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ORIGINAL ARTICLE

Microbiological Findings and Treatment of EBV-Associated Hemophagocytic Lymphohistiocytosis: A Case Report

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Abstract Epstein–Barr virus (EBV) is the major triggering factor for hemophagocytic syndrome or hemophagocytic lymphohistiocytosis (HLH). In patients with EBV-HLH, the EBV-infected T cells or natural killer cells are mostly mono- or oligoclonally proliferating, whereby hypercytokinemia plays a major role and causes hemophagocytosis, cellular damage, and dysfunction of various organs. This report describes the detection and treatment of EBV-associated HLH in the case of a 17-year-old male. Serum samples and skin swabs were tested for the presence of viral DNA using real-time PCR techniques. To confirm the molecular biological tests, electron microscopy was also performed. EBV DNA was detected with real-time PCR in both blood samples and skin swabs. The level of viral DNA constantly decreased during the applied therapy. The presence of the virus in the skin was confirmed by the appearance of herpes virus-like particles detected by electron microscopy in fluid taken from skin ulcerations. The results show that in terms of treatment, special therapeutic measures are required to control the cytokine storm generated by EBV and to suppress proliferating EBV genome-containing cells because the clinical course is often fulminate and results in a poor outcome. Therefore the potential of chemotherapy with a combination of steroids, etoposide, and cyclosporine to control HLH was assessed in the adolescent, who met the stringent diagnostic criteria for this reactive disorder of the mononuclear phagocyte system.

Keywords Epstein–Barr virus · Hemophagocytic lymphohistiocytosis · Real-time PCR

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Introduction

Epstein–Barr virus (EBV) is a member of the *γ-Herpesviridae* subfamily within the *Herpesviridae* family. Infections with EBV are ubiquitous, and over 90% of the adult population has IgG antibodies against its viral capsid antigen (VCA) and/or nuclear antigen (EBNA) (Kasahara and Yachie 2002; Okano 2000; Sullivan et al. 1985). Primary infections with EBV are characteristic of two age groups: children aged 2–5 years and adolescents and young adults 13–25 years old (Kasahara and Yachie 2002; Sullivan et al. 1985). Although EBV infections are widespread, they are rarely associated with clinical symptoms. An efficient immunological system is the main barrier containing the virus and preventing it from uncontrolled replication within susceptible cells. Clinically, the most popular variant of EBV infection, resulting from $CD21^+$ cell involvement, typically appears as infectious mononucleosis (Jenson 2000; Okano 2000). In contrast, in rare cases natural killer (NK) and activated T cell populations can be the predominant type of cellular targets for EBV. Two clinical syndromes, chronic active EBV infection (CAEBV) and hemophagocytic lymphohistiocytosis (HLH), can result from this variant of infection (Imashuku 2002). In CAEBV, the virus infects mainly NK cells and $CD4^+$ T lymphocytes, while patients with HLH have primarily infected $CD8^+$ T cells (Imashuku

et al. 2004; Kasahara and Yachie 2002). Cells harboring the EBV genome are localized in the lymph nodes, liver, and bone marrow (Imashuku 2002; Verbsky and Grossman 2006).

Thus EBV-associated HLH is a result of disturbance in the control of the inflammatory process, resulting in a hyperinflammatory response of improperly activated immunological cells infected with EBV (Imashuku 2002; Kasahara and Yachie 2002; Verbsky and Grossman 2006). Uncontrolled inflammation leads to the production of clinically significant amounts of proinflammatory cytokines (interferon-γ, interleukin (IL)-6, IL-10, and tumor necrosis factor-α) secreted by macrophages, histiocytes, and activated lymphocytes; this process is accompanied by functional paralysis of NK cells (Chuang et al. 2007; Imashuku 2002; Kasahara and Yachie 2002; Verbsky and Grossman 2006). Along with macrophage infiltration, it may lead to the presence of hemophagocytosis, hypercytokinemia, and, eventually, with disease progression, to marrow necrosis associated with severe cytopenia (Chuang et al. 2007; Imashuku 2002; Sullivan et al. 1985). The increase in phagocytic macrophage number leads to hypertriglyceridemia and hyperferritinemia (Imashuku 2002; Tiab et al. 2000; Verbsky and Grossman 2006). Clinically, at the late stage of HLH patients present the full range of symptoms: liver dysfunction, coagulopathy, hemorrhages, renal and respiratory distress, and sepsis-like disease (Imashuku et al. 2004; Kasahara and Yachie 2002; Sullivan et al. 1985).

