD4^+$, $CD8^+$, $CD56^+$, γδT 細胞の分画は Miltenyi 社磁気ビーズを用いて単離した。リンパ球表面マーカー発現解析は、蛍光標識モノクローナル抗体を結合させた後、ベックマン・コールター社 Cytomics FC500 を用いてフローサイトメトリーを行った。

4. $CD4^+$ 細胞の in vivo 除去実験

CD8 タイプ或いは NK タイプの患者 PBMC ($5×10^6$ cells) を移植した後、$100\,\mu g$ の OKT-4 抗体を静脈内投与した。さらに、翌日から 3 日連続して同量の抗体を投与した。

5. EBV の DNA 定量および遺伝子発現解析

EBV DNA は木村らの方法を用いてリアルタイム PCR 法により測定した。EBV 遺伝子の発現解析は、RT-PCR 法、免役化学染色、in situ hybridization（EBER の場合）によった。

（倫理面への配慮）

本研究は直接ヒトを対象とする医療行為を含まないが、CAEBV 患者由来ヒト細胞を利用するため、ヘルシンキ宣言に則った倫理的配慮を必要とする。患者本人あるいは保護者に対して、本研究に関する十分な説明を文書と口頭で行い、自由意思による同意書への署名を得ることによりインフォームドコンセントを取得した。試料および臨床情報は匿名化され、患者の個人情報は厳重に管理された。動物実験においては、動物実験指針を遵守し、動物愛護の観点から十分な配慮をした。本研究は国立成育医療研究センターおよび国立感染症研究所の倫理委員会および実験動物委員会の承認を得ている。

C. 研究結果

1. EBV 感染細胞を含むリンパ球分画の移植実験

前年度までの研究により、$CD4^+$, $CD8^+$, γδT, NK 細胞のそれぞれに EBV が感染する 4 タイプの CAEBV 全てにおいて、PBMC を移植することにより、EBV 感染 T 或いは NK 細胞を生着させることに成功している。そこで次に、それぞれのタイプの患者において、PBMC から $CD4^+$, $CD8^+$, γδT, 或いは NK 細胞を単離した後の移植を試みた。その結果、CD4 タイプの患者末梢血から $CD4^+$ 細胞を単離して移植した場合は EBV 感染 $CD4^+$ 細胞が生着したが、その他の場合、すなわち CD8 タイプの患者の $CD8^+$ 細胞分画、γδT タイプの患者のγδT 細胞分画、NK タイプの患者の $CD56^+$ 細胞分画を移植した場合は生着しなかった（図 1）。

2. PBMC から個々の細胞分画を除いた後の移植実験

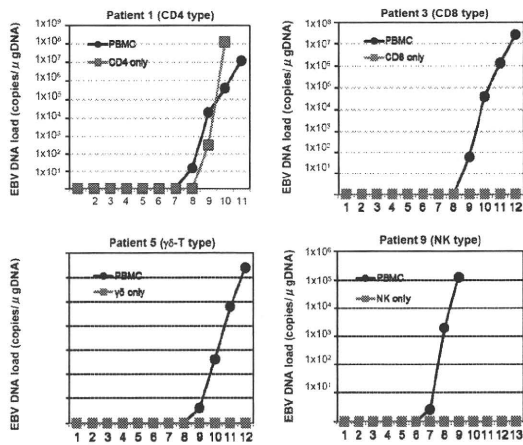次に、4 タイプのの CAEBV 患者において、PBMC から $CD4^+$, $CD8^+$, γδT, 或いは NK 細胞分画を除いた後の細胞を移植する

図1. CAEBV 患者 PBMC 或いは EBV DNA を含む細胞分画の移植実験. CD4 タイプを除いて、EBV 感染細胞を含む細胞分画のみの移植では生着しなかった。

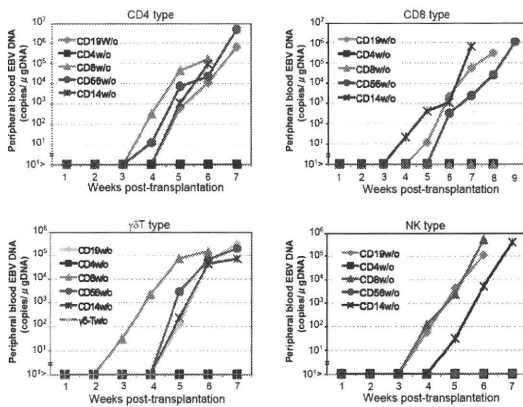図3. EBV 感染細胞を含む分画に CD4+細胞を加えて移植する実験. それぞれ単独では生着しえない CD8, γδT, NK 細胞分画に CD8+細胞を加えて移植すると生着する。

