Expression of Epstein-Barr Virus Gene Products in Burkitt’s Lymphoma in Northeast Brazil

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The Epstein-Barr Virus (EBV) is consistently found in tumor cells of Burkitt’s lymphoma (BL) endemic in central Africa and malaria is considered a pathogenic cofactor. In contrast, fewer than 20% of BL cases occurring in Western countries are EBV-associated. We have investigated 54 BL cases from Bahia, a tropical region of Northeast Brazil, for expression of EBV gene products by in situ hybridization and immunohistochemistry and performed typing of the EBV by polymerase chain reaction. Ten pediatric BL cases from Germany served as controls. New cases of malaria were not observed in the period and area of our study. Small nuclear EBV encoded transcripts, EBER, were found in tumor cells of 47 of 54 Brazilian cases (87%) but in only 2 of 10 German cases (20%). Type I latency of the EBV infection with absence of EBV-encoded proteins LMP1 and EBNA2 was found in 45 of 47 of the EBER-positive Brazilian cases. In two cases, occasional LMP1-containing tumor cells were found in the neighborhood of small Schistosoma mansoni granulomas and scars. BHLF1 transcripts associated with lytic EBV infection could be detected in few cells in 3 of the 40 EBER-positive Brazilian cases investigated. EBV type A was found in the majority of Brazilian BL cases (20 of 30 A-type, 7 of 30 B-type, and 3 of 30 not amplifiable). Our results indicate that the association of Bahian BL with EBV, but not the regional prevalence of malaria, is similar to endemic African BL. In two cases, type II latency was found in association with schistosomiasis, suggesting a role of this parasitosis in the induction of an EBV expression pattern that is unusual for BL. Because chronic schistosomiasis is associated with elevated Th2 cytokine expression resulting in reduced cell-mediated cytotoxicity, it seems possible that altered local immunity is responsible for this peculiar phenotype.

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BURKITT’S LYMPHOMA (BL), a high-grade B-cell neoplasm, was initially described by Dennis Burkitt in 1958 as a tumor involving the maxilla or mandible in African children. A possible association of this entity with a virus was already suggested by Burkitt in 1962. In fact, Epstein-Barr virus (EBV) was discovered only 2 years later in cell lines derived from BL samples from Uganda. EBV is found worldwide and is associated with a wide range of neoplasms, such as BL, nasopharyngeal carcinoma, Hodgkin’s disease, carcinoma of the stomach, and acquired immunodeficiency syndrome (AIDS)-related lymphomas. The frequency of the association of EBV infection with these neoplasms is extremely variable in different parts of the world. The strongest association of EBV infection (up to 96% of cases) with a lymphoma entity is observed in endemic BL (eBL) in central Africa. BL occurring in the United States and Western European countries (sporadic BL [sBL]) is morphologically and cytogenetically indistinguishable from eBL. However, eBL differs from sBL mainly by its more frequent association with EBV, a different preferential breakpoint location within the c-myc gene on chromosome 8, and its preferential jaw involvement.

Despite intensive research for more than 30 years, the precise role of EBV in the pathogenesis of BL is still not known. EBV infected cells may display three different types of latency according to the range of EBV encoded protein and RNA expression. Type I is characterized by exclusive expression of EBV encoded nuclear small nonpolyadenylated RNA (EBER) molecules and EBV encoded nuclear antigen (EBNA) 1. Type II shows in addition expression of latent membrane proteins (LMP). Type III displays additional expression of EBNA2 to EBNA6 by virtue of differential promoter usage. BL cells usually display latency type I but may convert to latency type III in culture. However, this latter phenotype has so far not been observed in primary tissues of BL. Type II latency may be found in EBV-infected nasopharyngeal carcinoma and tumor cells of Hodgkin’s disease. Very recently, expression of EBNA2 and LMP1 has also been described in eBL. It is well known that EBV is able to immortalize susceptible B cells in vitro, and this ability has been attributed to the expression of viral proteins such as EBNA2 and LMP1. However, expression of these proteins is not observed in most BL. Both a monoclonal pattern of EBV infection in BL and infection of all tumor cells indicate that the infection precedes the development of the tumor and thus possibly acts as a cofactor for the pathogenesis of this disease in endemic areas. For the lymphomagenesis of eBL, additional biologic factors (such as antigenic stimulation) are thought to be required. In Africa, the occurrence of an increased number of cells carrying EBV in patients with malaria indicates a possible permissive interaction between parasite and virus.

