

THE POSSIBLE INVOLVEMENT OF EPSTEIN - BARR VIRUS IN THE ETIOLOGY OF LEUKEMIA

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ABSTRACT

Background: Although considerable brainstorm has been known into Epstein-Barr virus EBV as an important etiologic factor in various tumors, virtually little is known about the relationship between EBV genes and leukemia. The actual cause of Leukemia, which is a serious cancer in Sudan, is still under scrutiny. Controversial hypotheses were proposed suggesting the role of physical as well as chemical and even biological factors as being responsible for Leukemia incidents. We hypothesized that EBV could be involved in the etiology of leukemia. We describe here the results of our attempt to find a possible link between leukemia and EBV. **Methods:** Real-time Polymerase Chain Reaction assay (q-PCR) has recently been used widely for detection of Cell-free EBVDNA in the plasma of patients with leukemia. To determine the possible correlation between plasma cell-free EBV DNA

Levels and the leukemia, we studied the plasma EBV DNA levels in patients with leukemia that were presented at Radiation and Isotope Centre Khartoum (RICK), Sudan during therapy. The concentrations of plasma cell-free EBV DNA of 128 leukemic patients, 17 patients with EBV-associated lymphoid malignancies during the course of therapy Burkitt's lymphoma, Hodgkin's disease, Nasopharyngeal carcinoma (NPC) as control positive and 15 healthy controls were determined by using the (q-PCR) assay with the Primer Design™ genesig quantification kit. **Results:** The results revealed that EBV DNA was detectable in a wide range of leukemia patients. Plasma EBV DNA was detected in 33/88 ALL patients,

28/40 CML patients, 15/17 patients with EBV-associated lymphoid malignancies, but not in any of 15 healthy control subjects. The median concentration of EBV DNA in leukemia and healthy control groups was 6561.00 and 0.00 copies/ml, respectively. **Conclusion:** Our findings provided evidence of the involvement of EBV in patients with leukemia. The results suggested that EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 p143 was observed in a significant proportion of patients with ALL. However, we could not exclude a correlation between these viral infections and later leukemogenesis in childhood ALL in Sudan.

KEYWORDS: EBV; real-time quantitative PCR; leukemia; Diagnosis; BNRF1.

INTRODUCTION

Leukemia is a cancer of the blood or bone marrow characterized by an abnormal increase of blood cells, usually leukocytes (white blood cells). Leukemia is a broad term covering a spectrum of diseases. In turn, it is part of, the even, broader group of diseases called hematological neoplasms. ^[1] EBV is a herpesvirus that infects and establishes a persistent infection in humans. Following a primary infection in healthy individuals, EBV infects and immortalizes B lymphocytes, which is followed by a lifelong viral latency. ^[2, 3] Proliferation of EBV infected B-cells is prevented and controlled by an adequate T-cell dependent specific immune response. However, strong reduction in the numbers of EBV-specific T lymphocytes may result in reactivation of the virus and, ultimately, the development of lymph proliferative disease (EBV-LPD). ^[4] The EBV BZLF1-encoded replication activator (ZEBRA) is a key mediator of reactivation from latency to the viral productive cycle. Among the EBV trans activators, the ZEBRA protein plays a crucial role in switching the virus from a latent to a productive mode. ^[5] This virus has been implicated in the development of a number of lymphoid malignancies. Burkitt's lymphoma was the first neoplasm found to be related to EBV, with a nearly 100% association in endemic areas, but less than 20% in low-incidence areas. ^[6] No single known cause for all of the different types of leukemia exists. The known causes, which are not generally factors within the control of the average person, account for relatively few cases. ^[7] Several studies have reported the presence of EBV DNA in the peripheral blood of patients with ALL. ^[8, 9] Most used either peripheral blood mononuclear cells or B-lymphocytes for EBV detection and showed increased levels of EBV DNA. Recently, much interest has been focused on the detection of tumour-derived DNA in the cell-free fractions, namely plasma or serum, of blood samples. ^[10, 11, 12] However, few