図2. PBMC から各種細胞分画を除いた後の移植実験. CD4 タイプの場合は、CD4+分画を除いた場合のみ生着が妨げられた。CD8, γδT, NK タイプの場合は、感染細胞を含む分画のみでなく、CD4+分画を除いた場合も生着が妨げられた。
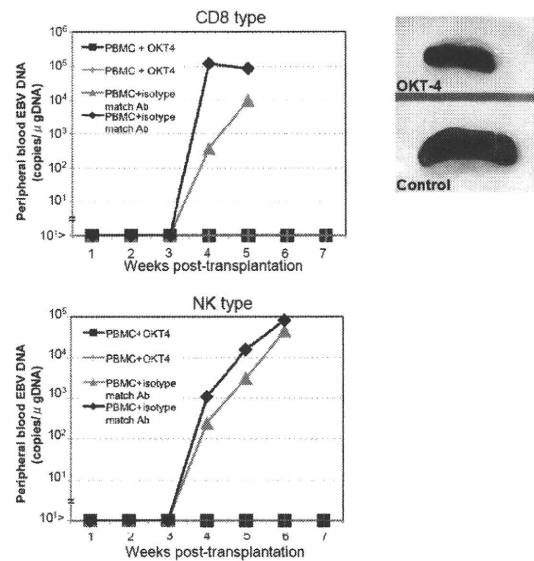
図4. OKT-4 抗体による CD4+細胞の in vivo 除去実験. CD8 或いは NK タイプの患者 PBMC を移植した後 OKT-4 抗体を投与すると生着が妨げられた。

実験を行った（図2）。CD4 タイプの患者においては、CD4+細胞を除いた場合に生着が妨げられたが、他の分画を除いても生着した。CD8 タイプの患者の場合は、CD4+或いは CD8+細胞を除いた場合に生着が妨げられたが、他の細胞を除いても生着した。γδT タイプの患者においては、CD4+或いは γδT 細胞を除くと生着が妨げられたが、他の細

胞を除いても生着した。NK 細胞タイプの患者においては、CD4+細胞或いは NK 細胞（CD56+）を除くと生着が妨げられたが、他の細胞を除いても生着した。以上の結果は、EBV 感染 T 或いは NK 細胞が NOG マウスに生着するためには、EBV 感染細胞の分画に加えて CD4+細胞が存在することが条件となることを示唆している。
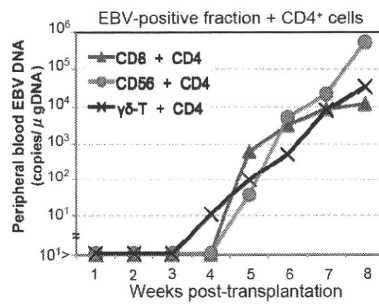
3. EBV 感染細胞に CD4+細胞を加えて移植

— 43 —

する実験

上記の実験により EBV 感染 T 及び NK 細胞の NOG マウスへの生着には、CD4$^+$細胞が重要な役割を果たすことが示唆された。そこで単独では生着できない CD8$^+$, γδT, 及び NK 細胞分画に CD4$^+$細胞を加えて移植する実験を行った。その結果、CD8$^+$細胞+CD4$^+$細胞、γδT 細胞+CD4$^+$細胞、NK 細胞+CD4$^+$細胞、のそれぞれの移植により EBV 感染 T 或いは NK 細胞が生着することが示された（図3）。

4. OKT-4 抗体による CD4$^+$細胞除去実験

EBV 感染 T 及び NK 細胞の生着における CD4$^+$細胞の役割をさらに検証するために、移植後のマウスに OKT-4 抗体を投与し、CD4$^+$細胞を除去した場合の生着の有無を調べた。CD8 タイプ或いは NK タイプの患者 PBMC を移植した後に、OKT-4 抗体を投与したところ、EBV 感染 T 或いは NK 細胞の生着が阻止された（図4）。

D. 考察

EBV の主要標的細胞は B 細胞及び上皮細胞であり、T 及び NK 細胞への感染は比較的稀である。T 及び NK 細胞における感染メカニズムや増殖誘発メカニズムは多くが不明のまま残されている。また、CAEBV における EBV 感染 T 或いは NK 細胞が腫瘍細胞の性格をもつかどうかについても議論が分かれている。今回の実験結果より、EBV 感染 T 及び NK 細胞の NOG マウスへの生着には CD4$^+$細胞が重要な役割を果たすことが示された。このことは CAEBV における EBV 感染 T 及び NK 細胞が完全な自律増殖能を有するものではなく、真の悪性細胞とは異なることを示唆している。CAEBV における EBV 感染細胞（特に T 細胞）は異型性に乏しく、EBV DNA 末端繰り返し配列の解析等によりモノクローナルな増殖が示唆される場合でも、患者が長期間にわたり良好な一般状態を保つことが稀ではない。これらのことから、CAEBV では、少なくとも初期においては悪性度の低い細胞が何らかの原因により免疫監視機構を逃れて増殖を続け、その結果として変異が蓄積されて生じる悪性細胞が後期にリンパ腫を発症させるというシナリオが考えられた。CD4$^+$細胞の作用メカニズムは現時点では不明であるが、CD4$^+$細胞が産生する IL-2 等のサイトカインの効果を今後検証する計画である。CD4$^+$細胞が重要な役割を果たすことが示されたことにより、この細胞を標的とする新規治療法の可能性が開かれた。

E. 結論

CEBV 患者由来の EBV 感染 T 及び NK 細胞の NOG マウスへの生着には CD4$^+$細胞が重要な役割を果たすことが示された。このことから少なくとも初期においては CAEBV における EBV 感染 T 及び NK 細胞は悪性度の低い細胞であることが示唆された。また、CD4$^+$細胞を標的とする新規治療法の可能性が示唆された。