Outside Africa, a high association of BL with EBV was found in New Guinea, a country with similar climatic conditions to Africa. It therefore appears likely that this association can also attain high proportions in regions of Latin America characterized by socioeconomic and climatic condi-

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tions similar to those in central Africa. Because there is limited data on the occurrence of EBV in BL in tropical areas of South America,25,26 we analyzed a large series of BL cases from Bahia, a region in Northeast Brazil, for the presence of EBV. This region is situated between 9° and 17° south of the equator and has climatic and socioeconomic conditions similar to central Africa, where BL is endemic. In addition, in analogy to the tropical areas of Africa, endemic parasitic diseases causing polyclonal B-cell proliferation and immunosuppression such as schistosomiasis and leishmaniasis frequently occur in Bahia.25,28

To detect EBV-infected cells, we used in situ hybridization for the presence of EBER1 and EBER2. The EBER molecules are by far the most abundant EBV RNAs in latently EBV infected cells29 and are thought to be expressed in all these cells. EBV strains can be categorized into two types (A or B), and different geographical prevalence of these strains has been observed. Because the B-type is related to eBL,29,31 we have performed polymerase chain reaction (PCR) to determine the frequency of each EBV strain. Immunohistologic studies were also performed to characterize the phenotype of tumor cells and the expression of LMP1, EBNA2, and BZLF1 trans-activator protein and to confirm the diagnosis.

MATERIALS AND METHODS

Tissues

Fifty-four consecutive cases of BL diagnosed at the pediatric Hospital Martagao Gesteira (Salvador-Bahia, Brazil) from 1976 through 1993 were analyzed. The specimens consisted of intestinal resection (11 cases), biopsies of liver metastasis with the primary site in the mesentery (5 cases), lymph nodes (2 cases), biopsies of intra-abdominal tumor not further specified (32 cases), and mandibular tumors (4 cases). Sixty-four percent of BL occurred in the ethnic group of Mestizos. Approximately 70% of the population in this region is composed of Mestizos, who are individuals with mixed European and non-European (black and/or Indian) ancestry.

Formalin-fixed and paraffin-embedded tissues were cut at 4 μm and stained with hematoxylin-eosin (H&E) and Giemsa solution. The diagnosis was verified in all cases by three independent histopathologists (I.A., A.B., and H.S.) who applied the criteria of the Revised European American Lymphoma Classification13 (see Results). As a control group, 10 cases of pediatric BL from Germany were drawn from the files of the Institute of Pathology, Klinikum Benjamin Franklin, Free University Berlin (Berlin, Germany). Fourteen paraffin-embedded tonsils from children between 2 and 14 years of age served as an additional control group. These samples were obtained from patients without clinically overt immune dysfunction and the patients were from the same region as the Brazilian BL cases. Tonsils displayed only slight to moderate follicular hyperplasia.

Immunohistology

The sections were stained by the immunoalkaline phosphatase (APAAP) method.27 The primary monoclonal reagents were CS1,4: a cocktail of four antibodies specific for LMP1, antibodies specific for B-cell–associated antigens CD20 (L26) and CD79a (JCB117), an antibody specific for EBNA2 (PE-2), an antibody specific for BZLF1 protein (B2-1), a polyclonal antibody specific for T cells (CD3), an antibody specific for the activator antigen CD30 (Ber-H2), and an antibody for bcl-2 protein. With the exception of JCB117, which was obtained from Dr D.Y. Mason (Oxford, UK), all antibodies were from DAKO (Glostrup, Denmark). Both CS1-4 and CD79a required microwave irradiation (10 minutes in 0.1 mol/L citrate buffer at 650 W) to antigen retrieval in paraffin sections. Cases of AIDS-associated B-cell lymphomas serving as positive controls consistently displayed specific nuclear staining for EBNA2 and BZLF1 protein during our experiments.

Probes

Probes specific for the EBER1 and EBER2 were prepared using the plasmids pJJ1 and pJJ2, kindly provided by Dr G. Niedobitek (University of Birmingham, Birmingham, UK).33 After linearization, antisense and sense (negative control) cRNA EBER probes were obtained by run-off transcription with incorporation of digoxigenin-labeled nucleotides.

