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Epstein–Barr Virus in a CD8-positive T-cell Lymphoma

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In contrast to its role in B-lymphomagenesis, Epstein–Barr Virus (EBV) only incidentally has been associated with T-cell lymphomas. In the present report we describe a fourth patient with EBV-related T-cell lymphoma. The patient presented with an angio-immunoblastic lymphadenopathy (AILD)-like T-cell lymphoma. Serology was compatible with chronic Epstein–Barr (EBV) infection. After a 1-year period of waxing and waning lymphadenopathy, this lymphoma evolved to an aggressive CD8$^+$ Immunoblastic T-cell lymphoma. A relationship with the chronic EBV infection was indicated by the finding of EBV genome in the tumor tissue by Southern blot analysis. Moreover, EBV nuclear antigen (EBNA) was detected in situ ***within individually defined CD8$^+$ tumor cells by two-color immunofluorescence. Two alternative possibilities, namely that EBV primarily played a role in lymphomagenesis of the AILD-like T-cell lymphoma or that the virus was an additional oncogenic event in the final process of tumor progression to the immunoblastic lymphoma, are discussed. (Am J Pathol 1990, 136:1093–1099)***

Epstein–Barr virus (EBV) has a special tropism for lymphoid and epithelial cells, in particular B lymphocytes, epithelium of the pharynx, salivary gland duct cells, and uterine cervix cells.[1–3] Infection has been related to benign diseases like infectious mononucleosis and chronic EBV infection,[4] but also has a role in the development of endemic Burkitt's lymphoma,[5] nasopharyngeal carcinoma, and thymic carcinoma.[6,7] The virus also has been implicated in the pathogenesis of B-lymphoproliferative disorders in immunodeficiency,[8–10] and more recently, also in the pathogenesis of T-cell lymphomas and Hodgkin's disease.[11,12] In the T-cell lymphomas described, all three patients had a history of chronic EBV infection.[11] The two lymphomas that could be tested had a CD4$^+$ phenotype.

We report on a patient who ultimately developed an EBV-containing CD8$^+$ immunoblastic T-cell lymphoma after initial presentation with a less aggressive T-cell lymphoma. The role of EBV in primary lymphomagenesis and secondary evolution to the high-grade lymphoma is discussed.

Materials and Methods

Lymph node biopsies were processed according to standard techniques. Direct immunofluorescence for the detection of immunoglobulins and indirect immunoperoxidase test for the detection of T- and B-cell–associated antigen with murine monoclonal antibodies were performed as described earlier.[13]

Epstein–Barr Virus Nuclear Antigen Tests and Two-color Immunofluorescence Tests

Anti-EBNA (EBV nuclear antigen) immunofluorescence was performed on frozen tissue sections and cytocentrifuged cells of the patient and Raji cells as positive controls. After fixation in acetone at −20°C for 10 minutes and methanol at −70°C for 30 minutes, slides were incubated with heat-inactivated (56°C for 30 minutes) human anti-EBNA positive (n = 5) and negative (n = 5) test sera, containing guinea pig complement. Guinea pig complement alone was used as a control for complement fixation. After incubation for 30 minutes at 37°C, the slides were washed and incubated with fluorescein-conjugated

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goat anti-guinea pig complement (GaGp/C3c FITC, Nordic, Tilburg, The Netherlands) diluted in physiologic barbiturate buffer pH 7.4, with 10% EBV-negative inactivated human serum, for 30 minutes at 37°C. For two-color immunofluorescence tests, slides were subsequently incubated with the Leu-2 monoclonal antibody, washed and incubated with a rhodamine-conjugated goat anti-mouse antiserum (Nordic, Tilburg, The Netherlands). After washing and mounting in buffered glycerol, slides were studied with a Leitz immunofluorescence microscope (Leitz-Diaplan, West Germany).

Antibody Studies for EBV and Other Viruses

Antibody tests for Epstein–Barr viral capsid antigen (VCA), early antigen (EA), and nuclear antigen (EBNA) were performed as described.[14] Tests for human T-cell lymphotropic virus type I (HTLV-I) and human immunodeficiency virus (HIV) were performed in the Central Laboratory of Blood Transfusion (CLB), Amsterdam, The Netherlands (sandwich enzyme-linked immunosorbent assay (ELISA), Dupont and Abbott, respectively).