F. 健康危機情報

該当なし。

G. 研究発表

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H．知的財産権の出願・登録状況
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ORIGINAL ARTICLE

Clinical features of adult-onset chronic active Epstein–Barr virus infection: a retrospective analysis

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Abstract We performed a retrospective analysis of patients with adult-onset chronic active Epstein–Barr virus infection (CAEBV). First, we analyzed five patients (aged 28–72) diagnosed at our hospitals with EBV-infected clonally proliferating T cells. Four patients were administered cyclophosphamide/doxorubicin/vincristine/prednisone (CHOP) chemotherapy, but no remarkable decrease of viral load was observed in three of the patients. The other patient died 19 days after initiation of CHOP treatment due to disease progression. Addition of high-dose cytarabine to the regimens of two of the patients was discontinued shortly after administration, due to the development of grade 4 pericardial effusion. Together, these regimens may be insufficient for treating adult-onset CAEBV. We next reviewed 23 adult-onset CAEBV patients, adding 18 previously reported patients to the five patients described in the present study. T cells were frequently infected (87%), whereas NK- and T-cell types are known to be almost equally prevalent in childhood-onset cases. The time duration from the onset of disease to initiation of treatment averaged 20 months. Reports showed that 12 patients died; seven patients died at an average of 8 months after initiation of treatment. Patients' disease courses seemed to be rapidly progressive and more aggressive than those of childhood-onset cases. More cases must be studied to clarify clinical features and establish an optimal treatment strategy.

Keywords Chronic active Epstein–Barr virus infection · Adult-onset · EBV-positive T-cell lymphoproliferative disorders of childhood · Chemotherapy · Clinical features

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1 Introduction

Chronic active Epstein–Barr virus infection (CAEBV) is a rare disease characterized by an infectious mononucleosis (IM)-like syndrome persisting for at least 6 months, and is associated with high titers of antibodies against EBV [1]. It shows a marked geographic preference for East Asia, and most reports are from Japan, Korea, and Taiwan. In these patients, T or NK cells are EBV-infected. Clonal expansion of EBV-infected cells has been reported in severe forms of CAEBV and is accompanied by high fever, hepatosplenomegaly, and pancytopenia. Because most of these patients were children and young adults, these severe cases of CAEBV with clonally proliferating T cells were termed EBV-positive T-cell lymphoproliferative disorders of childhood in the WHO classification revised in 2008 [2]. However, as reviewed here, some cases of adult-onset CAEBV have been reported. The placement of adult-onset CAEBV in the WHO classification, its clinical features and differences from pediatric cases, and a recommended treatment have not been determined.

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CAEBV prognosis is poor and CAEBV pathophysiology is considered to be an EBV infection of T or NK cells resulting in their activation and immortalization. Ohshima et al. [3] indicated that infected cells develop from polyclones and oligoclones, and finally become expanded monoclones to develop into aggressive diseases such as lymphomas or hemophagocytic syndrome. Although stem cell transplantation might be curative for CAEBV [4], no chemotherapeutic regimen has been identified with a confirmed effect on CAEBV. No prospective or retrospective analysis has been performed to evaluate the effects of treatment regimens for CAEBV, especially for adult cases.

In this study, we report 5 adult-onset patients with CAEBV. We will outline their clinical courses and the effects of chemotherapy as well as review the reported adult-onset cases in the literature and analyze their clinical features. The aims of this study are to investigate their clinical features in comparison with those of childhood-onset CAEBV and to discuss an optimal treatment strategy.

2 Materials and methods

2.1 Diagnostic criteria

CAEBV was diagnosed according to the criteria proposed by Okano et al. [1]. Briefly, these criteria are as follows: (1) EBV-related symptoms for more than 6 months, including fever, persistent hepatitis, extensive lymphadenopathy, hepatosplenomegaly, pancytopenia, hypersensitivity to mosquito bites (HMB), etc.; (2) increased quantity of EBV in either affected tissues or peripheral blood (PB) defined as EBV-DNA detected in tissue or PB samples by Southern blot hybridization, EBV encoded small RNA1 (EBER)-positive cells detected in tissue or PB samples, or an EBV-DNA level of $10^{2.5}$ copies/g of DNA detected in peripheral blood mononuclear cells (PBMCs); (3) EBV-infected cells confirmed as T or NK cells; and (4) no evidence of any prior immunologic abnormalities or other recent infection that might explain the condition. Criterion #3 was added to exclude EBV-positive B cell lymphoproliferative disorders. The time of diagnosis was defined as the time when the patient was found to meet the above criteria.

2.2 Detection of infected cells

Detection and isolation of infected cells was performed as described previously [5]. Briefly, PBMCs were isolated by Ficoll-Hypaque density gradient centrifugation. Then, CD19-, CD4-, CD8-, and CD56-positive cells were separated using antibody-conjugated magnetic beads (IMag Human Particles-DM; BD Biosciences, Sparks, MD, USA). The EBV-DNA of each fraction and of whole blood was quantified using a real-time quantitative polymerase chain reaction assay based on the TaqMan system (Applied Biosystems, Foster City, CA, USA). The fractions of blood with same or higher EBV-DNA titers than that of whole blood were designated as containing EBV-infected cells. The clonality of EBV was determined by Southern blotting using a terminal repeat probe.