literature points towards the controversial role of EBV in childhood leukemia. Employing sensitive and specific molecular tools like Real time PCR assay, EBV LMP1 gene transcriptional activity was observed in a significant proportion of patients with acute lymphoblastic leukemia. EBV infection in patients with lymphoid leukemia may be a factor involved in the high incidence of paediatric leukemia in the Sudan. ^[8] A study upon Finnish and Icelandic maternity cohorts to verify/falsify the association of maternal EBV infection with an increased risk of ALL in the offspring, (during 1983 to 2006 in Finland and 1997 to 2005 in Iceland), was carried out. It was concluded that there was no evidence of increased risk of ALL associated to EBV and the data argued against a role of EBV in ALL. ^[13] On the other hand, in 2003, Dr. Matti Lehtinen from National Public Health Institute, Oulu, Finland, and colleagues identified a link between maternal EBV reactivation and the development of ALL in offspring. They found some further confirmation to their findings, suggesting an association between maternal EBV infection and risk of Leukemia in the offspring, especially infants below 1 year of age. ^[9] Masao O, Takako S R, *et al* (2011) studied the etiology of cytomegalovirus (CMV), human herpesvirus-6 (HHV-6), and EBV reactivation and the potential for complications following cytotoxic chemotherapy in the absence of allogeneic transplantation. Their findings demonstrated that subclinical reactivation of CMV, HHV-6, and EBV were common in Adult T Cell Leukemia (ATL) patients receiving chemotherapy. ^[14] In fact, EBV does not directly cause a specific disease of its own, as is the case of other pathogens, but it plays a role in the etiology of several diseases, as for example cancer, rheumatoid, and a co-factor to several autoimmune diseases, to name but a few. This characteristic, logically, leads to the presumption that it is involved in leukemia incidents. A causative link between EBV and leukemia, a disease with a distinctly high incidence in Sudan, has been widely investigated. However, no full-length analysis of any subs train of EBV in this area has been reported. In the current study, we sought evidence whether EBV is associated with the development of childhood leukemia in the Sudan.

MATERIALS AND METHODS

Enrollment of patients

Patients with leukemia that were presented at Radiation and Isotope Centre Khartoum (RICK) Sudan have been studied for evidence of EBV infection. The patient's Consent and approval were requested by the (RICK) Ethics authorities to obtain clinical materials for research purposes. The patients were, beforehand diagnosed using several diagnostic parameters, including cell morphology and flowcytometry.

Sample collection

Plasma samples taken from 128 of treated leukemia patients, of different cohorts were collected upon their consent.

Extraction of EBV DNA

Cell-free EBV DNA from all plasma samples was extracted by using a Purification of DNA by phenol extraction and ethanol precipitation protocol (Oxford practical approach series) according to the user manual and kept at -20 °C until used. Briefly, a 200 µl plasma sample was added to equal volume phenol, and add an equal amount of 24:1 (v/v) chloroform-isoamyl alcohol, centrifuged at 8 000 rpm for 1 min to separate the aqueous phase (supplemented with 20 µl proteinase K) in a 1.5 ml microcentrifuge tube, mixed immediately and incubated at 65 °C for 2 hours. In order to precipitate the DNA, 20 µl of 3 M sodium acetate, pH 5.5 and 300 µl of absolute ethanol were added, followed by Incubation at -20 °C overnight. The precipitated DNA was recovered by centrifugation at 10 000 rpm for 15 min. Then the DNA was washed twice with 70% (v/v) ethanol to remove excess salt from the pellet. Finally, the DNA was resuspended in sterile TE, pH 8.0, and stored at -20 °C. The final volume of DNA obtained from each sample was 100 µl.

Detection of Plasma Cell-free EBV DNA

The Primer Design™ genesig Kit for Human Herpes Virus 4 EBV Genomes is designed for the in vitro quantification of EBV genomes. The target sequence within the BNRF1 gene has previously been shown to be a good genetic marker for EBV in other clinical real time PCR based studies.^[15] The kit is designed to have the broadest detection profile possible whilst remaining specific to the EBV genome. The primers and probe sequences in this kit have 100% homology with a broad range of clinically relevant reference sequences based on a comprehensive bioinformatics analysis.