EBV-DNA Hybridization

Restriction enzymes were from Boehringer Mannheim (Mannheim, West Germany) GmbH (*Bam*HI and *Dpn*I) and from New England Biolabs (*Mbo*I). Conditions for enzyme reactions (18 hours at 37°C) were as described.[15] Completeness of *Mbo*I and *Dpn*I cleavage was monitored by comparison of hybridization patterns obtained after digestion with 0.6 U and 2.4 U of enzyme per μg of DNA. Recombinant plasmids were propagated in *Escherichia coli* K-12 strain HB 101. Plasmid DNA was isolated as described.[16] Plasmid pFF 409-5,[17] which contains the 3.0 kb EBV *Bam*HI-W fragment, was a gift from Dr. G. Miller (Yale University, New Haven, CT). The *Bam*HI-W fragment was recloned at the *Bam*HI site of the high copy number vector pHV 624 (provided by Dr. I. Boros[18]) yielding pEBV-W. Isolation of cellular DNA, agarose gel electrophoresis, Southern blotting, and hybridization conditions were as described.[16] The 3.0-kb EBV *Bam*HI-W fragment was purified from *Bam*HI-digested pEBV-W by two cycles of gel-electrophoresis followed by isolation as described.[19] The purified fragment (500 ng) was labeled by nick-translation[20] with P^{32} dCTP (Amersham International) to a specific activity of approximately 10^8 cpm per μg, and was used as a probe.

Results

Case History

A 41-year-old Caribbean man presented in October 1983 with a 6-week history of malaise, intermittent fever (up to 40°C), and generalized lymphadenopathy. Physical examination revealed enlarged painful lymph nodes and splenomegaly. A computed tomography (CT) scan showed splenomegaly and mesenteric and retroperitoneal lymphadenopathy. The white blood cell count (WBC) was 9.2×10^9/1 with a normal differentiation. Renal and liver function tests were normal, as was the protein profile. The direct Coombs reaction was negative. Antibody tests for HTLV-I and HIV were negative. Serologic tests for EBV infection showed high gamma G immunoglobulin (IgG) (1:10.000), but negative gamma M immunoglobulin (IgM) antibody titers to EBV capsid antigen (EB-CA), high titers (1:640) to EBV early antigen (EB-EA), and low titers (1:32) to EBV nuclear antigen (EBNA). This profile is compatible with chronic EBV infection.[3] An inguinal lymph node biopsy showed nonspecific changes, but a cervical lymph node taken in December showed angio-immunoblastic lymphadenopathy (AILD). Prednisone therapy was given (initially 40 mg daily) until August 1984 and resulted in disappearance of lymphadenopathy and fever. In August 1984, EBV serology was still compatible with chronic EBV infection (anti-EA titer of 1:640) (Table 1).

In September 1984, the patient developed a transient and painful enlargement of the parotid glands, followed by generalized lymphadenopathy and fever. A lymph node biopsy showed AILD. Treatment with prednisone resulted in a partial response. In November 1984, he again developed painful enlargement of the parotid glands followed by generalized lymphadenopathy. An inguinal lymph node revealed AILD, but cervical lymph nodes showed a frankly malignant (polymorphic immunoblastic) lymphoma. Immunoblasts were found in blood and bone marrow smears. Polychemotherapy according to the CHOP regimen (cyclophosphamide, adriamycin, vincristine, prednisone) was given, but the patient died 2 weeks later. No permission for a postmortem examination was given.

