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Short Communication

Genotyping of Epstein-Barr Virus in Brazilian Burkitt's Lymphoma and Reactive Lymphoid Tissue

Type A with a High Prevalence of Deletions within the Latent Membrane Protein Gene

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Both Epstein-Barr virus (EBV) types A and B are found in endemic Burkitt's lymphoma (BL) occurring in equatorial Africa. We studied 17 cases of Brazilian BL previously demonstrated to be EBV-positive to determine the EBV type as well as the presence of a characteristic 30 bp deletion within the 3′ end of the latent membrane protein-1 (LMP-1) gene that may be important to the pathogenesis of several EBV-associated neoplasms. All cases in which the age was known were children. We found type A EBV in 13 of 14 (93%) evaluable cases, and type B in one case. The LMP-1 deletion was found in 12 of 15 (80%) evaluable cases, including the one case of type B EBV, and a similar high prevalence (59%) of the deletion was detected in EBV-positive normal and reactive lymphoid tissues from individuals from the same geographic region. The high proportion of cases associated with type A EBV suggests that immunodeficiency is not an important factor in the pathogenesis of Brazilian BL, in contrast to endemic African BL. The presence of the LMP-1 deletion in a high prevalence in the normal population in this region is unexplained. (Am J Pathol 1996, 148:17–23)

There is a strong association between the Epstein-Barr virus (EBV) and Burkitt's lymphoma (BL) occurring in equatorial Africa, a region of high incidence of this neoplasm (endemic BL).[1] In endemic BL, a latency type I pattern is seen, with expression of the Epstein-Barr nuclear antigen-1 (*EBNA-1*) gene, but no protein expression of the other EBNAs or latent membrane proteins (LMPs).[2] Analysis of the coding region of the *EBNA-2* gene in endemic BL has revealed a high prevalence of both EBV types A and B (or types 1 and 2).[3] This latter finding reflects the high prevalence of both types of EBV in the general population in equatorial Africa, and may suggest the presence of a subtle immunodeficiency–possibly due to holoendemic malaria, given that there is an increased frequency of type B EBV in various immunocompromised populations such as in patients with the acquired immunodeficiency syndrome (AIDS).[4] The role of EBV in the pathogenesis of EBV-associated endemic BL is still not clear, but it has been suggested that EBV infection results in the indefinite propagation of different EBV-transformed B cell clones, each with an increased probability of chro-

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mosomal translocation and subsequent c-*myc* deregulation.

Although previous studies had suggested an association between EBV and South American BL of 25 to 50%,[5,6] we recently identified evidence of EBV by EBV-encoded RNA (EBER) *in situ* hybridization in 71% of cases of BL in Brazil.[7] The incidence of BL in Brazil and other South American countries has been estimated to be intermediate between endemic BL and sporadic BL occurring in developed countries,[8] the latter having a low association with EBV that ranges from 5 to 30%.[9,10]

In this followup study, we have investigated the genotype of the EBV in our previously studied cases of EBV-associated Brazilian BL. We have studied the cases for type A *versus* type B EBV, to compare the cases with endemic BL occurring in equatorial Africa. In addition, we have studied the *LMP-1* (*BNLF-1*) gene for evidence of a characteristic deletion at the 3′ end of the gene that has been reported to be detected in a subset of of cases of Hodgkin's disease, nasopharyngeal carcinoma, and peripheral T-cell lymphoma.[11–16] This 30-bp deletion results in the absence of 10 amino acids (amino acids 343 to 352) from the cytoplasmic domain of LMP-1, a change that may make LMP-1 more tumorigenic.[14,17] For comparison, we have also studied normal and reactive lymphoid tissues.

Materials and Methods

Cases

The cases of BL have been previously published.[7] Twenty-four cases of BL from Brazil were previously studied by EBER *in situ* hybridization, with 17 cases determined to be EBV-positive;[7] these latter cases constitute the cases in the current study. In all 17 cases, all or nearly all of the neoplastic cells were EBER-1-positive. In addition, 39 cases of normal or reactive tonsillar tissues from Brazilian patients were studied for comparison.