EBV-induced HLH is diagnosed most often in East Asian countries (Japan, Taiwan), but it is also noted in Western countries, and in the majority of cases, HLH appears in apparently immunocompetent children or young adults, which indicates that HLH is a result of acute primary infection (Fisman 2000; Imashuku 2002; Imashuku et al. 2004). In Europe, EBV-associated HLH seems to be less frequent, but it should be noted that the epidemiology of the disease is difficult to describe because of diagnostic difficulties and the relatively small number of the centers prepared to recognize HLH and share the results (Fisman 2000). EBV-associated HLH is burdened with significant mortality (literature data reports mortality rates from 25 to 100%, depending on the surveying group) (Fisman 2000; Kasahara and Yachie 2002; Verbsky and Grossman 2006). The introduction of a proper therapeutic procedure and diagnostic criteria (HLH-94, HLH-04) seems to decrease the mortality significantly; thus the rapid and accurate identification of EBV-associated HLH becomes a matter of importance (Fisman 2000; Imashuku et al. 2001; Imashuku et al. 2004; Tiab et al. 2000; Verbsky and Grossman 2006).

We believe this article reports the first virologically documented EBV-associated case of HLH in Poland.

Case Report

A 17-year-old immunocompetent male Caucasian presented to a family physician in the middle of May of 2007 because of pharyngotonsillitis, fever (39°C), lymphadenopathy, and hepatosplenomegaly. Viral infection of the upper respiratory tract was initially diagnosed. For 5 days the patient received ambulatory symptomatic treatment and, because of persisting symptoms (high-grade fever (40°C) and the appearance of maculopapular mucosal and cutaneous rash), the patient was admitted to a local hospital, where infectious mononucleosis was diagnosed on the basis of clinical symptoms and heterophilic antibodies present in the patient's serum. After 2 days the patient was transferred to the pediatric ward of the central provincial hospital where, during his 14-day stay, worsening of symptoms was observed: the development of mucosal and cutaneous changes with a drift toward vesicles and ulcerations, secondary hemorrhagic skin changes, hemorrhages, and erosions on the oral cavity mucosa. Lymphadenopathy, hepatosplenomegaly, jaundice, and a small amount of fluid in the pleural cavity and pericardium were also noted. Additional examinations revealed increasing leukopenia, thrombocytopenia, hypoproteinemia, and traits of liver dysfunction (AspAT: 869 U/l, AlAT: 404 U/l, bilirubin: 8.8 mg/dl). Cerebrospinal fluid examination and myelogram results presented a normal picture. Empirical therapy was introduced, including acyclovir, inosine pranobex, and broad-spectrum antibacterial agents. Immunoglobulins and steroids were also administered, without the desired clinical effect. Because of the patient's worsening status, i.e. increase in pancytopenia and further signs of liver dysfunction, the patient was transferred to the Hepatology Clinics and, shortly thereafter, to the Hematology and Oncology Clinics of Warsaw Medical University Clinical Hospital (WMUCH).