Quantitative EBV-specific PCR

DNA from plasma was amplified using Taqman PCR primers selective for the EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 p143. BNRF1 p143 was used as the target gene in this assay. PCR amplification and detection was performed with (Swift Spectrum 48 Thermal Cycler) detection system (ESCO). In the PCR reaction, a fluorogenic EBV/p143-specific probe was added with a FAM reported molecule attached to the 59th end and a TAMRA quencher linked at the 39th end to detect amplified DNA. Real

time measurements were taken and a threshold cycle (Ct) value was calculated for each sample by determining the point at which the fluorescence exceeded a threshold limit of 50. Each run contained a negative control (no template or EBV negative DNA), a positive control (a known amount of EBV copies). The standard curve was created automatically by the (ESCO) (Swift Spectrum 48 Thermal Cycler) Detection System Software, by plotting the Ct values against each standard of known concentration.

Statistical analysis

Data management was done by using the Statistical Package for Social Sciences (SPSS version 11.5; SPSS Inc, Chicago, IL). SPSS was used for analysis and to perform Fisher exact test for statistical significance (*P* value). The 95% confidence level was used. Comparison of plasma EBV DNA concentrations between controls and patients were performed using the Mann-Whitney U-test. *P* value < 0.05 was considered statistically significant.

RESULTS

Development of Real-Time Quantitative PCR

To determine the dynamic range of real-time quantitative PCR, serial dilutions of DNA were made and subjected to analysis by the EBV real-time quantitative PCR system. Figure 1 shows the amplification curve result obtained using the BNRF1 region PCR system. The system was sensitive enough to detect 5 copies of EBV DNA. Figure 2 shows a plot of the threshold cycle (CT) of the EBV BNRF1 region PCR which plotted on a common logarithmic scale. The threshold cycle (CT) was set at 10 serial dilutions (SDs) above the mean baseline fluorescence calculated from cycles 1–15 and was proportional to the starting target copy number (logarithmic scale) used for amplification. The linearity of the graph demonstrates the large dynamic range and the accuracy of real-time quantitative PCR.

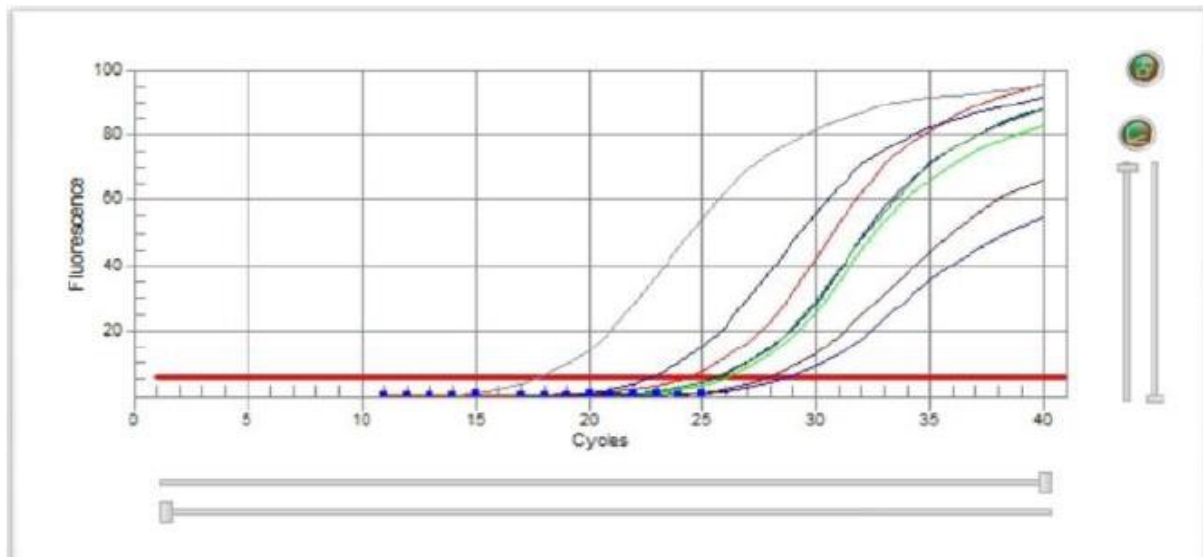


Figure 1: The q-PCR amplification plot of standard control of EBV DNA, the point that the fluorescence signal increased above baseline is the threshold cycle (CT). Each plot corresponds to a particular input target quantity marked by a corresponding symbol. The X axis denotes the cycle number of a quantitative PCR reaction. The Y axis denotes the fluorescence intensity.