Pathology of Lymph Nodes

Revision of the lymph node biopsies taken between November 1983 and October 1984 showed an AILD-like T-cell lymphoma with absence of follicles and small clusters of immunoblasts (Figure 1). Limited histochemical tests showed polyclonally derived B lymphocytes and plasma cells and dispersed T lymphocytes. Chromosomal analy-

Table 1. *Clinical Course and Laboratory Data*

	Oct. '83	Dec. '83	Aug. '84	Sept. '84	Oct. '84	Nov. '84
Lymphadenopathy	+++	+++	—	+++	+	+++
Parotid enlargement	—	—	—	++*	—	++*
Histology	Reactive	AILD			AILD	Immunoblastic
Treatment		Prednisone			Prednisone-CHOP	
Serology						
IgG EB-CA	1:10.000		1:10.000			1:10.000
IgM EB-CA	Negative		Negative			Negative
IgA EB-CA	—		1:20			1:10
IgG EB-EA	1:640		1:160			1:160
Ig EBNA	Positive		1:64			1:16
Ig EBNA1			1:32			1:16
Ig EBNA2			1:32			1:8
HTLVI	Negative				Negative	
HIV	Negative				Negative	
IgG CMV	Negative				Negative	
IgM CMV	Negative				—	
Mumps					Negative	
EBNA intracellular	Negative				30% pos.;	50% pos.
EBV DNA						Positive

* Parotid enlargement preceeded lymph node enlargement.

sis of unstimulated suspended lymph node cells (December 1983) revealed a 6q− (q21) abnormality in three out of 16 metaphases. Chromosomal analysis of Phytohemagglutinin (PHA)-stimulated peripheral blood cells (November 1984) revealed a 6q− in two and a t (6;12) (921;q24) in 1 of 23 metaphases (Dr. G. C. Beverstock, Department of Human Genetics, Leiden University, The Netherlands).

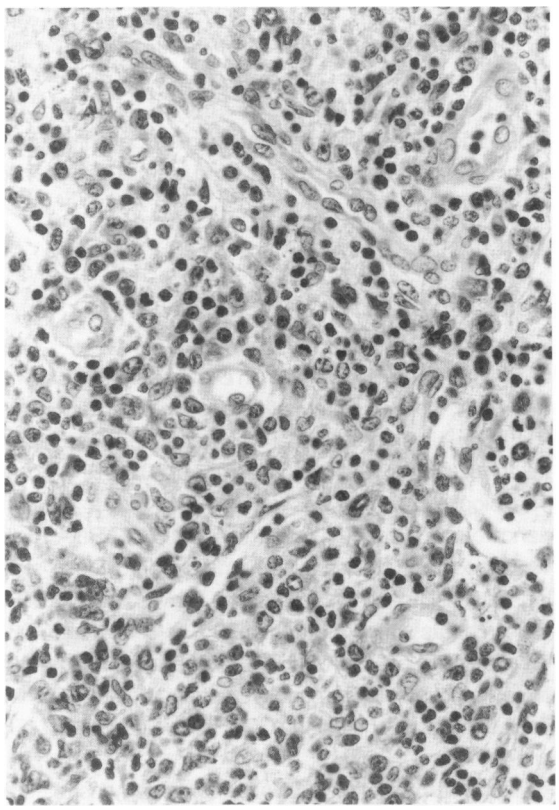

Figure 1. *Lymph node biopsy with AILD-like T-cell lymphoma, taken in December 1983. Prominent high endothelial venules with an infiltrate of small lymphocytes, plasma cells, and isolated or clustered immunoblasts (in particular in the lower side) are shown (H & E, original magnification ×400).*

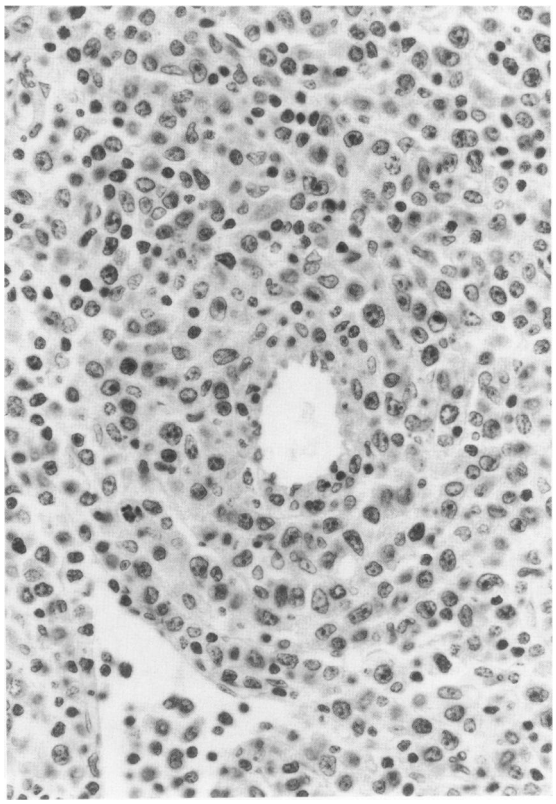

Figure 2. *Lymph node biopsy taken in December 1984. The picture is dominated by polymorphic immunoblasts with a clear cytoplasm. Note infiltration of the vascular wall (H & E, original magnification ×400).*