At the time of admission to the Hematology and Oncology Ward of WMUCH, the patient presented persistent increased body temperature without considerable changes between morning and evening measurements (38.6–40.0°C), hemorrhagic ulcerations affecting large areas of the body (Fig. 1), hepato- and splenomegaly, jaundice, bleeding from the nose and gums, worsened general physical state, and weight loss. Laboratory findings revealed increased hepatic markers (AspAT: 794 U/l, AlAT: 300 U/l, bilirubin: 15.7 mg/dl), blood coagulability deficiency (platelet count: 95×10^3 μl^{-1}), peripheral blood pancytopenia with lymphopenia (leukocytes: 0.67×10^3 μl^{-1}, erythrocytes: 2.68×10^6 μl^{-1}), and also highly increased levels of ferritin (>20,000 ng/ml), D-dimers (24,800 ng/ml), LDH (7,300 U/l), GGTP (442 U/l), triglycerides (596 ng/dl), and C-reactive protein (28.4 mg/l). In the phenotypic examination of bone marrow,

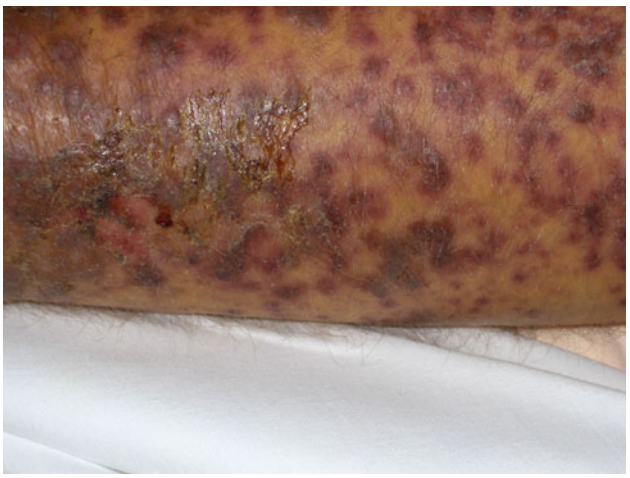

Fig. 1 Ulcerative hemorrhagic skin changes observed on the patient's arm on day +2

hemophagocytosis was observed with an increased rate of $CD8^+$ cells and decreased numbers of $CD4^+$ and NK cells.

Materials and Methods

Blood for virological examinations was taken for 5 weeks, starting on the first day after admission, and swabs from ulcerative skin changes were taken every 7 days. Whole DNA was isolated from blood serum and skin swabs using a High Pure Viral Nucleic Acid Kit (Roche Diagnostics) according to the manufacturer's procedure. In the serum and skin swab samples the presence of DNA of herpes simplex viruses type 1/2 (HSV-1/2), varicella-zoster virus (VZV), EBV, cytomegalovirus (CMV), parvovirus B19, and human herpesvirus 6 (HHV-6) was tested. The examinations were performed using the real-time PCR technique in a LightCycler 2.0 apparatus with the use of qualitative detection kits for HSV-1/2 and VZV (HSV 1/2 Qual Kit, VZV Qual Kit) and quantitative detection kits for parvovirus B19, EBV, and CMV (EBV Quant Kit, CMV Quant Kit). All tests were produced by Roche Diagnostics except for the kits for HHV-6 detection, which were supplied by ALPCO.

The detection of anti-EBV antibodies (anti-VCA, anti-EA, and anti-EBNA in IgM and IgG classes) was performed in serum samples parallel to viral DNA testing using ELISA (HYCOR).

Viral particle detection in the sample taken the first day after vesicular hemorrhagic skin change was performed using electron microscopy.

Bacteriological findings included routine examinations of peripheral blood samples and intravenous catheters, the detection of *Clostridium difficile* toxins in stool samples, screening tests for vancomycin-resistant enterococci (VRE), and bacteria isolation from skin changes.

In the period taken into consideration, 36 peripheral blood samples were taken for bacteriological and mycological investigations. Besides peripheral blood, blood from catheters was examined four times and fluids used for transfusion were examined for bacterial presence once. The isolation of bacteria from the patient's blood as well as platelet concentrate and plasma used for transfusion was performed using a BacT/Alert automated system (bioMerieux) with subsequent standard bacteriological culture and identification techniques.