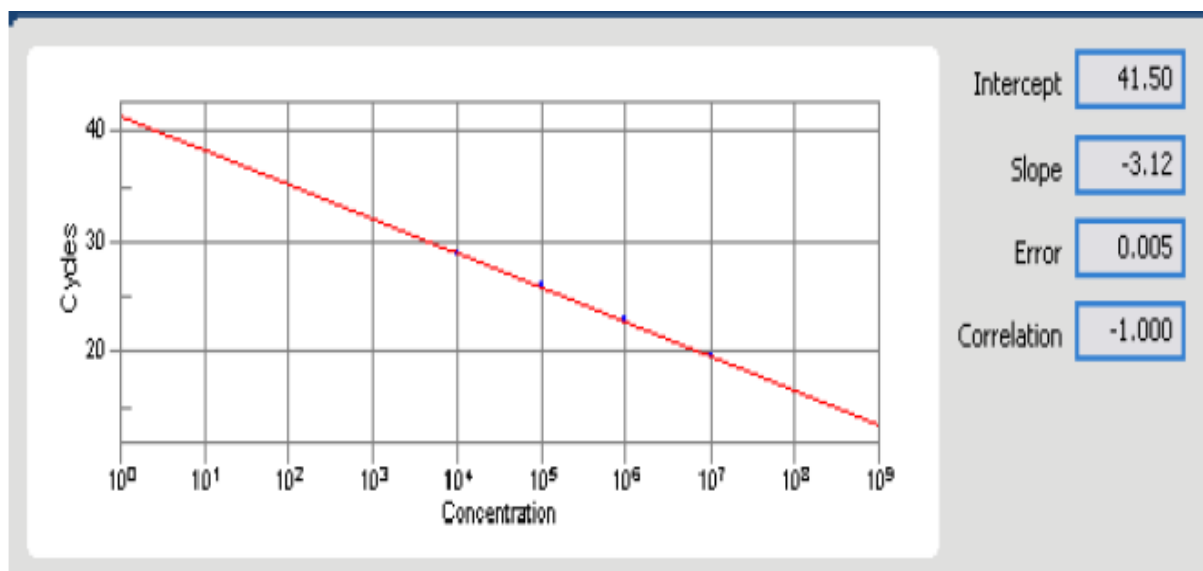


Figure 2: plot of the threshold cycle (CT) against the input target quantity (common log scale). The input target quantity was expressed as copies of EBV DNA. The correlation coefficient is 0.994.

Detection of Plasma EBV DNA in leukemic Groups and Controls

EBV DNA genome encoding the non-glycosylated membrane protein BNRF1 p143 was successfully detected by real-time PCR in 61 (47.6%) of the 128 leukemia cases, 15 (88.2%) of the 17 patients with EBV-associated lymphoid malignancies the control positive with the

mean level of 19683 ± 2187 copies/ml, and in none of the 15 healthy controls with the mean level of 0 copies/ ml ($P < 0001$). The mean level of plasma cell-free EBV DNA in leukemic patients was significantly higher than healthy controls at $p < 0.001$. The median concentration of plasma cell-free EBV DNA in leukemia groups was 6561.00 copies/ml whereas in healthy controls group was 0.0 copies/ml. The plasma cell-free EBV-DNA levels in healthy controls and leukemic Patients are summarized in Table 1. In terms of gender; the majority of patients were male 80 (62.5%) while 50 (37.5%) were females. The distribution of EBV BNRF1 p143 in different leukemic lymphomas subtypes is as follows: 33 (37.5%) of 88 ALL, and 28 (70%) of 40 CML (Figure 3). This was significantly higher ($P < 0.01$; chi-square test) compared with the incidence in the control group (0 of 15 individuals; 0%). When we compared plasma EBV DNA levels in these patients, we found that the median titers were significantly higher ($P < 0.001$; Mann–Whitney rank sum test) in patients with leukemia (6561.00 copies/ ml). In this study, patients with EBV⁺ leukemic lymphomas in different age ranges were as follows: 23 (17.7%) of 75 for age range 1-9 years, 19 (14.6%) of 32 for 10-19 years, and 2 (1.5%) of 2 for 20-29 years, 6 (4.6%) of 8 for 30-39 years, 4 (3.1%) of 4 for 40-39 years, 7 (5.4%) of 9 for 50-80 years. Eighty eight pediatric patients that underwent chemotherapy treatment at RICK comprised the ALL study group. The plasma cell-free EBV DNA was successfully detected in 12 (31.5%) of 38 complete remission (CR (1), 12 (42.8%) of 28 complete remission (CR (2), 5 (38.4%) of 13 complete remission (CR(3) and 4 (44.4%) of 9 follow-up (Figure 4).