Polymerase Chain Reaction Studies for EBV A and B Typing

Genomic DNA was extracted from 5-μm sections cut from formalin-fixed, paraffin-embedded tissue blocks using standard methods. Two 20-base oligonucleotide primers flanking a region of the EBNA-2 differing between type A and type B EBV were used, as previously reported[18]: 5′-AGGCTGCCCACCCTGAGGAT-3′ and 5′GCCACCTGGCAGCCCTAAAG-3′. Each reaction was performed with 2 μl of extracted DNA in a 50 μl mixture containing 50 pmol of each primer, 0.2 mmol/L of each deoxynucleotide triphosphate, 1.5 mmol/L of Mg^{2+}, and 2.5 units of *Taq* polymerase (Perkin Elmer Cetus, Norwalk, CT). After an initial denaturation for 5 minutes at 94°C, 45 cycles were performed as follows: 94°C for 1 minute, 56°C for 1 minute, and 72°C for 1 minute. A final extension at 72°C for 7 minutes completed the polymerase chain reaction (PCR) amplification. The amplified products were electrophoresed in 7% polyacrylamide gels with ox174-puc 19/*Hae*III size markers. After Southern blot transfer, hybridization was performed using a ^{32}P-labeled internal probe. Using this method, the primers amplified a product of 168 bp for EBV type A and a product of 184 bp for EBV type B.

PCR Studies for LMP-1 Gene Deletion

Two 20-base oligonucleotide primers flanking the site of the characteristic 30-bp deletion were used, as previously published[12,19]: 5′-CGGAGGAGGTGGAAAACAAA-3′ and 5′-GTGGGGGTCGTCATCATCTC-3′. The reaction was carried out similarly as described above, except that 50 cycles were performed as follows: 94°C for 1 minute, 61°C for 1 minute, and 72°C for 1 minute. The polyacrylamide gel was visualized with ethidium bromide. Using this method, the amplified germline product of the 3′ end of the EBV *LMP-1* gene is 161 bp, whereas a product containing the characteristic deletion is 131 bp. The results were confirmed by slot-blot hybridization using a ^{32}P internal probe (5′-GCCGTCATGGCCGGAATCAT-3′) that is specific for the deleted segment, which hybridizes against the germline but not deleted LMP gene, as well as a probe (5′-GGCGGGCCCTGGTCACCTCC-3′) that is specific for a region flanking the deleted segment, and thus hybridizes against both the germline and deleted LMP genes.

Immunohistochemical Studies for LMP-1 Protein

Immunohistochemical studies were performed using formalin-fixed, paraffin-embedded tissue sections and previously published methods, including heat-induced epitope retrieval.[20] After enzymatic digestion, the CS1–4 cocktail of antibodies (Dako, Carpinteria, CA) was used at a dilution of 1:25 to 1:50. Reactivity was detected using avidin-biotin technique and 3′,3′-diaminobenzidine-tetrahydrochlo-

