Short Communication

Cytokine Profile Changes During the Initial Stage of Treatment for Childhood Epstein–Barr Virus–Associated Hemophagocytic Lymphohistiocytosis

Running title: Cytokine profile in EBV-HLH treatment

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Abstract

Hemophagocytic lymphohistiocytosis (HLH) associated with Epstein–Barr virus (EBV) infection ranges from self-limiting to severe/aggressive or fatal. We report the case of a 4-year-old boy with EBV-HLH with persistent fever, severe pancytopenia, hypertriglyceridemia, and hypofibrinogenemia. The levels of plasma cytokines and chemokines were measured using a Bio-Plex system on Days 1, 2, 3, 4, 5, and 8 following hospital admission. The administration of steroid and high-dose intravenous immunoglobulin (1 g/kg) did not alleviate the fever or reduce cytokine production; however, following etoposide (a known antineoplastic agent) administration, the fever decreased immediately, and the patient’s general condition improved. The present study revealed that IL-6, IL-10, IL-8, MCP-1, IFN-γ, and TNF-α were suppressed by etoposide administration. In particular, production of IFN-γ sharply declined from 1,104.1 pg/mL to 101.5 pg/mL, and the IL-6 level also decreased from 229.8 pg/mL to 11.0 pg/mL the day after the initial etoposide administration. Subsequently, there was no recurrence of symptoms with dexamethasone, etoposide, and cyclosporine A treatment. The present case demonstrates that early etoposide administration is critical for successful treatment and indicates that etoposide exhibits a prompt inhibitory effect on cytokine production.
Key words: Epstein–Barr virus, hemophagocytic lymphohistiocytosis, immunochemotherapy, cytokine, etoposide

Introduction

Primary Epstein–Barr virus (EBV) infection is usually asymptomatic but may cause infectious mononucleosis (IM). Hemophagocytic lymphohistiocytosis (HLH) in children is characterized by persistent fever, splenomegaly with cytopenia, hypertriglyceridemia, and hypofibrinogenemia. Infiltration of histiocytes with hemophagocytic activity is usually observed in the bone marrow. HLH is generally divided into two types: primary and secondary HLH. The former is caused by genetic defects such as those in PRF1 (which encodes perforin)\(^1\). EBV-associated HLH (EBV-HLH) is considered a major subtype of secondary HLH. Recently, the pathogenesis of HLH has been shown to involve impaired activation of T lymphocytes following stimulation by immune responses, resulting in large quantities of inflammatory cytokines that promote macrophage infiltration and cytokine network formation \(^1\). Studies on cytokine profiles in HLH patients have demonstrated elevated concentrations of many pro-inflammatory cytokines such as interferon gamma (INF-\(\gamma\)), tumor necrosis factor alpha (TNF-\(\alpha\)), and interleukin-6 (IL-6) \(^2\).

Here, we report the case of a 4-year-old boy in whom we investigated cytokine profile
changes in the first 8 days of EBV-HLH treatment. To our knowledge, there are no published reports on cytokine profile changes during initial-stage treatment of childhood EBV-HLH.

Case Report

A 4-year-old boy was admitted with a 10-day history of persistent high fever, cervical lymph node swelling, and general malaise. There was no significant illness in his medical or family history. On admission, blood examination revealed a white blood cell count of 0.9×10^9/L including 1% atypical lymphocytes, a hemoglobin level of 6.6 g/dL, and a platelet cell count of 2.4×10^9/L. Coagulation examination showed a fibrinogen level of 83.6 mg/dL (170–400 mg/dL) and a fibrin degradation product level (FDP) of 28.7 µg/mL (<10 µg/mL) (normal ranges in parentheses). Other laboratory findings were as follows: total bilirubin 0.3 mg/dL (<0.85 mg/dL), aspartate aminotransferase (AST) 217 IU/L (<40 IU/L), alanine aminotransferase (ALT) 43 IU/L (<28 IU/L), lactate dehydrogenase (LDH) 4,021 IU/L (<350 IU/L), blood urea nitrogen (BUN) 10.6 mg/dL (<19.5 mg/dL), creatinine 0.37 mg/dL (<0.42 mg/dL), triglyceride 369 mg/dL (<149 mg/dL) ferritin 15,954 ng/mL (<140 ng/mL), soluble interleukin 2 receptor (sIL2-R) 13,237 U/mL (<496 U/mL), EBV
viral capsid antigen (EBV-VCA) IgM titer 10, EBV-VCA IgG titer 160, and EBV-associated nuclear antigen (EBNA) negative. Bone marrow aspiration at initial diagnosis showed hypoplastic marrow with marked hemophagocytosis. Cytogenetic analysis of the bone marrow showed 46,XY (20/20). EBV DNA was positive in peripheral blood (5×10^6 copies/mL) and we diagnosed EBV-HLH after excluding other malignancies.