Table 2. *Immunophenotype of Immunoblastic Lymphoma*

T-cell markers		B-cell markers		Other markers	
CD1 (OKT6)	–	CD20 (B 1)	–	HLA-DR	+
CD2 (LEU5)	+	CD22 (Dako panB)	–		
CD3 (LEU4, OKT3)	+	Immunoglobulins	–		
CD4 (LEU3a)	–				
CD5 (LEU1)	–				
CD7 (WT1)	+				
CD8 (LEU2)	+				

A biopsy taken in October 1984 contained larger clusters of atypical immunoblasts. Unfortunately, no frozen material has been left for additional immunohistochemistry and analysis of T-cell receptor rearrangements.

Lymph node biopsies taken in November and in December 1984 showed necrosis and diffuse infiltration by large atypical immunoblasts (Figure 2). The tumor cells had an HLA-DR$^+$, CD4$_-$, CD8$^+$ phenotype (Table 2; Figure 3). Their malignant character was underlined by the lack of reactivity for CD5 and by the presence of a 6q– chromosomal abnormality as found both in suspended lymph node cells and cultured peripheral blood lymphocytes. Notably, B cells, as determined by the reactivity for CD20 and CD22 monoclonal antibodies, constituted less than 5% of cells and were only found in small easily recognizable clusters.

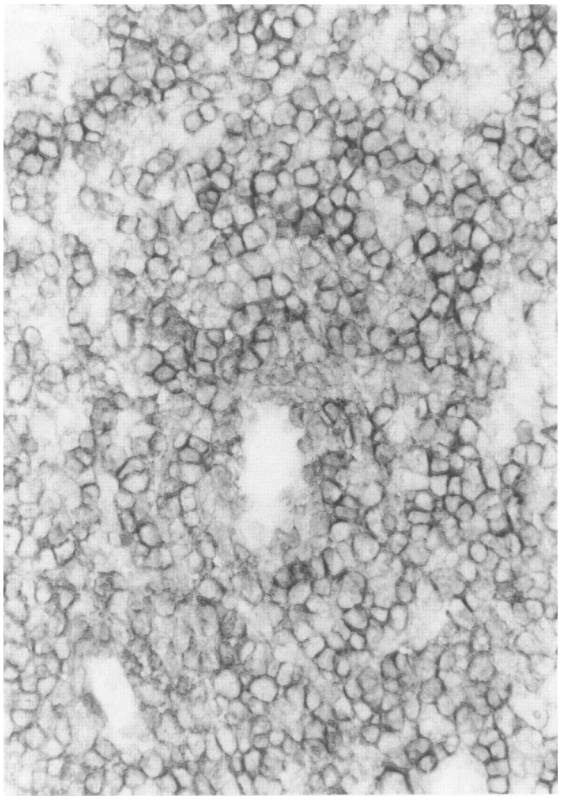

Figure 3. *Immunoperoxidase staining for CD8 (Leu 2) on a frozen tissue section of the lymph node biopsy taken in December 1984. Note that almost all cells are positive. Infiltration of the vascular wall is shown in the center (Hematoxylin counter staining, original magnification ×400).*

