J Infect Chemother 22 (2016) 268–271

Contents lists available at ScienceDirect

Journal of Infection and Chemotherapy

journal homepage: http://www.elsevier.com/locate/jic

Note

Diagnostic values for the viral load in peripheral blood mononuclear cells of patients with chronic active Epstein–Barr virus disease

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ARTICLE INFO

Article history:
Received 14 September 2015
Received in revised form
15 October 2015
Accepted 6 November 2015
Available online 17 December 2015

Keywords:
Chronic active Epstein–Barr virus disease
CAEBV
Viral load
Diagnosis
Peripheral blood mononuclear cells

ABSTRACT

Chronic active Epstein–Barr virus disease (CAEBV) is a distinct EBV-associated lymphoproliferative disease with a poor prognosis. Although the viral load in blood samples has been widely used for diagnosing CAEBV, well-defined viral load thresholds to guide clinicians are currently lacking. The aim of the present study was to determine standardized diagnostic values for EBV load in blood samples of CAEBV patients using the World Health Organization international standard for reporting. Levels of EBV DNA in 103 peripheral blood mononuclear cells (PBMCs) and 95 plasma/serum samples from 107 cases with CAEBV were quantified and expressed in international units. Receiver operating characteristic curves were analyzed to assess the most appropriate cut-off values for levels of EBV DNA to distinguish CAEBV from EBV-associated infectious mononucleosis (IM) and controls with past EBV infection. Levels of EBV DNA in PBMCs were significantly higher in the CAEBV group (median, $10^{4.2}$ IU/μgDNA) compared to the IM (median, $10^{2.1}$ IU/μgDNA) and control groups. An inconsistent qualitative result was seen in 13 of 86 CAEBV patients; in these, EBV-DNA was positive in PBMCs, but negative in plasma. Diagnostic cut-off values for viral load in PBMCs from CAEBV patients, as compared to those of healthy controls and IM patients, were $10^{2.0}$ IU/μgDNA and $10^{3.2}$ IU/μgDNA, respectively. For diagnostic purposes, the viral load of PBMCs was better than of plasma/serum. A diagnostic cut-off EBV load for CAEBV may be useful for the management of CAEBV patients.

Epstein–Barr virus (EBV) causes primary EBV infections and can also cause chronic infections in apparently immunocompetent individuals [1]. Chronic active EBV disease (CAEBV) was initially thought to be a progressive EBV infection of B cells as the primary target, but the term recently has referred to aggressive EBV-associated T cell, NK cell, or B-cell lymphoproliferative diseases (LPDs), mainly affecting persons of Asian origin [2–4]. These diseases are characterized by chronic or recurrent infectious mononucleosis (IM)-like symptoms [5], and hematopoietic stem cell transplantation (HSCT) may be the only curative therapy [6,7]. Because shorter time from onset to HSCT significantly increases survival [3,7], confirming the diagnosis of CAEBV during the early stages of the disease is important. Although the viral load in blood samples has been widely used for diagnosis of CAEBV, there is currently a lack of well-defined viral load thresholds to guide clinicians. Recently, the first World Health Organization (WHO) International Standard for EBV for Nucleic Acid Amplification Techniques was released for standardization of the quantitative PCR assay [8].

A total of 107 patients with CAEBV, ranging in age from 1 to 50 (median 8) years, were enrolled in this study. CAEBV was defined according to previously proposed criteria [5], as follows: (1) EBV-related symptoms for at least 3 months; (2) an increased EBV load in either the affected tissue or peripheral blood; and (3) lack of evidence of other diseases that could explain the condition. Peripheral blood drawn at the time of diagnosis was used for viral DNA quantification. Among the 107 enrolled patients, peripheral blood mononuclear cells (PBMCs) from 103 and plasma/serum samples from 95 were available (PBMCs and plasma were simultaneously separated in 91 patients). The EBV-associated IM group (IM group) was defined as patients with primary EBV infection and fever, pharyngitis, cervical lymphadenopathy, and more than 10%

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http://dx.doi.org/10.1016/j.jiac.2015.11.002
1341-321X/

atypical lymphocytes among peripheral white blood cells. Primary EBV infection was defined as patients having positive anti-viral capsid antigen IgM and EBV DNA in blood samples at diagnosis. With respect to samples from IM, PBMCs from 37 patients and plasma from 42 patients were available (PBMCs and plasma were simultaneously separated in 37 patients). PBMCs for controls were also obtained from 39 healthy volunteers who were seropositive for anti-viral capsid antigen IgG. Plasma/serum samples for controls were obtained from 6 healthy volunteers (of the above 39 healthy volunteers) with past EBV infection and 175 pediatric patients with a variety of health complaints, including upper respiratory tract infection, bronchitis, pneumonia, bronchial asthma, gastroenteritis, fever of unknown origin, and treated epilepsy. None had any underlying immunodeficiency or was receiving any immunosuppressive therapy. Blood samples were taken for clinical purposes. The study design and purpose were approved by the institutional review board of Nagoya University (2010-881). Written, informed consent was provided by study participants and/or their legal guardians prior to enrollment.

Viral DNA was quantified as previously described [9]. Our system was standardized using the first WHO international standard for EBV, which comprises a whole-virus preparation of the EBV B95-8 strain [8]. To compare the levels of EBV DNA among the 3 groups, one-way analysis of variance with the Games-Howell procedure was used for pairwise comparisons. Pearson's correlation coefficient analysis was used to assess the relationship between DNA copy number in PBMCs and plasma/serum samples. Receiver operating characteristic (ROC) curves were analyzed to assess the most appropriate cut-off values for levels of EBV DNA to distinguish CAEBV from the other study groups (IM or control). Differences with $P < 0.05$ were defined as significant.