Clostridium difficile A and B toxins were detected using an immunoenzymatic technique (Techlab). For VRE screening, bacteria from stool samples were cultured on Mueller–Hinton agar supplied with vancomycin (6 μg/ml).

Results

Microbiological Findings and the Patient's Management

All results of virological findings (CMV, VZV, HSV-1/2, parvovirus B19, HHV-6) were negative in all the examined samples, except for EBV. Results of EBV DNA detection in serum samples were positive within the 2-week period. The level of EBV DNAemia constantly decreased, ranging from 145,000 copies/ml in the first examined sample to 5,700 copies/ml in the last positive sample on day +15 (Fig. 2). EBV DNA was also detected in three consecutive swabs taken from ulcerated regions of the patient's skin at 1-week intervals, starting with the first day after admission. The presence of the virus in the skin was confirmed by the appearance of herpes virus-like particles detected in skin ulceration fluid with electron microscopy (Fig. 3). The results of EBV immunology indicated a low production of specific antibodies and a constant decrease in detectable antibody levels (anti-VCA IgG and anti-EBNA IgG), preceded in a short period by a short increase in anti-VCA IgG level. The examined antibodies from other groups (a-VCA IgM, a-EA IgM and IgG, also a-EBNA IgM) remained at undetectable levels (Fig. 2).

No bacteria or fungi were isolated from the blood samples (both peripheral blood and from catheters) or from transfusion materials during the whole examined period. The results of HIV and hepatotropic viruses (HBV, HCV) immunology were also negative.

In two stool samples, toxin A of *C. difficile* was detected and a toxinogenic strain of *C. difficile* was isolated. Skin swabs taken from ulcerations for bacteriological/mycological laboratory testing were negative by culture under both aerobic and anaerobic conditions.

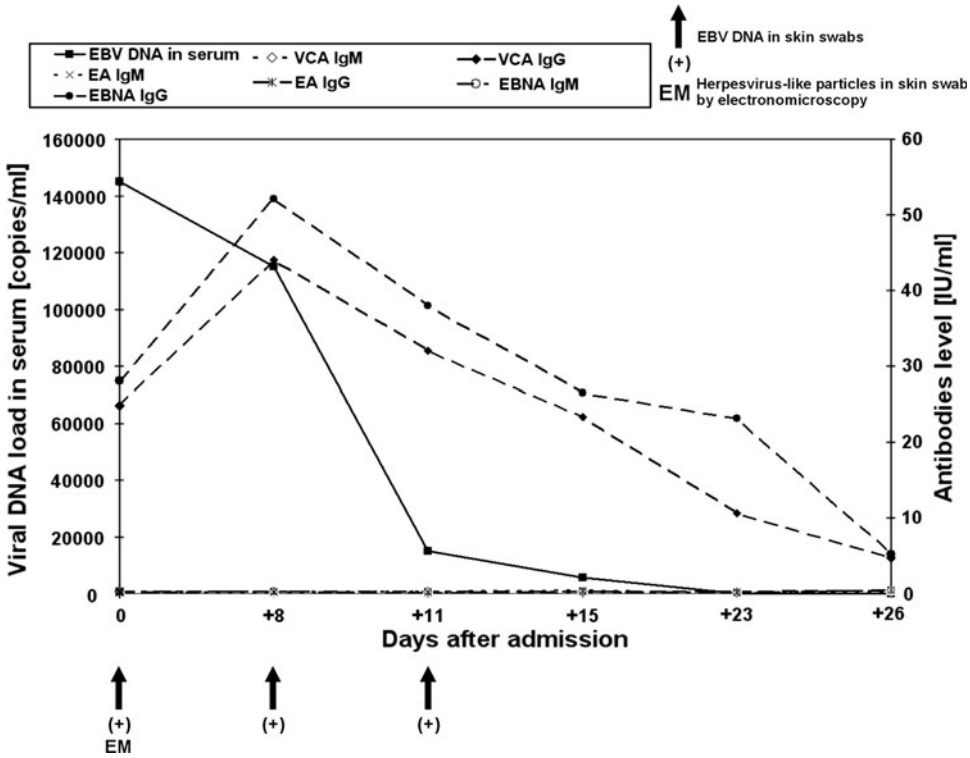

Fig. 2 Changes in EBV-DNA load in plasma samples and detectable anti-EBV antibody levels