2.3 Treatment protocol

Koyama and colleagues recently reported 2 pediatric patients with EBV-positive T-/NK-cell lymphoproliferative disorders and HMB who were successfully treated with sequential chemotherapy consisting of cyclosporine A and prednisolone (CP), followed by CHOP, Capizzi, and HDCA regimens [6], as described below:

CP: cyclosporine A (3 mg/kg/day), (prednisolone 1–2 mg/kg/day).

CHOP: cyclophosphamide (750 mg/m^2 on day 1), doxorubicin hydrochloride (50 mg/m^2 on day 1), vincristine (1.4 mg/m^2 on day 1), and prednisolone (100 mg on days 1–5).

Capizzi regimen: cytosine arabinoside (cytarabine) (3 g/m^2 every 12 h on days 1 and 2), L-asparaginase (6,000 U/m^2 on day 2), prednisolone (30 mg/m^2 on days 1 and 2).

We treated our patients according to this protocol.

The study complied with the principles of the Declaration of Helsinki and was approved by the Ethical Committee of the Tokyo Medical and Dental University. Written informed consent was obtained from each patient.

3 Results

3.1 Case reports

3.1.1 Case 1

A 48-year-old male was admitted to our hospital due to sustained fever which lasted for 1 year, liver dysfunction, and lymphadenopathy. Histology of his cervical lymph node revealed infiltration of EBER-positive cells without atypia; therefore, a diagnosis of lymphoma could not be made. As shown in Table 1, the EBV-DNA level in PB was significantly elevated in mononuclear cells. EBV-infected cells were identified as clonally proliferating CD4-positive T cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. From these findings, a diagnosis of CAEBV was made.

CP and CHOP treatment produced a transient resolution of clinical symptoms, but achieved only one log reduction of viral load (Table 1). Grade 4 neutropenia was sustained

Table 1 Effects and adverse events of chemotherapy of chronic active Epstein–Barr virus infection

Case	Age (years)	Sex	Cell type	EBV-DNA (copies/µg DNA)			Adverse events			
							CHOP	HDCA		
				On admission	After CHOP	After HDCA	Grade 4	Grade 3	Grade 4	
1	48	M	CD4	2.3×10^5	1.8×10^4	8.5×10^2	Neutropenia (4 days)			Fever, pericardial effusion
2	28	M	CD8	4.2×10^4	5.6×10^4	2.4×10^{4a}	Neutropenia (8 days)	Fever		Pericardial effusion
3	37	F	CD8	1.0×10^7	2.8×10^6	b	Neutropenia (9 days)		b	
4	72	F	CD4	9.5×10^4	Dead	b	Neutropenia (5 days)		b	

F female, *M* male, *EBV* Epstein–Barr virus, *HDCA* high-dose cytarabine

[a] The titer was examined after adding L-asparaginase

[b] High-dose cytarabine was not performed for Cases 3 and 4

for 4 days (Table 1). We judged that the effect of CHOP was insufficient, and 4.8 g of cytarabine was administered according to the Capizzi regimen. However, we discontinued the 3rd and the 4th cytarabine doses because of grade 4 high fever and grade 4 pericardial effusion. L-Asparaginase was administered after resolution of these events. Although the EBV-DNA level in PB decreased drastically, a Guillain-Barré-like neuropathy developed. The EBV-DNA level was elevated in the cerebrospinal fluid (CSF), and CD4-positive cells were detected in the fluid. Magnetic resonance imaging (MRI) revealed no abnormal lesion in the brain. However, we concluded that EBV-infected CD4-positive cells had infiltrated the CSF, and an intrathecal MTX injection was administered until cells were no longer detectable. BMT was performed from a HLA-matched unrelated donor with reduced intensity conditioning therapy (fludarabine 25 mg/m²/day, 4 days; melphalan 80 mg/m²/day, 1 day; total body irradiation, 2 Gy × 2), and engraftment was confirmed 1 month later. EBV-DNA became undetectable in both PB and CSF 1 month after BMT. However, the EBV-DNA level rose to 1.2×10^3 copies/g DNA 1 year after BMT. The patient showed no symptoms and the EBV-DNA level decreased without treatment within 2 months. He has remained in remission for 25 months.

3.1.2 Case 2

Two years prior to admission, a 28-year-old male developed fever, mediastinal lymphadenopathy, and polyneuropathy. Biopsies of lymph node lesions were performed at a previous hospital. CD3-positive lymphocyte infiltrations were observed in the specimens, and they were EBER-positive; however, a histological diagnosis of lymphoma was not made due to lack of atypia. However, the anti-EBV antibody titer (Table 2) and the EBV-DNA level in PB were significantly elevated in mononuclear cells. In addition, EBV-infected cells were detected and identified as clonally proliferating CD8-positive T cells (Table 1). From

these findings, a diagnosis of CAEBV was made. To evaluate the cause of neuropathy, a sural nerve biopsy was performed and an infiltration of CD8-positive cells was observed in the vascular wall. From these results, we concluded that his neuropathy was due to CAEBV. On examination of his CSF, CD8-positive cells and elevated EBV-DNA levels were detected in the fluid. Although MRI revealed no abnormal lesion in the brain, we concluded that EBV-infected CD8-positive cells had infiltrated the fluid.

