CASE REPORT

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.

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Materials and Methods

A sample from a patient with infectious mononucleosis (IM) was used as control to measure CTL activity.

Establishment of spontaneous LCL

EBV-infected spontaneous lymphoblastoid cell lines (LCL) were established from CD19-positive B cells in the peripheral blood of this patient and the control patient in the acute phase IM. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Hypaque density gradient centrifugation. Then CD19-positive cells were separated from these cells using anti-CD19 antibody conjugated magnetic beads (IMag Human CD19 Particles-DM; BD Biosciences, San Jose, CA). We cultured the cells in 10% FCS RPMI to establish an LCL. The EBV infection in the LCL was confirmed by RT-PCR.

Measurement of EBV-specific CTL activity

ELISPOT assay was performed with the IMMUNOCYTO IFN-γ ELISPOT kit (Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) following instructions supplied by the manufacturer as described previously (6). Briefly, CD8-positive T cells were isolated from PBMCs with the IMag anti-human CD8 Particles-DM (BD Biosciences). Samples were obtained from this patient in remission and the control IM patient after recovery phase, respectively. The viral loads at harvest were similar in the two; 8.0×10^2 copies/μgDNA in the patient, and 5.0×10^2 copies/μgDNA in the control. Mixture of these CD8-positive T cells and the LCL were incubated with IL-2 in microplates coated with antibody to IFN-γ for 17h. Captured IFN-γ was detected by biotinated antibody to IFN-γ and alkaline phosphatase conjugated streptavidin, and visualized by reaction with the BCIP/NBT Chromogen Substrate.

The study was approved by the ethical committee of Tokyo Medical and Dental University and written informed consent was obtained from this patient and the control IM patient.

Case Report

An 88-year-old man with no remarkable history was diagnosed with AIHA of unknown cause 9 years prior to admission. On two occasions (1 and 7 years before admission) his anemia had worsened. The triggers of developing anemia of both occasions could not be detected. Oral PSL of 30 mg/day (0.5 mg/kg) was started during his hospitalizations on both occasions, and he recovered soon. During the first hospitalization, PSL was discontinued successfully after AIHA improved; however, during his second hospitalization anemia relapsed as the PSL was tapered; therefore, he had been administered 15 mg of PSL every other day until this admission.

The clinical course of this admission is shown in Fig. 1. Two days before admission, the patient developed fever. As his symptoms became worse, he was admitted to the previous hospital. Although no focus of infection could be detected, sulbactam/ampicillin (ABPC/SBT) was initiated at 3 g/day. His hemoglobin (Hb) concentration was 8.6 g/dL on admission, but on the second hospital day, it drastically decreased to 3.6 g/dL, following which he was transferred to our hospital for further investigation and treatment of ane-
On admission, his body temperature was 37.9°C. On physical examination, he had severe anemia visible in the conjunctiva, however no jaundice was detected. His chest was normal. He had no lymphadenopathy or hepatosplenomegaly. He had marked normocytic normoclonic anemia; red blood cell (RBC), 128×10⁴/μL; Hb, 3.9 g/dL; mean corpuscular volume, 93.8 fl; mean corpuscular hemoglobin concentration, 32.5%. The number of reticulocytes was lower than previous blood examinations. His peripheral blood leukocytes were normal in number with no appearance of atypical lymphocytes. Platelets were normal, too. LDH (480 IU/L) and conjugated bilirubin (3.1 mg/dL) were elevated (Fig. 1). Both direct and indirect Coombs tests were positive, and haptoglobin was undetectable. Cold agglutinin was negative. From these findings, the patient was diagnosed with a hemolytic crisis of AIHA. Because fever preceded the progression of anemia, we suspected infection as a trigger of this crisis and blood was obtained for bacterial examination and detection of viral DNA. The results showed a significant increase in EBV DNA copies, 2.3×10⁵ copies/μg DNA (Table 1). Anti-EBV I gG and anti-EBV I gM were positive, and anti-EBV I gM was not high titer. We considered these findings revealed reactivation of EBV. Parvo virus B19 DNA was not detected. Bacterial examination, and the antigens and antibodies for the hepatitis B and C virus were negative, too (data not shown).

After RBC transfusion, we immediately increased the PSL to 50 mg/day (1 mg/kg). His LDH and bilirubin, then decreased gradually, suggesting that the hemolysis had resolved. However, it was more than 5 days before the numbers of reticulocytes and RBCs began to increase. ABPC/SBT was discontinued on hospital day 7 when his fever resolved. We started to taper the PSL on hospital day 11, and he was discharged on hospital day 19 receiving 30 mg/day of PSL. Anemia improved and PSL was tapered to 10 mg without recurrence of AIHA. EBV DNA copies decreased to 1.6×10⁵ copies/μg DNA. We examined the bone marrow later, and neither dysplasia nor chromosomal abnormality could be detected.