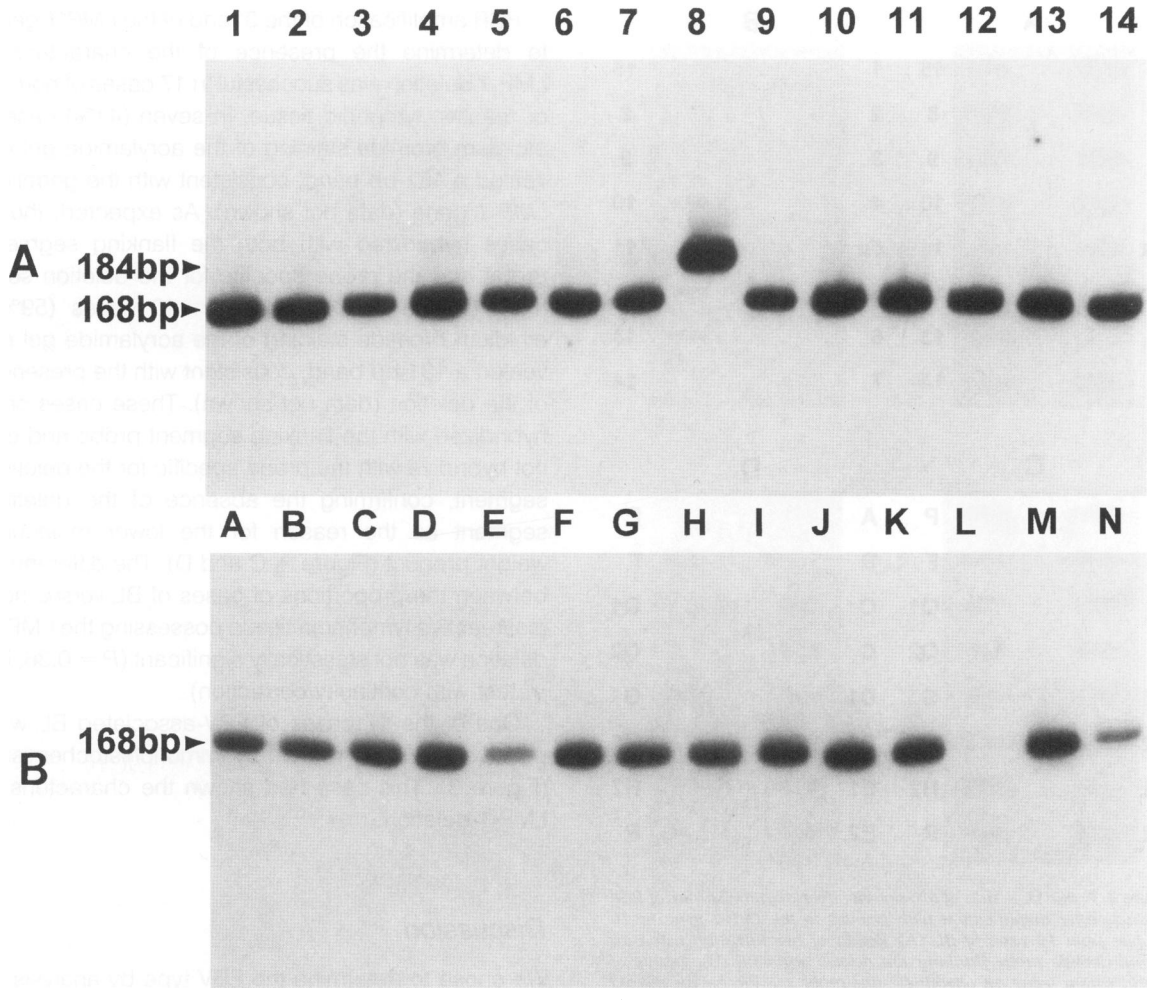

Figure 1. (Top) *Results of Southern blot hybridization of PCR products after amplification with primers to the EBNA-2 gene to determine the EBV type in representative cases of BL. In 13 cases, a 168 bp product is obtained, consistent with EBV type A, whereas in 1 case, a 184 bp product is obtained, consistent with EBV type B.* (Bottom) *Results of a similar study of representative cases of normal or reactive tonsillar tissue. In all but one case, a 168 bp product is obtained, consistent with EBV type A. In one case, no product is obtained. Although this could be due to DNA degradation, the extracted DNA from this case was amplifiable using primers for the LMP-1 gene. It is possible that the lack of amplification using EBNA-2 primers was due to deletion of this region of the gene (see Discussion).*

ride dihydrate as the chromogen (Biotek, Santa Barbara, CA).

Results

The age was available in 13 of the 17 cases of BL; all were children 13 years and under, with a median age of 6 years. Of the 15 cases in which information about gender was available, there were 7 males and 8 females. Of 39 cases of normal or reactive tonsillar tissue, 36 were from patients 13 years and under, with a median age of 6 years. Of the 17 cases from which EBV was identified by PCR (using the LMP-1 primers), 15 were 13 years and under, with a median age of 7 years; there were 8 males and 9 females.