In Situ Hybridization

Under RNase-free conditions, in situ hybridization for the detection of EBER transcripts was performed as described previously.34 In brief, dewaxed and rehydrated paraffin sections were exposed to 0.2 N HCl and 0.6 mg/mL pronase (Boehringer Mannheim, Mannheim, Germany), followed by postfixation with 4% paraformaldehyde and dehydration through graded ethanols. Slides were hybridized to 0.1 μg of labeled probes overnight at 50°C. EBER1- and EBER2-specific probes were combined to increase sensitivity. After washing, digoxigenin-labeled EBER probes were detected using a monoclonal digoxigenin-specific antibody, alkaline phosphatase conjugate (Boehringer Mannheim), and an alkaline phosphatase detection system (DAKO). The positive signals were localized exclusively in the nuclei, with the exception of mitotic figures showing cytoplasmic staining. In situ hybridization experiments with the sense (control) probe did not show any signal (see Fig 1). In situ hybridization with fluorescein isothiocyanate (FITC)-labeled oligonucleotides for BHLF1 transcripts was performed according to the protocol provided by the manufacturer (DAKO).

Primers for PCR

Two primers in the EBNA2 region were selected for EBV typing (E2 up: 5'-AGGCTGCCCCACCTTGGAGAT-3' [48170-48189]; E2 low: 5'-GCCACCTGGACGGCTAAAG-3' [48339-48320]).34 These primer encompassed a region containing a 16-bp deletion in EBV type A (B95-8). The cell lines AG876 and B95-8 served as positive controls for EBV strain B and A, respectively,35 yielding an amplification product with 186-bp and 170-bp fragment length for AG876 and B95-8, respectively. In some cases, a seminested reamplification was performed using the E2 up primer and a 19-base primer (E2R low 5'-GCTGCCCACTGGGCAGAAA AT-3' [48280-48262]). The length of the second amplification product was 127 bp and 111 bp for the AG876 and B95-8, respectively. Negative controls consisted of water and a cell line negative for EBV (HUT-102). Primers were synthesized on an Applied Biosystems 381A DNA synthesizer (Applied Biosystems, Weiterstadt, Germany) and purified by high-performance liquid chromatography.36

PCR Amplification

DNA was extracted using the QIAGEN Tissue-kit according to the protocol provided by the manufacturer. Aliquots of 100 ng genomic DNA were amplified in a total reaction volume of 0.1 mL containing 10 mmol/L Tris-HCl, pH 8.3, 50 mmol/L KCl, 1.5 mmol/L MgSO4, 0.001% gelatin, 200 μmol/L each of dATP, dCTP, dGTP, and dTTP, 0.2 μmol/L of primer, and 2.5 U of Taq DNA polymerase
Fig 1. Brazilian BL. (A) Giant cells engulf *S mansoni* egg remains located within the tumor (Giemsa stain). (B) LMP1 expression in tumor cells near an *S mansoni* egg. (C) LMP1-positive tumor cells in scars of a BL associated with *S mansoni*; (inset) high-power magnification (×400) of the LMP1-positive tumor cells (immunohistology using the APAAP technique). (D) Expression of EBER molecules in virtually all tumor cells of a Brazilian BL case (in situ hybridization using digoxigenin-labeled RNA probes and the alkaline-phosphatase procedure). (E) In situ hybridization with sense (control) probe does not show any signals in a Brazilian BL case; the *S mansoni* egg in the illustration contains miracidia and shows a lateral spine typical of the species *mansoni*. (F) In situ hybridization for BHLF1 shows positive nucleus in a BL cell. Original magnifications ×200 (A); ×200 (B); ×100 (C); ×200 (D); ×200 (E); and ×400 (F).