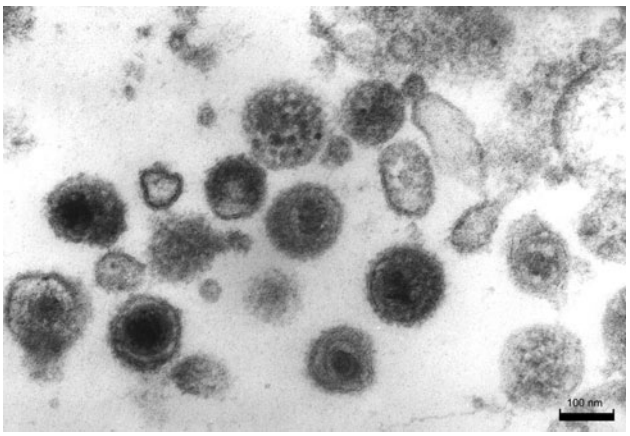

Fig. 3 Electron microscopic image of herpesvirus-like particles from skin swabs taken from ulcerative changes on +1 day

On the basis of the clinical signs and the positive results of EBV DNA detection in the patient's blood and skin samples, EBV-associated HLH was diagnosed.

Treatment according to the HLH-94 standard with use of etoposide (150 mg/m^2 twice a week) and dexamethasone (10 mg/m^2 for the first 2 weeks and 5 mg/m^2 for the next 2 weeks) was introduced in the fourth week after presentation of the first clinical symptoms. Plasmaphereses and platelet and RBC concentrate transfusions were also introduced as well as infusions of cyclosporine starting on day +3 for three subsequent weeks, until improvement in the morphological blood parameters. Fever persisted for 22 days after treatment introduction and skin changes started to disappear from the tenth day after admission to the Hematology Clinics. The patient's status constantly improved; the results of liver tests normalized as well as other clinical laboratory parameters, except for D-dimers and GGTP serum levels, which remained significantly increased (Table 1). After the introduction of treatment, a decreasing level of EBV DNA copies in routine testing was also observed, with eventual negativization of EBV DNA results in the serum sample taken on day +23 (Fig. 2). At

Table 1 Changes in clinical parameters between days 0 and +28 after patient admission to the Hematology and Oncology Ward of WMUCH

Parameter	Day 0	Day +28
RBC count ($\times 10^6$ μl^{-1})	2.68	3.44
WBC count ($\times 10^3$ μl^{-1})	0.67	3.9
Platelet count ($\times 10^3$ μl^{-1})	95	560
D-dimers (ng/ml)	24,800	44,208
Ferritin serum level (ng/ml)	>20,000	1,434
Triglycerides (mg/dl)	596	247
Lactate dehydrogenase (LDH) (U/l)	7,300	597
AspAt serum level (U/l)	794	52
AlAt serum level (U/l)	300	64
Bilirubin serum level (mg/dl)	15.7	1.6
Gamma-glutamyl transpeptidase (GGTP) (U/l)	442	574
CRP (mg/l)	28.4	2.4

present the patient is under the observation of the Hematology Clinics day-care unit and his general status is satisfying.

Discussion

At the most intensive stage of disease, the patient presented almost the classical set of symptoms characteristic of HLH. According the HLH-2004 protocol, the diagnosis of HLH can be established if five out of eight diagnostic criteria are fulfilled. These criteria are: fever, splenomegaly, bicytopenia, hypertriglyceridemia and/or hypofibrinogenemia, hemophagocytosis, low or absent NK cell activity, hyperferritinemia, and increased soluble CD25 levels (Henter et al. 2004). In the described case, six of them were met and elevated serum aminotransferases and extensive hemorrhagic ulcerative changes on the skin and mucosa were also observed.