PCR amplification of the *EBNA-2* gene to determine the type of EBV was successful in 14 of the 17 cases of EBV-positive BL. In 13 cases (93%), a 168-bp product was identified on Southern blot, consistent with type A EBV, whereas in one case (8%), a 184-bp product was present, consistent with type B EBV (Figure 1A). In three cases, there was no amplification product, including one case that was amplifiable for the *LMP-1* gene (see below). Of the 14 cases of normal or reactive lymphoid tissues that showed successful amplification using the EBNA-2 primers, all showed a 168-bp product, consistent with type A EBV (Figure 1B).

PCR amplification of the 3′ end of the *LMP-1* gene to determine the presence of the characteristic LMP-1 deletion was successful in 15 of the 17 cases

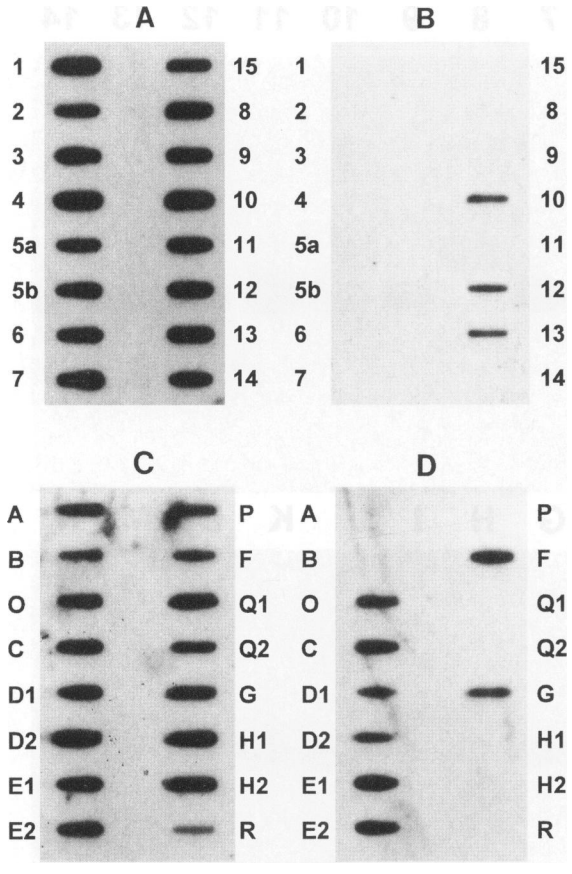

Figure 2. A *and* B: *Results of two slot-blot hybridization studies of PCR products after amplification with primers to the LMP-1 gene in 16 samples from 15 cases of BL.* (A) *Results of hybridization using an oligonucleotide probe flanking the deleted segment.* (B) *Results of hybridization using an oligonucleotide probe specific for the deleted segment. The absence of hybridization using this latter probe in all but three samples is consistent with the occurrence of the deletion in these 13 specimens.* C *and* D: *Results of two slot-blot hybridization studies of PCR products after amplification with primers to the LMP-1 gene in 16 samples from 12 representative cases of normal or reactive tonsils.* (C) *Results of hybridization using an oligonucleotide probe flanking the deleted segment.* (D) *Results of hybridization using an oligonucleotide probe specific for the deleted segment. The absence of hybridization using this latter probe in eight samples from six cases is consistent with the occurrence of the deletion in these eight specimens.*

of EBV-positive BL. In three cases (20%), ethidium bromide staining of the acrylamide gel revealed a 161-bp band, consistent with the germline *LMP-1* gene (data not shown). As expected, these cases hybridized with both the flanking segment probe and the probe specific for the deletion segment (Figure 2, A and B). In 12 cases (80%), including the case of type B EBV, ethidium bromide staining of the acrylamide gel revealed a 131-bp band, consistent with the presence of the deletion (data not shown). These cases only hybridized with the flanking segment probe and did not hybridize with the probe specific for the deletion segment, confirming the absence of the deletion segment as the reason for the lower molecular weight product (Figure 2, A and B).