Chemotherapy, including an intrathecal MTX injection, was performed. CP and CHOP treatments were first administered. Vincristine was discontinued due to neuropathy. He had transient resolution of clinical symptoms, but without a remarkable decrease in viral load (Table 1). Grade 4 neutropenia was sustained for 8 days (Table 1). We judged that the effect was insufficient, and 5.2 g of cytarabine was administered as part of the Capizzi regimen. However, we discontinued the 3rd and 4th cytarabine doses due to a grade 3 high fever and grade 4 pericardial effusion. We performed a BMT from an unrelated donor with 1 allele-mismatched HLA in DR locus, with reduced intensity of the conditioning therapy (fludarabine 25 mg/m²/day, 4 days; melphalan 80 mg/m²/day, 1 day; total body irradiation, 2 Gy × 2), and engraftment was confirmed 1 month later. EBV-DNA became undetectable in PB and CSF 1 month after BMT. However, the EBV-DNA level in PB rose to 1.6×10^4 copies/g DNA 1 year after BMT. He had no symptoms and we detected that the EBV-infected cells were B cells without clonal expansion. The EBV-DNA level decreased without any treatment and has been changing between negative and 4×10^2 copies/g DNA up to the present. We believe that he has remained in remission for 22 months.

3.1.3 Case 3

A 37-year-old female was diagnosed with systemic lupus erythematosus at the age of 24, and 13 years later developed recurrent IM-like symptoms with fever and cervical lymphadenopathy and was transferred to our hospital. Her

Table 2 Reported cases of adult-onset chronic active Epstein–Barr virus infection: clinical features

No.	Sex	Age (years)	Age of onset (years)	Infected cell	Anti-EBV antibodies and EBV-DNA in PB					Clonality	Symptoms and clinical findings on onset	References
					VCA-IgG	EA-IgG	EBNA	VCA-IgM	EBV-DNA			
1	M	24	22	NK cell	5120	5120	40	ND	ND	mono	Fever, LD, hepatosplenomegely, papules	[7]
2	M	36	35	CD4–CD8–T	2560	2560	10	2	ND	mono	Fever, fatigue, facial erythema, parotitis, dry eyes, dry oral cavity	[8]
3	F	25	23	CD4+	>10240	2560	10	ND	103.9 copies/µg	mono	ND	[9]
4	M	29	27	T cell	2560	2560	20	ND	103.6 copies/µg	ND	ND	[9]
5	M	28	26	CD3+	2300 AU/mL	1800 AU/mL	(−)	ND	Positive	ND	Uveitis, iridocyclitis, cardiomyopathy, perimyocarditis, esthesia, paresis	[10]
6	F	35	35	CD3+	18.4^a	7.8^a	11.9^a	0.1^a	ND	ND	Fever, arthralgia, jaundice, dyspnea	[11]
7	F	57	56	CD4+	5120	1280	10	ND	ND	mono	Fever, eyelid swelling, papules	[12]
8	F	27	24	γδT	2560	320	ND	ND	ND	mono	Fever, LD	[13]
9	F	69	68	CD56+	ND	ND	ND	ND	1.5×10^4 copies/mL	ND	Fever, LD	[14]
10	M	26	24	T cell	1280	ND	40	<10	ND	mono	Fever, weight loss, LD	[15]
11	F	71	68–69	CD3+CD4–/8–T	2560	1280	20	<10	(+)	oligo	Fever, weight loss, proximal extremities weakness	[16]
12	M	56	54	CD3+CD4–/8–T	320	1280	10	<10	(+)	oligo	Fever, dysarthria, dysphagia, the proximal extremities weakness	[16]
13	M	27	27	CD3+	ND	ND	ND	ND	140×10^2 copies/mL	ND	LA, motor paralysis, sensory disturbance, involuntary movements	[17]
14	M	52	52	CD8+	Positive	ND	Negative	Positive	9.0×10^4 copies/mL	poly	Fever, night sweats, general malaise	[18]
15	M	59	58	NK cell	(+)	ND	(−)	(−)	2.7×10^3 copies/mL	ND	Fever	[18]
16	F	26	26	T cell	ND	ND	ND	ND	ND	ND	ND	[19]
17	M	45	40	T cell	10240	640	10	ND	ND	ND	Fever, dyspnea, general malaise, hepatosplenomegaly	[20]
18	M	35	33	CD3+	10240	2560	80	<10	7.6×10^3 copies/µg	mono	Fatigue, HMB	[21]
19	M	48	47	CD4+	1280	640	40	<10	2.3×10^5 copies/µg	mono	Fever, LD, LA	OC 1
20	M	28	26	CD8+	2560	2560	<10	<10	4.2×10^4 copies/µg	mono	ND	OC 2
21	F	37	36	CD8+	10240	10240	40	<10	1.0×10^7 copies/µg	mono	Fever, LD, LA	OC 3
22	F	72	72	CD4+	11.2^a	6.9^a	3.3^a	0^a	9.5×10^4 copies/µg	mono	Fever, diarrhea, LD, body weight loss	OC 4
23	F	62	61	CD4+	2560	2560	40	<10	3.2×10^4 copies/µg	mono	Fever, LD, LA, body weight loss	OC 5