Dexamethasone (10 mg/m^2) administration was started on admission and high-dose intravenous immunoglobulin (1 g/kg) was administered on Day 3. Despite this, the fever persisted, and the laboratory findings revealed thrombocytopenia and severe neutropenia (180/μL); the ferritin level increased to 46,580 ng/mL, about three times the level on Day 1 (on hospitalization), and LDH also increased to 6,912 IU/L (Fig. 1). We then administrated etoposide on Day 4. Prior to etoposide therapy, the levels of IL-6, IFN-γ, and monocyte chemoattractant protein (MCP-1) were increasing, and IL-10 and TNF-α levels were unchanged with high expression from Day 3 to Day 4. However, following etoposide therapy the fever was reduced immediately, and the expression of several critical cytokines sharply decreased from Day 4 to Day 5 (Fig. 1 and Table 1).

Thereafter, the patient received dexamethasone, etoposide, and cyclosporine A. The final administration of etoposide was on Day 25 (a total of 6 administrations, total dose of 600 mg/m^2), and DEX was still administered at 5 mg/m^2. DEX dosage was reduced
from Day 43 to 2.5 mg/m². After confirming that there was no recurrence of the symptoms and that the copy number of EBV DNA on Day 49 was negative, the patient was discharged. No serious adverse events were noted during the treatment. He was subsequently discharged on Day 56. Since then his general health has been good, with no recurrence of symptoms in the year following treatment.

Methods

We analyzed plasma cytokine and chemokine levels using a Bio-Plex system on Days 1, 2, 3, 4, 5, and 8 following hospital admission. Serum aliquots (50 μL) were collected from peripheral blood and analyzed using the Bio-Plex Pro™ Human Cytokine Grp 1 Panel 27-plex Assay according to the manufacturer’s protocol (Bio-Rad, Hercules, CA, USA). Cytokine and chemokine concentrations were calculated using Bio-Plex Manager 3.0 software (Bio-Rad, Tokyo, Japan) with a five-parameter curve-fitting algorithm applied for standard curve calculations.

Discussion

Our aim was to evaluate the effect of therapeutic drugs on cytokine production and clinical symptoms by measuring cytokine production in the very early stages of EBV-
HLH treatment. It was found that etoposide plays a critical role in the treatment of severe EBV- HLH. It is well known that EBV- HLH manifests as uncontrolled activation of the immune system and hypercytokinemia. Imashuku et al reported that etoposide treatment within 4 weeks of HLH diagnosis improved the prognosis of patients with EBV- HLH. Although hypercytokinemia ceases with treatment, there are no reports of cytokine measurements being made at short regular intervals (i.e. daily) during therapeutic drug administration in the early stages of treatment.

In our patient both the symptoms and serum cytokine levels worsened even when steroid and immunoglobulin therapies were administered in the initial treatment stage; however, fever was reduced, and cytokine production was markedly suppressed on the day following initial etoposide administration. Early use of immunoglobulin and/or corticosteroid is sometimes useful for controlling HLH activity, with transient effect.