PCR amplification of the 3′ end of the *LMP-1* gene to determine the presence of the characteristic LMP-1 deletion was successful in 17 cases of normal or reactive lymphoid tissue. In seven (41%) cases, ethidium bromide staining of the acrylamide gel revealed a 161-bp band, consistent with the germline *LMP-1* gene (data not shown). As expected, these cases hybridized with both the flanking segment probe and the probe specific for the deletion segment (Figure 2, C and D). In 10 cases (59%), ethidium bromide staining of the acrylamide gel revealed a 131-bp band, consistent with the presence of the deletion (data not shown). These cases only hybridized with the flanking segment probe and did not hybridize with the probe specific for the deletion segment, confirming the absence of the deletion segment as the reason for the lower molecular weight product (Figure 2, C and D). The differences between the proportions of cases of BL *versus* normal/reactive lymphoma tissue possessing the LMP-1 deletion was not statistically significant ($P = 0.36$, by χ^2 test with continuity correction).

One of the 17 cases of EBV-associated BL was positive for LMP-1 protein by immunohistochemistry (Figure 3). This case had shown the characteristic LMP-1 deletion.

Discussion

We chose to determine the EBV type by analysis of the *EBNA-2* gene. In the region spanning the PCR primers we used, there is a 16-bp deletion in type A, but not type B, EBV. A minority of groups have studied several genes to analyze the EBV type, because it is known that types A and B differ at several genes, including *EBNA-2*, *EBNA-3a*, *EBNA-3b*, and *EBNA-3c*[21]; however, such studies have demonstrated consistency between the multiple analyses in almost all cases.[22] By studying the *EBNA-2* gene, we determined that 93% of the Brazilian BL and 100% of the cases of normal or reactive lymphoid tissue contained type A EBV and only one case, a BL, contained type B EBV; no dual infections were found. In one case of BL and three cases of reactive lymphoid tissue, amplification was not detected, despite the fact that an EBV LMP-1 amplification product was found using a similar PCR protocol. Deletions in the *EBNA-2* gene have been described, and could have accounted for the negative results in these cases.

Our results differ from what has been described in BL occurring in equatorial Africa. In those cases, both types A and B EBV have been identified at high frequency.[3] Type B EBV is also identified at high

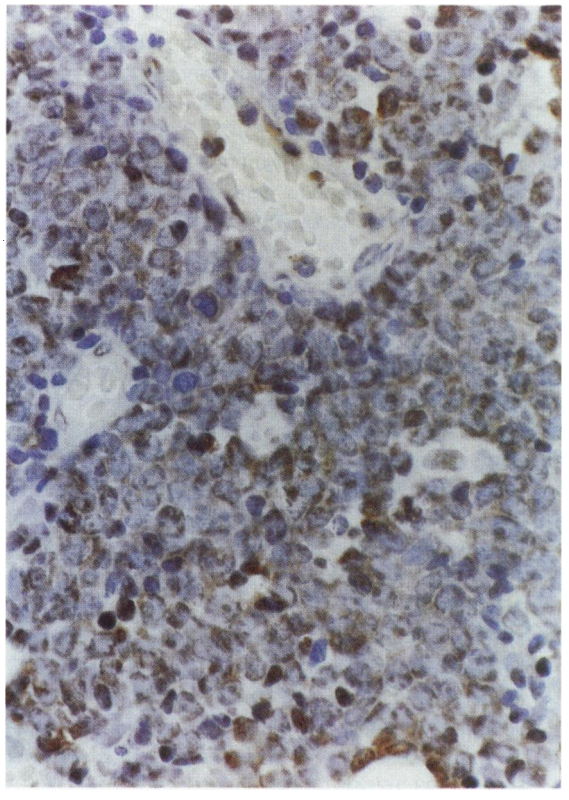

Figure 3. *BL (case 5), immunohistochemistry for LMP-1 protein. This was the only case to show positivity for LMP-1 protein; note the primarily cytoplasmic staining.*