EBV Epstein–Barr virus, PB peripheral blood, M male, F female, ND not described, LD liver dysfunction, LA lymphadenopathy, HMB hypersensitivity to mosquito bites, OC our case, mono monoclonal, poly polyclonal, oligo oligoclonal

a It was measured by enzyme immuno assay. Cut off value was 0.5

clinical course has been reported previously [5]. As shown in Table 1, the EBV-DNA level in PB was elevated in mononuclear cells. Her infected cells were clonally proliferating CD8-positive T cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. CP and CHOP treatments produced a transient resolution of clinical symptoms, but without a remarkable decrease in viral load (Table 1). Grade 4 neutropenia was sustained for 9 days (Table 1). BMT was performed from a HLA-matched unrelated donor with reduced intensity conditioning therapy (fludarabine 37.5 mg/m^2/day, 5 days; melphalan 60 mg/m^2/day, 2 days; total body irradiation 2 Gy $\times$ 2). Engraftment was confirmed 1 month after BMT and EBV-DNA in PB became undetectable 2 months after BMT. EBV-DNA remained undetectable for almost 1 year; however, her disease relapsed and chemotherapy was initiated 4 years after BMT.

3.1.4 Case 4

A 72-year-old female was admitted to our hospital due to persistent diarrhea which lasted for 6 months, liver dysfunction, and body weight loss. Biopsies of liver and small intestine were performed and a diagnosis of EBV-positive T-cell lymphoproliferative disorder was made. As shown in Table 1, the EBV-DNA level in PB was elevated in mononuclear cells. The infected cells in the PB were clonally proliferating CD4-positive cells. As shown in Table 2, the anti-EBV antibody titer in PB was significantly elevated. Treatment with CP followed by CHOP was initiated. Grade 4 neutropenia appeared and was sustained for 5 days (Table 1). Unfortunately, her disease progressed and she died 19 days after CHOP initiation.

3.1.5 Case 5

A 62-year-old female, who had been suffering from fever, liver dysfunction, and body weight loss for 8 months, admitted to our hospital. A BM biopsy revealed hemophagocytosis with infiltration of EBV-positive cells. Hepatosplenomegaly was detected by computerized tomography. As shown in Table 2, the anti-EBV antibody titer and EBV-DNA level in PB were elevated in mononuclear cells. EBV-infected cells were clonally proliferating CD4-positive T cells. The diagnosis of CAEBV was made from these results. Both CSF and brain MRI revealed normal findings. She is now in preparation for chemotherapy.

3.2 Analysis of reported cases

To investigate clinical features of adult-onset CAEBV, we retrospectively analyzed the reported cases. We selected patients who met the criteria as described in Sect. 2, and

whose onsets were clear after 20 years of age. Eighteen reported patients were selected as shown in Table 2 [7–21]. Although the anti-EBV antibody titer and EBV-DNA level were not described in the report, Case 16 was diagnosed according to the guidelines. After addition of our 5 cases, we analyzed the clinical features of these 23 patients. The age of onset ranged from 22 to 72 years (median 36), in 13 male and 10 female patients. The clinical findings of the onset were described as including fever: 17 cases, liver lesion (dysfunction or hepatomegaly): 10 cases, lymphadenopathy: 5 cases, neurological symptoms: 4 cases, and cardiopulmonary symptoms: 3 cases. HMB was detected in 1 patient. These features were similar to those of childhood-onset cases. The infected cells were NK cells (including CD56-positive cells) in 3 patients, CD4-positive cells in 5 patients, and CD8-positive cells in 3 patients. Three patients had infected T cells that were negative for CD4 and CD8. One patient's infected cells were $\gamma\delta$-T cells. The other 8 patients were CD3-positive, or "T cell" type, and all together, 20 (87%) cases were T-cell type. VCA-IgG was significantly elevated in all described cases. VCA-IgM was undetectable or low except in Case 14, whereas EA-IgG was elevated in most cases. These findings suggested that their infections were not primary, but developed through reactivation of EBV. The elevation of EBV-DNA titer in PB was confirmed in 12 patients. We also confirmed EBV-DNA in PBMC in our 5 patients as in child-onset cases. Clonality was examined in 15 patients. Twelve cases were monoclonal. Their outcomes are summarized in Table 3. The duration from onset to treatment initiation ranged from 6 to 60 months (mean 20 months). Twenty-one patients had their clinical courses documented and 12 (57%) of these patients died. Most patients died from disease progression. The major causes of death were multiple organ failure (4 patients) and hemorrhage (3 patients). Among 8 elderly patients (aged > 50 years; Table 3), 6 patients died form disease progression. Their mortality was 75% besides the younger patients' mortality (aged < 50 years) was 30%. The duration from the onset or treatment initiation to death ranged from 7 to 63 months (mean 27 months) or 4 days to 38 months (mean 8 months), respectively.

4 Discussion

Only a small number of adult CAEBV cases have been reported in the literature; hence, their clinical features are still unclear and treatment strategies have not been evaluated. However, after the diagnostic criteria were suggested in 2005 [1], the diagnosis of CAEBV, especially in adults, has been increasing.