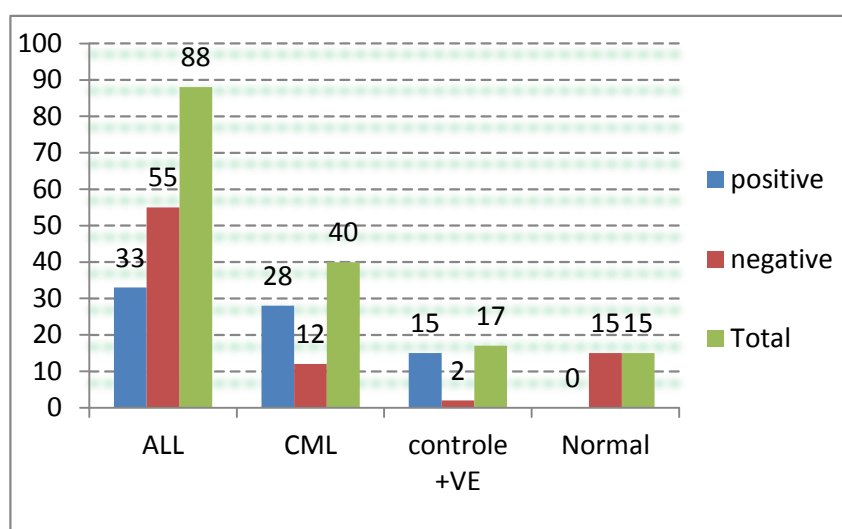


Figure 3: Description of EBV BNRF1 in Different Leukemic Lymphomas Subtypes.

Table.1: The plasma cell-free EBV-DNA levels in healthy controls and leukemic Patients.

Leukemic and control groups	EBV viral load in terms of copies/cell						Total of patients
	0	2187	6561	19683	59049	177147	
ALL	55	9	10	6	7	1	88
CML	12	1	8	12	7	0	40
Positive control (NPC,BL,HL)	2	0	0	2	10	3	17
Negative control	15	0	0	0	0	0	15
Total of copies	48	10	18	20	24	4	160

ALL=Acute lymphoblastic leukemia, CML = Chronic myelogenous leukemia, NPC= Nasopharyngeal carcinoma, BL=Burkitt's lymphoma, HL=Hodgkin's disease.

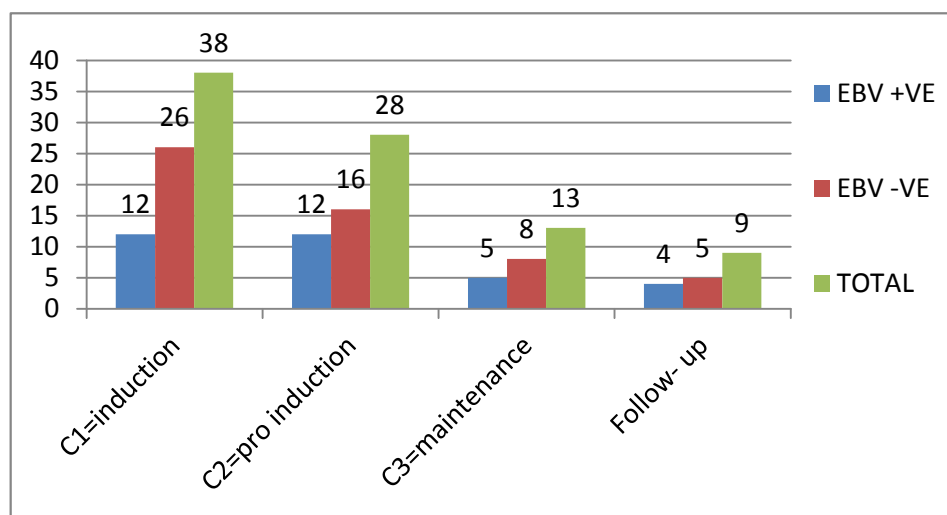


Figure 4: Description of EBV BNRF1 in Different treatment stages.