(Perkin-Elmer, Norwalk, CT). The mixture was subjected to 35 cycles of amplification (30 seconds at 96°C, 30 seconds at 60°C, and 1 minute at 72°C) in a Thermocycler (TC 9600; Perkin-Elmer). Before cycling, the samples were denatured at 96°C for 2 minutes. After the last cycle, the polymerization step was extended by 10 minutes. PCR-amplified products were analyzed in 6% polyacrylamide gel stained with ethidium-bromide. The specificity of the PCR products was determined by direct DNA-sequencing using the Dye Terminator Cycle Sequencing procedure (Perkin-Elmer) and an automatic fluorescence DNA sequencer (373A; Applied Biosystems,
Table 1. Clinical Data From the Brazilian BL Cases

<table>
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<tr>
<th>No. of Cases</th>
<th>Sex</th>
<th>BL Localization</th>
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Abbreviations: l, intestinal; Ab NS, abdominal not specified; PLN, peripheral lymph node.

Weiterstadt, Germany). The PCR products were purified by low melting agarose before the sequence reaction.

Statistical Methods

The Student's t-test was used for statistical analyses.

RESULTS

Clinical Data

The clinical data from the Brazilian BL cases are summarized in Table 1. All cases occurred in children between 1 to 14 years of age, with the median age being 6 years. Thirty-four patients were male and 18 were female (ratio 1.8:1). The abdomen was the most frequent site of tumor involvement (48 cases), followed by the mandible (4 cases) and the peripheral lymph node (2 cases). In two cases, some of the clinical data were not available. The median ages of the control groups were 6.5 years (tonsils) and 9.1 years (German pediatric BL cases).

Histopathologic Findings

All 54 cases fulfilled the morphologic criteria for BL as defined by the REAL classification. The tumors were composed of monomorphic, medium-sized lymphoid cells with round nuclei, multiple nucleoli, and relatively abundant basophilic cytoplasm, which gave the cells a cohesive appearance. In some specimens this aspect was only observed at the periphery. Mitotic figures were numerous. The classic stary-sky pattern was present in most of the cases. All cases displayed a diffuse growth pattern, with the exception of one case with a follicular pattern. This case was a mesenteric lymph node in the drainage area of a diffusely growing intestinal tumor. This lymph node showed EBER-positive neoplastic cells confined to germinal centers. In two cases, frequent small circumscribed granulomas with centrally placed S mansoni ova were observed between tumor cells (Fig 1). The cellular infiltrate around the eggs was composed of giant cells, lymphocytes, and scattered eosinophils. In other areas in these cases, scars without eggs or only with egg residue and giant cells were observed.

The control tonsils showed nonspecific activation of the lymphoid tissue with mild follicular hyperplasia. The BL occurring in German children displayed morphologic features identical to the Brazilian BL cases.

Immunohistochemistry

All cases of BL displayed a B-cell phenotype with expression of both CD20 and CD79a. The T-cell antigen CD3 was observed in only a few small reactive lymphocytes. In the two cases with S mansoni eggs, many small T lymphocytes were found around the eggs or in scars (data not shown). The LMP1 protein could not be detected in any of the BL samples, with the exception of the two cases that also showed S mansoni egg granulomas. In these cases, two to five LMP1-positive tumor cells were localized around granulomas (Fig 1) and in scars. EBNA2, BZLF1 protein, and CD30 were not observed in tumor cells of BL cases and bcl-2 expression was restricted to small lymphoid cells morphologically compatible with the reactive T cells.

In Situ Hybridization

EBER. Forty-seven of the 54 Brazilian BL cases (87%) showed strong nuclear signal specific for EBER in virtually all tumor cells (Fig 1), whereas the remaining seven cases were completely negative for EBER. The age distribution of EBER-positive and negative Brazilian BL cases is presented in Fig 2. The median age for EBER-positive cases was 5.2 years and for EBER-negative cases was 9.6 years (P < .046). The control tonsils showed EBER-positive cells in 8 of 14 cases (57.1%). In the majority (6 of 8) of the positive tonsils,
the EBER-positive cells were confined to the extrafollicular zone. In four of these cases, only a few small positive lymphocytes were observed (4 to 14 cells/cm²); in the other two cases, the number of positive cells exceeded 60 cells/cm², and some of them displayed a blastic appearance. The two remaining positive cases showed a mild expansion of EBER-positive medium and large lymphoid cells in one single germinal center (data not shown). Only 2 of 10 cases of BL occurring in German children proved to express EBER (20%) and, as observed in the Brazilian BL cases, all tumor cells were positive for EBER. The percentage of EBER-positive cases among the Brazilian BL cases was significantly higher than that observed in both control groups (P < .046 for tonsils and P < .001 for BL occurring in Germany).