Except for EBV, the single infectious agent detected during the entire course of HLH, there was no other infection detected during the course of clinical symptoms presented by the patient. A vancomycin-resistant strain of enterococcus and a toxin-producing strain of *C. difficile* detected in stool samples seemed to be rather an effect of prolonged hospital stay and antibiotic therapy than a factor influencing the patient's status.

Despite EBV DNA presence in the serum samples during HLH, much lower levels of viral DNA were detected in the material taken from skin swabs (Fig. 1). This is surprising, since literature data contain information about extremely strong lymphotropism of EBV. Possible explanations for EBV presence in the skin can be explained either as a result of transfer of complete viral particles from serum to skin tissue with serous fluid or, even more likely, intumescences of EBV-infected T cells and/or histiocytes to the skin (Chuang et al. 2007; Iwatsuki et al. 2000). With time and the applied treatment, the viral DNA level gradually decreased, eventually reaching undetectable values, which was accompanied by constant progress in the patient's laboratory parameters. Two of the most important biochemical parameters which remained at significantly high levels after 4 weeks of treatment were D-dimers and GGTP. As the increased GGTP level may be explained by the slow ability of liver tissue to regenerate after probable massive damage caused during the course of EBV-HLH, the dramatically elevated level of D-dimers may be a delayed result of a prolonged generalized inflammatory process.

Treatment of HLH with etoposide and dexamethasone depends mostly on the EBV load in the blood and its effectiveness decreases along with the time passed from the beginning of infection (Chuang et al. 2007; Imashuku 2002; Imashuku et al. 2004; Tiab et al. 2000; Verbsky and Grossman 2006). Without the introduction of treatment, the disease is fatal in almost all cases (Kasahara and Yachie 2002; Verbsky and Grossman 2006). Relying on the few literature reports, the described therapy is effective when introduced within the 4-week period after the first symptoms of EBV infection (Imashuku et al. 2001; Imashuku 2002; Imashuku et al. 2004; Janka 2007; Verbsky and Grossman 2006). Because of the possibility of the appearance of symptoms similar to those observed in HLH in many other clinical syndromes, this condition is often not fulfilled. In such situations, molecular biological methods become a very useful tool. These methods, besides their ability to detect viral DNA at very low levels, allow the monitoring of treatment efficacy and are obviously helpful in differentiating the diagnosis with the goal of excluding other pathogens.

In HLH cases, pathological examination of tissues is recommended to establish a proper diagnosis, but the findings may be difficult to interpret. Bone marrow examination in the early stages is not often diagnostic and may only show erythroid hyperplasia, so a false-negative result is common in up to two-thirds of cases. The specimens show a mixed lymphohistiocytic infiltrate and accumulation with the presence of the activated form of macrophages. In the cerebrospinal fluid, mild to moderate pleocytosis may be seen and most of the observed cells are lymphocytes and macrophages (Henter et al. 2004).

In recent years the pathophysiology of the HLH was recognized as a direct life-threatening condition in young people, and rules for its identification and treatment were developed (Chuang et al. 2007; Fisman 2000; Imashuku 2002; Imashuku et al. 2004). It is to be noted that the management rules established for children are also fully acceptable in adult patients (Fisman 2000; Imashuku 2002; Janka 2007; Verbsky and Grossman 2006). When there is a suspicion of HLH, patients should be redirected to specialized hematology centers possessing proper abilities, both to perform complete diagnostic procedures and to introduce efficient treatment methods immediately. Differential diagnosis should distinguish the EBV-driven X-linked lymphoproliferative syndrome (XLP syndrome) and also the rare Chediak-Higashi and Griscelli syndromes (Henter et al. 2004).

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