Adult cases consist of 2 groups, adult-onset and childhood-onset, and according to our analysis, their clinical

Table 3 Reported cases of adult-onset chronic active Epstein–Barr virus infection: clinical courses

No.	Sex	Age at diagnosis (years)	Age at onset (years)	Duration from onset to treatment initiation	Treatment	Outcome (duration from treatment initiation to death)	Duration from onset to death	Cause of death	References
1	M	24	22	24 MO	CHOP/HDCA/allo PBSCT (Sib)	Dead (6 M)	30 M	Pulmonary hemorrhage	[7]
2	M	36	35	12 MO	ND	ND			[8]
3	F	25	23	24 MO	VP16/PSL/CPA	Alive^a			[9]
4	M	29	27	24 MO	VP16/PSL/CPA	Alive^a			[9]
5	M	28	26	24 MO	IVIG, PSL + CY	Dead		Heart failure due to infiltration	[10]
6	F	35	35	6 MO	mPSL	Dead (1 MO)	7 M	Respiratory failure	[11]
7	F	57	56	12 MO	PSL, CHOP, CY + VP16	Dead (38 MO)	50 M	MOF due to disease progression	[12]
8	F	27	24	31 MO	allo RIST (Sib)	Alive (180 D)			[13]
9	F	69	68	21 MO	Chemotherapy (ND)	Alive (12 MO)			[14]
10	M	26	24	24–36 MO	Observation	Dead	36 M	Respiratory failure	15
11	F	71	68–69	24–36 MO	PSL + CY	Dead		DIC	[16]
12	M	56	54	24 MO	CPA + VCR + CY	Dead		Septic shock	[16]
13	M	33	27	36 MO	Steroid pulse therapy	Dead		Hemorrhagic shock due to HPS	[17]
14	M	52	52	6 MO	Steroid	Dead (4 D)	6 M	MOF due to disease progression	[18]
15	M	59	58	12 MO	CPA + VP16 + DEX/allo RIST (Sib)	Dead (3 M)	15 M	Gastric bleeding	[18]
16	F	26	26	ND	allo RIST (UR)	Alive^a			[19]
17	M	43	40	60 MO	VP16/PSL/CPA	Dead (3 M)	63 M	MOF, DIC	[20]
18	M	35	33	24 MO	ND	ND			[21]
19	M	48	47	12 MO	CHOP/HDCA/allo RIST (UR BM)	Alive in CR (25 MO)			OC 1
20	M	28	26	24 MO	CHOP/HDCA/allo RIST (UR BM)	Alive in CR (21 MO)			OC 2
21	F	37	36	6 MO	CHOP/allo RIST (UR BM)	Alive in relapse (60 MO)			OC 3
22	F	72	72	6 MO	CHOP	Dead (19 D)	7 M	MOF due to disease progression	OC 4
23	F	62	61	8 MO	In preparation				OC 5

PB peripheral blood, *M* male, *MO* month, *ND* not described, *HDCA* high-dose cytarabine, *allo*, allogeneic, *PBSCT* peripheral blood stem cell transplantation, *Sib* sibling, *F* female, *PSL* prednisolone, *CPA* cyclosporin A, *IVIG* intravenous immunoglobulin, *CY* cyclophosphamide, *mPSL* methyl-prednisolone, *RIST* reduced intensity stem cell transplantation, *MOF* multiple organ failure, *VCR* vincristine, *D* day, *DIC* disseminated intravascular coagulation, *HPS* hemophagocytic syndrome, *DEX* dexamethasone, *UR* unrelated, *BM* bone marrow, *CR* complete remission, *OC* our case

^a Observation period was not described

symptoms, EBV-DNA titers in PB, and EBV antibodies were similar. EBV-DNA was detected in PBMC in our 5 patients as in child-onset cases. However, their clinical courses were different. In adult-onset cases, the duration from onset to treatment initiation averaged 20 months. Death occurred in 12 cases (57%) and the duration from the onset of the disease or initiation of treatment to death averaged 27 or 8 months, respectively, in the patients whose clinical courses were documented. In contrast, Kimura et al. [9] reviewed 30 Japanese patients consisting of children and young adults and reported that young patients could be observed for 12–336 months (mean 71 months) without treatment. They also reported that the probability of survival at 5 years was 0.68 ± 0.06 for young patients. These data indicate that adult-onset cases may progress more rapidly and their prognosis may be poorer than those of younger patients.

Kimura et al. also analyzed 82 CAEBV patients (aged 9–53 years, median 11.3 years) and reported that the patients aged >8 years or with the T-cell type disease had a poor prognosis [22]. As shown in Table 2, 87% of the adult-onset patients were of the T-cell-infected type, whereas Kimura [9] reported that NK- and T-cell types were almost equal in prevalence (T cell 16/30, NK cell 12/30) in childhood-onset cases. Among adult-onset cases, 4 cases were considered to be γδ-T-cell type (Cases 2, 8, 11, and 12). Because the cause of T-cell dominancy is unknown, it may be one reason for the high mortality in adult cases. In addition, the elderly patients (aged > 50 years; Table 3) may have demonstrated aggressive disease courses and poor prognosis. The mortality of the elderly was significantly higher than the younger's. Analysis of CAEBV patients by age or infected cell types is needed to clarify the relation between these factors and pathophysiology of the disease.