*BHLF1*. These experiments showed few positive nuclei in neoplastic cells in 3 of the 40 Brazilian BL cases, whereas the tonsil specimens and German BL cases remained negative. Three infectious mononucleosis cases serving as control displayed few labeled lymphoid cells within the crypt epithelium in agreement with previously published findings.

**PCR for EBV Typing**

EBV-specific amplificates of either EBV A- or B-type were achieved in 27 of the 30 EBER-positive BL Bahian cases investigated. A 111-bp (type A) and 127-bp (type B) PCR product was obtained in 20 and in 7 of these cases, respectively (Fig 3). In three BL cases, the DNA was not amplifiable. The tonsil specimens contained EBV type A in 6 cases and type B in 2 cases.

**DISCUSSION**

Our study on EBV infection of BL cases from Bahia, a region in Northeast Brazil, which represents the first extensive investigation of BL in this area, showed the following main findings: (1) 87% of BL cases expressed the EBER molecules, this frequency being significantly higher than in a control group of tonsils from the same region and in German pediatric BL cases; (2) EBV type A predominated both in tonsils and BL specimens from Brazil; (3) EBER-associated BL patients were significantly younger than patients with BL not associated with EBV; (4) type I latency predominates in EBV-associated Brazilian BL cases; and (5) LMP1 expression occurred in 2 of 47 Brazilian BL cases and was associated with *S. mansoni* infection at the site of the tumor.

Bahia appears to show a higher frequency of EBV association of BL (47/54) in comparison with Southeast Brazil (7/12) and Argentina. This difference is most likely related to different socioeconomic and climatic conditions. The frequency of EBV infection in BL in Northeast Brazil is similar to that observed in North Africa, which appears to be intermediate between the frequency of endemic cases and sporadic BL. The preferential abdominal tumor localization and infrequent jaw involvement of our Brazilian cases is also comparable to cases from North Africa. This suggests that common pathogenetical mechanisms are at work in both regions; however, the precise nature of these mechanisms remains to be elucidated. The observation of EBER-associated cases occurring predominantly in very young children is compatible with the findings in other studies. Presumably an effective immune control reduces with time the number of EBV-infected cells that can be the target of neoplastic transformation.

The EBV typing by PCR also showed a pattern intermediate between that observed in eBL (approximately 40%) and North American cases (approximately 10%). A high frequency of B-type strain has been observed in HIV-associated B-cell lymphomas, and this strain has been considered an important pathogen in immunocompromised patients. The percentage of EBV type-B (25%) in Bahian BL is similar to that observed in normal tonsils and may thus reflect the distribution of the two virus types in the Bahian population rather than indicate an underlying severe immunodeficiency. The finding of EBV B-type in (4/12) Hodgkin’s disease cases that occurred in immunocompetent children from Argentina is also consistent with the interpretation of our results.

*Plasmodium falciparum* malaria is thought to be a cofactor in the development of BL in Africa. This hypothesis is mainly based on a similar geographical distribution of both diseases and the occurrence of polyclonal B-cell proliferation and increased numbers of EBV-infected cells in patients with malaria. Other epidemiologic evidence linking malaria with the etiology of eBL includes the reduction of BL with malaria prophylaxis and the peak age incidence of BL closely following the peak age incidence for malaria in endemic areas. In addition, the observation that experimental malaria infection of laboratory animals enhances the oncogenic potential of tumor viruses supports the notion about the role of malaria as a cofactor in the etiology of eBL. However, for our Brazilian cases, *falciparum* malaria is not likely to be involved in the pathogenesis of BL because no such infections were described in this period in the area in which the patients of our study lived. Our results suggest that a high association between BL and EBV infection may occur in the absence of malaria and therefore question the necessity of malaria in particular for the development of BL.

All our cases are from areas in which *S. mansoni* infection is endemic. Like malaria, *S. mansoni* infection occurs early in children in endemic areas and is associated with polyclonal B-cell proliferation. Mice simultaneously infected with *S. mansoni* and vaccinia virus show a delay of the viral
clearance. Interestingly, the intestinal wall is a common site of *S. mansoni* egg deposition and is also the preferential primary location of the BL. Because most of our Brazilian BL cases are archival materials and clinical data on *S. mansoni* infection are not available, further investigation of the association between *S. mansoni* infection and BL is clearly necessary.