The levels of EBV DNA in PBMCs were significantly higher in the CAEBV group (median, $10^{4.2}$ IU/μgDNA) than in the IM (median, $10^{2.1}$ IU/μgDNA) and control groups (Fig. 1A). The levels of plasma/serum EBV DNA were significantly higher in the CAEBV group (median, $10^{3.5}$ IU/mL) than in the control group. However, there was no significant difference in serum/plasma levels of EBV DNA between the CAEBV and IM groups (Fig. 1B). Next, the correlation between the viral load of PBMCs and that of plasma/serum in the same blood samples of CAEBV patients was analyzed. An inconsistent qualitative result was seen in 13 of 86 patients; in these, EBV-DNA was positive in PBMCs but negative in plasma. No significant correlation was found between the amounts in PBMCs and those in plasma/serum (Fig. 1C).

The ROC curves were used to compare the CAEBV and control groups; for EBV DNA in PBMCs, the cutoff value was $10^{2.0}$ (=100) IU/μgDNA (sensitivity: 99.0%, specificity: 97.4%) (Fig. 2A) and in plasma/serum, the cutoff value was $10^{0.2}$ IU/mL (sensitivity: 85.3%, specificity: 97.8%) (Fig. 2B). The ROC curves were used to compare the CAEBV and IM groups; for EBV DNA in PBMCs, the cutoff value was $10^{3.2}$ (=1700) IU/μgDNA (sensitivity: 81.6%, specificity: 86.5%) (Fig. 2C) and in plasma/serum, the cutoff value was $10^{3.6}$ IU/mL (sensitivity: 49.5%, specificity: 83.3%) (Fig. 2D).

The clinical utility of viral load measurements using quantitative PCR assays for the diagnosis and management of EBV-associated malignancies has been described [10]. However, the heterogeneity of the quantitative PCR assay for EBV makes its use difficult for comparing viral load measurements among different laboratories and to develop uniform therapeutic strategies. Indeed, more than 25 different in-house PCR assays have been developed, and about 10 different commercial reagents and systems for EBV detection by real-time PCR are available in different countries [11]. Multicenter studies revealed variability in the performance of different assays for EBV [12]. With the recent development of the first international EBV WHO standard to calibrate routine laboratory assays [8],

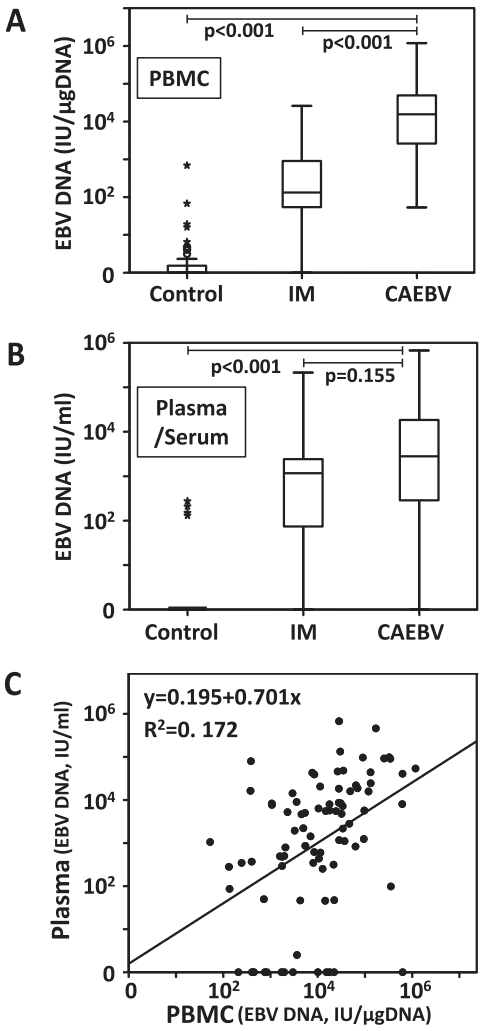

Fig. 1. Levels of Epstein–Barr virus (EBV) DNA in chronic active EBV disease (CAEBV), infectious mononucleosis (IM), and controls. A. Comparison of the levels of EBV DNA in peripheral mononuclear cell (PBMCs) among the study groups (CAEBV, n = 103; IM, n = 37, controls, n = 39). **B**. Comparison of the levels of EBV DNA in PBMCs among the study groups (CAEBV, n = 95; IM, n = 42, controls, n = 181). Boxes show the minimum, maximum, and median levels for each group, together with the lower and upper quartile. Asterisks outside the range represent outliers. **C**. Correlation between the levels of EBV DNA in PBMCs and those in plasma/serum in CAEBV patients.

previous difficulties of inter-laboratory comparison of EBV load will be overcome. In proposed guidelines for CAEBV, more than $10^{2.5}$ copies/μgDNA in PBMCs is the proposed cut-off value for diagnosis based on results obtained in a single laboratory [5]. Because of the increasing recognition of CAEBV, evaluation of EBV load in a large number of patients using a standardized quantitative PCR assay is needed.

Currently there is no consensus regarding the optimal blood component to examine. EBV-infected cell-associated DNA from infected T/NK cells constitutes the majority of EBV DNA in PBMCs from CAEBV [10]. Otherwise, cell-free EBV DNA, derived from apoptotic cells infected with EBV in affected organs, is a relatively major component in plasma/serum, although cell-associated EBV-DNA is also present. In the early stage of the disease, the amount of cell-free virus may fluctuate because the amount of infiltrating EBV-infected cells in the affected organ is small. Thus, CAEBV patients usually have high viral loads in their PBMCs [13], but viral