No reports have reviewed the effects of chemotherapy in adult-onset CAEBV. As shown in Table 3, 2 patients (Cases 6 and 14) were treated with steroids alone. The treatment had no effect and the patients died within a month after initiation of treatment. Regarding our 4 patients, CP followed by CHOP treatment improved the clinical profiles of the patients. However, CHOP treatment was insufficient to eradicate EBV-infected cells in 3 patients and could not suppress disease progression in 1 patient. Grade 4 neutropenia was detected in all of our patients. Because the rate of developing grade 4 neutropenia after CHOP treatment is reported as 35% [23], the rate in our CAEBV patients might be high. The effects of CHOP treatment were also considered to be insufficient to eradicate EBV-infected cells in Cases 1 and 7 (Table 3). This chemo-resistant property of CAEBV may be an important factor distinguishing CAEBV from other lymphomas. One of the reasons for the ineffectiveness of

CHOP treatment may be an upregulated p-glycoprotein (p-gp) function in EBV-infected T or NK cells (unpublished data). Because doxorubicin and vincristine, included in the CHOP regimen, are removed by p-gp, they may not exercise a sufficiently potent effect on CAEBV cells.

Despite pre-administration of prednisolone, high-dose cytarabine (HDCA), included in the Capizzi regimen, produced a severe grade 4 advanced effect in our 2 cases (Cases 1 and 2). In the original report, the Capizzi regimen, consisting of HDCA and L-asparaginase, produced a grade 4 fever in 34% of patients; however, grade 4 cardiopulmonary disease was demonstrated in <1% of patients [24]. Ek et al. [25] reported that administration of 2 g/m^2 of cytarabine every 12 h to children with leukemia and lymphoma resulted in 13 of 16 patients developing a temperature of >38°C. They also reported that inflammatory cytokines such as tumor necrosis factor-α, interleukin-6, and interferon-γ were elevated; however, no serious disease occurred in these patients [25]. These cytokines can be produced and secreted by T cells, which are significantly activated in CAEBV, indicating that severe acute onset reactions resulting from administration of cytarabine may be peculiar to CAEBV patients, especially patients of the T-cell-infected type. More investigation is needed to evaluate the effect and safety of cytarabine in CAEBV treatment and whether the development of severe adverse events depends on patients' age or infected cell type.

In conclusion, adult-onset CAEBV seems to be more aggressive and has different clinical features from those of childhood-onset cases. Treatment with CHOP may be insufficient, and HDCA may produce severe adverse events. Further study is needed to clarify clinical features and to establish optimal treatments for CAEBV in children and adults.

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INTERNAL MEDICINE

Autoimmune Hemolytic Anemia Accompanied by Reactivation of an Epstein-Barr Virus Infection with Suppressed CTL Response to EBV-infected Cells in an Elderly Man

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Abstract

An 88-year-old man with autoimmune hemolytic anemia (AIHA) who had been treated with low dose prednisolone developed a sudden worsening of his anemia accompanied by reactivation of Epstein-Barr virus (EBV). We established EBV-infected spontaneous lymphoblastoid cell lines (LCL), performed an enzyme-linked immunosorbent spot assay, and confirmed a significantly suppressed EBV-specific cytotoxic T-cell (CTL) response to the LCL. EBV reactivation might have been brought about by suppressed CTL activity which could have been due to low dose PSL administration or aging. Since the EBV-DNA titer decreased as AIHA improved, we concluded that EBV might have played a role in the development of anemia.

Key words: autoimmune hemolytic anemia, Epstein-Barr virus, reactivation, enzyme-linked immunosorbent spot assay

Introduction

Autoimmune hemolytic anemia (AIHA) is an acquired hemolytic anemia in which pathologic antibodies destroy erythrocytes. Various diseases and conditions are responsible for the development of AIHA including viral infection, autoimmune disease, immune deficiency status, lymphoproliferative disorders, other malignancies, drugs and so on.

Here, we report a man AIHA patient accompanied by reactivation of Epstein-Barr virus (EBV). EBV is a human disease pathogen which is especially common in Asian countries including Japan. Almost 90% of people acquire the viral infection during their childhood or adolescence (1). Once infected, the infection persists and becomes latent in B-cells. If the immune system is suppressed by transplantation, lymphoma, or HIV infection, EBV is reactivated and can cause lymphoproliferative disorders (LPD) (2). Recently, Oyama and colleagues evaluated EBV-associated B-cell LPD and speculated that EBV can be reactivated by age-related

immunological deterioration resulting in LPD, which they called age-related EBV-associated B-cell LPD (3). The disorder now is defined as EBV-positive diffuse large B-cell lymphoma of the elderly according to the new World Health Organization (WHO) classification (4). However there had been no report proving suppressed EBV-specific cytotoxic T cell (CTL) activity in such patients.

A primary EBV infection is known to cause AIHA (5). We considered that EBV reactivation of the present patient might have played a role in the development of AIHA crisis because his crisis was accompanied by elevation of the EBV-DNA titer, and it improved as the EBV-DNA titer decreased. In addition, we investigated and detected actually suppressed EBV-specific CTL activity by enzyme-linked immunosorbent spot (ELISPOT) assay. Aging, as well as prednisolone (PSL) administration could have been reasons for the reactivation of EBV.