To investigate the cause of reactivation of EBV, we tried to detect EBV-specific CTL activity after achieving remission. First, we performed flow cytometric analysis of PBMCs and detected that they consisted of 5.3% of CD19-positive, 39.8% of CD4-positive, 29.6% of CD8-positive, and 23.5% of CD56-positive cells. CD19-positive cells were markedly decreased. Second, we tried and successfully established LCL from CD19-positive cells in PBMCs. Since these cells proliferated spontaneously in vitro and were positive for EBV as those of the control patient of acute phase IM, it was speculated that the EBV-infected cells of the patient were B-cells and might be activated and immortal in vivo. From these results, we considered that his state was the same as LPD. To investigate CTL activity, we performed ELISPOT assay against these cells. As shown in Fig. 2D, significant suppression of CTL activity was detected and considered the cause of EBV reactivation.

### Discussion

The patient had been diagnosed with AIHA and had been administered low dose PSL for about one year. On his admission, he suddenly developed worsening AIHA with fever. ABPC/SBT, which had been started one day before admission, could have been the cause of hemolysis. However, it was resolved despite continuing the administration of ABPC/SBT. On the other hand, the crisis was accompanied by reactivation of EBV. The EBV-DNA titer was closely associated with the course of anemia, which suggested that EBV reactivation might have played a role in the development of anemia.

Erythropoietic failure due to EBV infection can be developed by two major mechanisms; one is hemolysis, the other is suppression of erythropoiesis itself. AIHA is accompanied by a 0.1-3% EBV infection rate (7) but usually by a primary EBV infection. To the best of our knowledge, this is the first case of AIHA accompanied by reactivation of an EBV infection. The mechanism of the development of AIHA due to an EBV infection has not been clarified. Antibodies against EBV may cross-react with antigens expressed on the surface of RBCs and directly attack and destroy...
them. Riboldi et al. (8) reported that EBV-infected B cells produce anti-i IgM cold agglutinin, which cross-reacts with an RBC surface antigen in vitro. Anti-i IgM cold agglutinins are autoantibodies mainly responsible for the development of AIHA (9), suggesting that the RBCs in our patient, who also had EBV-infected B cells, might have been destroyed by this mechanism. Actually we could not detect cold agglutinin in the patient, but we examined it after increasing the PSL up to 1 mg/day. Increasing the dose was effective possibly by suppressing the EBV-infected B cells which produced antibodies, including cold agglutinin.

The other possible mechanism of erythropoietic failure due to EBV is suppression of erythropoiesis in the bone marrow. Pure red cell aplasia (PRCA) can occur with an EBV infection (10) and five such cases have been reported to occur due to reactivation of EBV (11-15). There was no reactive increase in reticulocytes on admission in this patient which suggested that the erythroid cells might be dominantly suppressed. The pathogenesis of EBV-induced PRCA has not been determined. Erythroid cells do not express the CD21 molecule which is known as the target of EBV infection. However EBV can interact with other molecules including HLA-class II (16) which is expressed on the surface of erythroid cells. In addition, EBV infection induces T-cell activation and its cytokine production (17). Produced cytokines may suppress hematopoietic cells including immature erythroid cells (18). Further study is necessary to prove these hypotheses and identify whether EBV infection suppresses erythroid cells directly or indirectly.

The EBV-infected LCL was successfully established from CD19-positive cells in PBMC. Furthermore, an ELISPOT assay proved that the CTL activity was suppressed against the LCL. In addition, as clonality could not be proved due to the lack of cells, it was considered that the cells might become immortal and his status might be EBV-associated B-cell LPD.

The present patient had received low dose PSL for about one year. Although a relationship between low dose administration of PSL and EBV reactivation has not been reported, it may suppress CTL activity and produce reactivation of EBV, just as methotrexate. Cases should be accumulated to examine this possibility. Another possible cause of EBV reactivation is aging. This disorder was summarized by Oyama et al. in 2003 (3) and it is now defined as EBV-positive diffuse large B-cell lymphoma of the elderly in the new WHO classification (4). It is an EBV-positive clonal B-cell proliferation that occurs in patients over the age of 50 who are not immunodeficient and who do not have prior lymphoma. It had been speculated that its pathogenesis was due to immunological deterioration by aging. We actually detected CTL suppression against EBV and it was compatible with the hypothesis.

The present patient highlighted the fact that EBV reactivation can cause not only lymphoma but also various diseases including AIHA. Further investigation should be conducted to clarify their clinical features and determine the mechanism of the EBV reactivation.

References