BL has been considered the prototypic lymphoma associated with latency type I, characterized by a restriction of EBV latent gene expression to EBER molecules and EBNA1.15 Very recent publications report the expression of LMP1 or EBNA2 in AIDS-associated BL and in some eBL from Africa as well BZLF1 expression in African eBL,18,24 challenging the concept of consistent latency type I in BL. However, the precise mechanism of the genesis of this phenotype remains unclear. In our cases, LMP1 was found in some tumor cells of BL in scars or around the *S. mansoni* granuloma in two cases, whereas EBNA2 and BZLF1 protein were not found in any of the cases investigated. The reason for the lack of expression of EBNA2 and BZLF1 is not clear. The finding of BHLF1 transcripts associated with lytic infection in three cases, which is in agreement with previous studies,29 suggests that technical factors, ie, lack of the preservation of antigens due to inadequate fixation conditions in tissues from Brazil, may be the reason for these differences.

The close association between LMP1 expression and intratumoral *S. mansoni* infection strongly suggests a relationship between parasite infection and EBV gene expression pattern. The ability of BL cell lines to acquire LMP1 expression in cell culture, ie, in the absence of immunosurveillance, is well documented.16 In vitro LMP1 has been shown to be the target of a cytotoxic response leading to the elimination of LMP1-positive cells.54 This phenomenon is likely to explain why in most cases the phenotype conversion occurring in cell culture is not observed in BL cases in vivo. On the other hand, expression of LMP1 in two BL cases suggests that an immune defect results in an inability to delete the LMP1-positive cells in these cases, *S. mansoni* might induce such a local immunodeficiency. In support of this hypothesis, later stages of *S. mansoni* infection, such as the ones observed in our cases with scar formation, are consistently associated with a Th2 pattern of cytokine expression characterized by increased secretion of both IL-4 and IL-10.52 Both of these interleukins have been shown to suppress macrophage and T-cell functions, including activity of cytotoxic T cells. In addition, mice coinfected with *S. mansoni* and vaccinia virus show a downregulation of virus-specific cytotoxic T-lymphotocyte responses.46 Local expression of Th2 cytokines may therefore result in an inability of the cell-mediated cytotoxicity to delete LMP1 expressing cells and may allow BL cells to acquire an LMP1-positive phenotype. Although unlikely, it cannot be ruled out, however, that *S. mansoni* directly induces EBV gene expression.

LMP1 has been shown to be an inducer of CD30 and bcl-2 expression in cell culture systems.28 However, in vivo LMP1 expression is regularly accompanied by CD30 expression in only certain lesions such as Hodgkin’s disease,17 but not in other diseases, such as AIDS-associated lymphoma or endemic BL in Africa.16,49 Similarly, expression of LMP1 has been shown to be not necessarily associated with bcl-2 positivity in both Hodgkin’s disease and AIDS-associated lymphomas.49,53 Therefore, the absence of expression of CD30 and bcl-2 in our LMP1-positive BL cases is well in line with generally accepted concepts that LMP1 expression in vivo is not always associated with enhanced CD30 and bcl-2 immunohistologic staining.

In conclusion, we have shown a high association of EBV with BL in a tropical area of Brazil, suggesting that EBV may play a role in the development of this disease in this region. The frequency of this association in the present study is comparable to that observed in North Africa and is slightly lower than that observed in Central Africa, where BL is endemic. The expression of LMP1 in some BL cases suggests that local cytotoxic immune responses are suppressed and, because LMP1-positive cases showed a coinfection with *S. mansoni*, secretion of Th2 cytokines known to be associated with chronic schistosomiasis is thought to be the cause of this local immunosuppression. As previously suggested by others,41 our data corroborate the hypothesis that EBV association with BL is probably determined by the age at which EBV infection occurs. Possibly concurrent infections involving the intestinal wall (visceral leishmaniasis and schistosomiasis) could act as local cofactors for the development of BL with preferential abdominal presentation by inducing a polyclonal B-cell proliferation and an inability to clear the EBV. However, prospective studies are necessary to support this notion.

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