In a recent retrospective nationwide survey, more than half of the patients with EBV- HLH were treated with immunochemotherapy based on the HLH-94/HLH-2004 protocol in Japan which includes dexamethasone, etoposide, and cyclosporine A, while 30% were treated with a corticosteroid-based therapy, and 10% with only supportive therapy, resulting in complete remission in 90% of the patients following initial therapy. Among several prognostic factors, patients with both hyperbilirubinemia (>1.8 mg/dL) and
hyperferritinemia (>20,300 ng/mL) at the time of diagnosis had significantly poorer outcomes than those with low serum bilirubin and ferritin levels. A disease state in which CD8-positive cytotoxic T lymphocytes (CTL) are the main EBV-infected cells is often classified as EBV-HLH. The presence of clonal CTL proliferation suggests that CTL over-activation by direct infection with EBV is considered to be a basic pathological condition. We examined several cytokines during early-stage treatment on Days 1, 2, 4, 5, and 8. Han reported that IL-6, IL-10, and INF-γ levels were significantly increased in EBV-HLH patients. In our patient, the administration of steroid and intravenous immunoglobulin did not reduce fever, and cytokine production and pancytopenia progressed, and the ferritin level increased to 46,580 ng/mL; however, IL-6, IL-10, IL-8, MCP-1, IFN-γ, and TNF-α levels were suppressed by etoposide administration. IL-6, IFN-γ, and TNF-α are pro-inflammatory cytokines that are regarded as the “gatekeepers of inflammation”. IL-8, a CXC chemokine, activates neutrophils, and MCP-1 is involved in monocyte/macrophage infiltration, both of which have been reported to be significantly elevated in the active phase in HLH. In our patient, the IFN-γ level notably decreased from 1,104.1 pg/mL on Day 4 to 101.5 pg/mL on Day 5. On Day 8, the day after a second dose of etoposide was administered, it had fallen to 14.8 pg/mL. The IL6 level also decreased from 229.8 pg/mL on Day 4 to 11.0 pg/mL on Day 5 to 4.5 pg/mL on Day 8; the
MCP-1 level also decreased similarly. These results indicate that etoposide exhibits a prompt inhibitory effect on cytokine production. Furthermore, suppression of the inflammatory cytokines IL-17 and RANTES from Day 2 to Day 3 was considered to be due to the effect of steroid treatment. However, on Day 8, the levels of RANTES and PDGF were re-elevated, suggesting that it may be necessary to continue treatment. Among the anti-inflammatory cytokines, IL-10, IL-4, and IL-13 showed little change. However, the level of IL-1Ra, a receptor antagonist of the inflammatory cytokine IL-1, increased from the start of treatment and reached the highest level on Day 8. It is an interesting result that the value of IL-18 did not increase. It has been shown that IL-1Ra suppresses IL-1 (α and β) activity by competitively inhibiting the binding of IL-1 to its receptor. Thus, if IL-1Ra is produced in significant excess relative to IL-1, IL-1 activity could be suppressed and, consequently, the inflammatory response might be suppressed as well.

To date, the role of IFN-γ in EBV-HLH has not been fully elucidated. Research on primary HLH model in mice that are perforin-deficient has shown that anti-IFN-γ antibodies were effective for treatment, and IFN-γ is considered to be a driver cytokine for the onset of HLH. Interestingly, mouse experiments have recently demonstrated that etoposide selectively and rapidly removes activated T cells. Our analysis suggests
that this phenomenon is also involved in the actual clinical treatment due to the rapid decrease in inflammatory cytokines following etoposide administration. Ishii recommends initial treatment with high-dose immunoglobulin and/or corticosteroid therapy for all patients with EBV-HLH, followed by immunochemotherapy for those resistant to initial therapy or with several risk factors. In our case, when no initial treatment response was obtained, early administration of etoposide was found to be effective in the immediate suppression of several cytokines. The mechanism of action of etoposide (a known antineoplastic agent) in the treatment of EBV-HLH is not fully understood. The present data may advance our understanding; however, data from multiple cases are required to confirm the effects of etoposide on cytokine kinetics in early-stage EBV-HLH treatment.