frequency in normal individuals in equatorial Africa.[3] In contrast, type A EBV is almost exclusively found in the peripheral blood of people from developed Western countries, where only 3% of individuals carry type B EBV.[4,23] Type A EBV is much more efficient in immortalizing B cells than type B,[24] and some investigators hypothesize that type B EBV can only effectively transform B cells in immunodeficient populations. Patients with AIDS have an increased prevalence of infection with type B EBV, and both AIDS-related non-Hodgkin's lymphoma and Hodgkin's disease also have an increased frequency of type B infection.[4,25–27] However, although patients on immunosuppression after organ transplantation have a relatively high prevalence of type B in their blood,[23] posttransplantation lymphoproliferative disorders arising in this setting contain exclusively type A EBV.[22] In our cases, virtually all type A was identified in BL, reflecting the predominant subtype identified in reactive tissues from the local population. We cannot completely rule out the presence of type B EBV in the population, because type B may be present in the oropharynx, without gaining entry to the peripheral blood and lymphoid tissue.[4,28] Our results are somewhat similar to recent studies of BL occurring in Egypt and Turkey, in which 10 of 12 cases, and 12 of 13 cases, respectively, were EBV type A, while the remaining cases were type B.[29,30]

The LMP-1 protein upregulates a variety of host genes,[31] inhibiting apoptosis via induction of *bcl-2*.[32] LMP-1 is essential for transformation of B lymphocytes *in vitro*,[33] and it is considered to be a viral oncogene, because it can transform rodent fibroblasts.[34] A characteristic 30-bp deletion has been identified at the 3′ end of the *LMP* gene first identified in nasopharyngeal carcinoma.[13,14] Transfectants of these isolates have been found to be aggressive when inoculated into severe combined immunodeficiency or nude mice, suggesting that the presence of this deletion may be associated with more aggressive behavior when found in tumors.[14,17] In support of this hypothesis, Knecht and colleagues[11] found that this deletion was found in a subset of cases of European Hodgkin's disease associated with numerous or anaplastic Reed-Sternberg cells and cases with necrosis. In nasopharyngeal carcinoma and Hodgkin's disease, the LMP-1 protein is expressed in the majority of cases (latency pattern II), while the protein is not typically expressed in BL (latency pattern I),[2] raising doubt whether the presence of LMP-1 deletions could be of any importance in the latter neoplasm. All but one of the cases in the current study were negative for LMP-1 protein by immunohistochemical studies; this is similar to the results of a recent study.[35] However, it is possible that LMP-1 protein is expressed and is important only during neoplastic transformation, and is no longer expressed at a later point in time, as it may not be critical to maintenance of the neoplastic state.

In the current series of Brazilian BL, we found clear evidence of the characteristic *LMP-1* gene deletion in 80% of cases. In these cases, the amplification products were found to be 30 bp smaller than expected, consistent with presence of the deletion. More importantly, the amplification products showed strong hybridization with an oligonucleotide probe flanking the site of the deletion, but showed no hybridization with a probe homologous to the deletion segment. The one case of BL that was EBV type B contained the LMP-1 deletion. In one study of nasopharyngeal carcinoma, the deletion was found only in type A and not type B strains.[16] However, Sandrej and colleagues[15] have also reported the presence of the deletion in type B strains, suggesting that this change may have mutated independently through the evolution of EBV strains. In our previous study of LMP-1 deletions in Hodgkin's disease, we noted the

occurrence of the deletion in two cases at one but not other sites, consistent with its occurrence after neoplastic transformation.[19]

Equally surprising as the high incidence of the LMP-1 deletion in BL tissues was the finding of the characteristic deletion in a similar proportion, ~60%, of cases of normal or reactive lymphoid tissues, suggesting that this genotype is common in the Brazilian population. Although there is yet little data on the prevalence of the deletion in normal individuals from developed Western countries, it is thought that it is not a frequent occurrence. For example, in the previously mentioned European Hodgkin's disease study, the deletion was found in only about 10% of cases,[11] and the deletion was seen in only three of nine cases of acute infectious mononucleosis in Europe.[15] However, a high frequency of LMP-1 deletions has been found in a series of cases of Danish (60%) and Malaysian (100%) peripheral T-cell lymphomas, suggesting that the deletion may be important in the pathogenesis of these neoplasms.[15] It is possible that the presence of the 30 bp deletion within the *LMP-1* gene is responsible for the high frequency of EBV-associated BL seen in this Brazilian population. In this regard, to our knowledge there are no data on the frequency of the LMP-1 deletion in equatorial Africa, both within the general population as well as in tissues involved by BL.

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