Abbreviations: (CR (1) = complete remission (induction), (CR (2) = complete remission (pro induction), (CR (3) = complete remission (maintenance).

DISCUSSION

A defining feature of herpesviruses is their ability to maintain a latent infection with the virus genome retained in host cells without production of infectious virions. EBV targets B lymphocytes through the CD21 receptor and establishes a latent infection both in vivo and in vitro.^[23] In recent years some convincing leads have been obtained on a causal relationship between EBV and a variety of childhood leukemia. EBV can be considered as the prototype of oncogenic viruses that behave as direct transforming agents. In fact, in classical EBV-associated tumors, the virus genome is present in virtually all neoplastic cells, which show the expression of viral RNAs and proteins that variously contribute to the induction of the

transformed phenotype. On the basis of these features and of the strict association with distinct tumor types, EBV has been classified as a group I carcinogen. The presence of this herpes virus has been variably detected in a broad spectrum of other tumors for which a causal role of EBV seems unlikely. These tumors include also chronic lymphocytic leukemia, yet the authors reviewed data suggesting a possible role of EBV as a direct or micro-environmental progression factor in a fraction of CLL ^[24] Leukemia, like other cancers, results from somatic mutations in the DNA. Certain mutations produce leukemia by activating oncogenes or deactivating tumor suppressor genes, and thereby disrupting the regulation of cell death, differentiation or division. These mutations may occur spontaneously or as a result of exposure to radiation or carcinogenic substances, and are likely to be influenced by genetic factors ^[25] Among adults, the known causes are natural and artificial ionizing radiation, a few viruses such as Human T-lymphotropic virus, Epstein-Bar virus, Cytomegalovirus and some chemicals, notably benzene and alkylating chemotherapy agents for previous malignancies. ^[26] The results of our study revealed a relative correlation between plasma cell-free EBV DNA levels and the leukemia in Sudanese patients. The results were correlated with those of, Ahmed *et al*, Matti L, *et al*, Lu Y *et al* and Sehgal S *et al*. ^[8,9,27,28] but not Tedeschi R *et al*.^[13] Ahmed *et al*, observed that EBV LMP1 gene transcriptional activity was in a significant proportion of Sudanese patients with acute lymphoblastic leukemia. They suggested that EBV may be a factor involved in the high incidence of paediatric leukemia in the Sudan. Matti *et al* (2006) in Finland identified a link between maternal EBV reactivation and the development of ALL in offspring. The paucity of such important information prompted us to perform a serial analysis of circulating EBV DNA levels at multiple time points during treatment using real-time quantitative PCR assay. This reports is supported by our findings, and the incidence of EBV in pediatric leukemia in the Sudan might be higher than this because we only detected BNRF1 gene, which could probably detect the majority of cases associated with EBV. Our samples were taken from patients receiving treatment, so the EBV viral load levels might decline to 0 copies/cell after completion of radiotherapy even in cases of cancers already known that the EBV is the causative agent like NPC. ^[29,30] Furthermore, Kenny *et al* reported that all patients who responded to therapy demonstrated a significant reduction of plasma EBV DNA to low or undetectable levels, whereas in patients with ineffective therapy, disease progression was associated with a rapid increase in plasma EBV DNA levels. ^[31] The disagreement might be due to the differences in the source of the cell-free EBV DNA and the differences in the type of samples that selected for the individual studies.

CONCLUSION

In conclusion, the results obtained suggest that plasma cell-free EBV DNA is a highly specific marker for a screening and diagnosis of EBV-associated diseases. The feasibility of using quantitative analysis to determine the possible relationship between the clinical outcome and the level of plasma cell-free EBV DNA are warranted features in childhood ALL. Further investigation on the link between maternal EBV reactivation and the development of ALL in offspring. needs to be explored. Nor latent infection nor congenital infection cannot be excluded.

Ethics: This study was approved by Sudan Medical specialization Ethics Review Board, Sudan.

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Competing interests: The authors have stated that they have no conflicts of interest.

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