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Conflict of Interest: The authors declare that they have no conflicts of interest.
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Figure Legend

Fig. 1: Clinical course in the present case showing sequential changes in ferritin levels and fever status during treatment. Day 1 denotes hospital admission. BT, body temperature; DEX, dexamethasone; IVIG: intravenous immunoglobulin; Hb, hemoglobin; LDH, lactate dehydrogenase; Plt, platelet transfusion; RCC, red blood cell transfusion; VP-16, etoposide; WBC, white blood cell.
Table 1. Analysis of serum cytokine levels

| Cytokine (pg/mL) | Day 1 | Day 2 | Day 3 | Day 4 | Day 5 | Day 8 |
|------------------|-------|-------|-------|-------|-------|-------|
| IFN-γ            | 790.6 | 1259.8| 825.9 | 1104.1| 101.5 | 14.2  |
| IL-6             | 117.6 | 36.2  | 25.1  | 229.8 | 11.0  | 4.5   |
| IL-10            | 225.4 | 20.1  | 12.9  | 12.2  | 7.5   | 5.9   |
| TNF-α            | 5.7   | 74.4  | 34.4  | 57.4  | 52.5  | 14.3  |
| MCP-1            | 434.0 | 480.3 | 322.8 | 1152.2| 284.0 | 93.4  |
| IL-8             | 34.0  | 169.4 | 92.4  | 228.2 | 128.1 | 19.5  |
| IL-13            | 2.1   | 4.0   | 1.3   | 1.3   | 1.6   | 5.9   |
| II-17            | 5.2   | 55.8  | 20.3  | 36.1  | 36.8  | 16.5  |
| RANTES           | 482.3 | 3788.5| 546.1 | 405.8 | 260.4 | 1004.2|
| IL-1β            | 1.3   | 3.6   | 0.86  | 2.59  | 2.0   | 0.8   |
| IL-1Ra           | 1077.2| 1426.2| 817.1 | 3345.9| 2007.3| 4957.8|
| G-CSF            | 17.9  | 102.4 | 21.0  | 68.4  | 39.5  | 19.8  |
| GM-CSF           | 146.3 | 172.6 | 177.8 | 172.9 | 162.7 | 135.5 |
| IL-12            | OOR<  | 9.5   | OOR<  | 81.0  | 41.8  | OOR<  |
| PDGF-β           | 81.4  | 115.2 | 67.3  | 82.6  | 45.1  | 301.8 |
| MIP-1β           | 88.9  | 253.2 | 71.4  | 103.0 | 49.1  | 32.1  |
| IP-10            | 11321.3| 80694.3| 74015.6| 78186.0| 76922.3| 3138.6|
| FGF basic        | 30.1  | 91.1  | 52.2  | 63.2  | 70.0  | 40.4  |
| Eotaxin          | 11.7  | 47.6  | 30.5  | 37.8  | 46.3  | 35.0  |
| MIP-1α           | 5.0   | 8.7   | 2.5   | 4.3   | 2.1   | 0.5   |
| VEGF             | 24.3  | 29.2  | 17.3  | 22.1  | 28.7  | 20.1  |
| IL-9             | 22.17 | 80.16 | 18.7  | 26.23 | 16.0  | 21.2  |
| IL-15            | OOR<  | 50.79 | OOR<  | 80.99 | 41.85 | OOR<  |
| IL-7             | 1.39  | 3.02  | 1.60  | 3.21  | 1.80  | 2.62  |
| IL-2             | OOR<  | 3.60  | OOR<  | 2.96  | 2.23  | OOR<  |
| IL-4             | 0.55  | 1.48  | 0.58  | 1.04  | 0.99  | 1.81  |
| IL-5             | OOR<  | OOR<  | OOR<  | OOR<  | OOR<  | OOR<  |

*On Days 1, 2, 3, 4, 5, and 8 following hospitalization.

IFN-γ, interferon gamma; IL, interleukin; MCP, monocyte chemoattractant protein; TNFα, tumor necrosis factor alpha; RANTES, regulated on activation normal T Cell expressed and secreted; G-CSF, granulocyte-colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; PDGF, platelet-derived growth factor; MIP, macrophage inflammatory protein; VEGF, vascular endothelial growth factor; OOR, out of range.