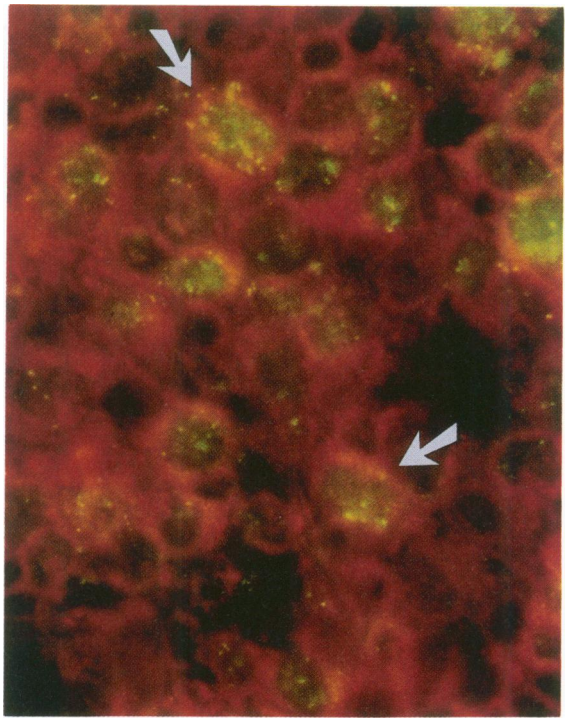

Figure 4. *Two-color immunofluorescence of CD8$^+$ EBNA$^+$ tumor cells. CD8$^+$ (Leu2) tumor cells are identified by rhodamin (red). EBNA is identified by fluorescein (green). The arrows indicate double-positive (CD8$^+$ EBNA$^+$) cells. Note that other CD8$^+$ cells are EBNA (original magnification ×700).*

Detection of EBNA and EBV-DNA

The lymph node biopsy taken in December 1983 showed no EBNA$^+$ cells by immunofluorescence. An EBNA$^+$ cell line from this same material was generated. The biopsy taken in October 1984 contained about 30% EBNA$^+$ cells. The biopsy taken in December 1984, with a predominance of CD8$^+$ cells (>90%) and about 50% EBNA$^+$ cells, was used for further analysis. Immunoperoxidase stainings in serial frozen tissue sections showed many EBNA$^+$ cells in areas where more than 90% of cells were CD8$^+$. The presence of EBNA within CD8$^+$ cells was further confirmed by two-color immunofluorescence tests, which revealed that about half of the CD8$^+$ cells were EBNA$^+$ (Figure 4).

In the same material, EBV DNA was demonstrated by Southern blot analysis (Figure 5). After *Bam*HI digestion of lymphoma DNA, a 3.0-kb fragment comigrated with the EBV *Bam*HI-W fragment from marker DNA. To ascertain that the hybridization signals were not due to contamina-

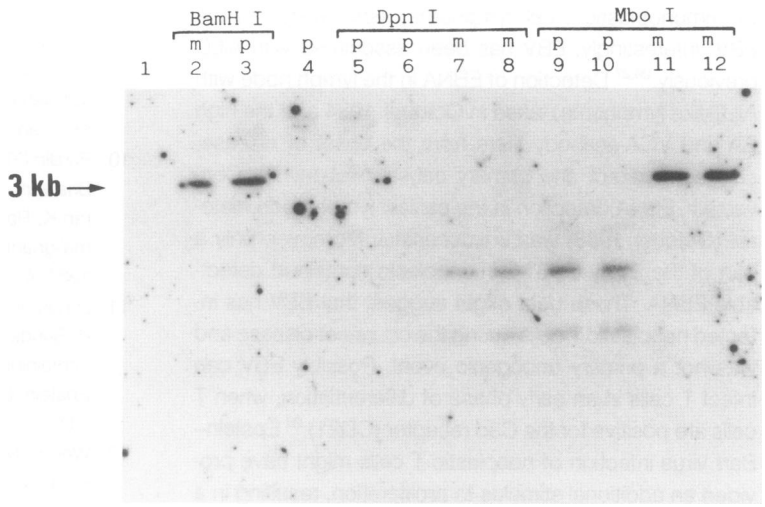

Figure 5. *Southern blot analysis. Patient lymphoma DNA (p; 5 μg/lane) was either left undigested* (lane 4) *or was digested (18 hours at 37°C) with BamHI* (lane 3), *DpnI* (lane 5, *3 U/5 μg;* lane 6, *12 U/5 μg) or MboI* (lane 9, *3 U/5 μg;* lane 10, *12 U/5 μg). Marker DNA (m; human placenta DNA, predigested with PstI, to which was added BamHI-digested pEBV-W DNA to approximately 50 copies/cellular genome; 5 μg/lane) was digested with BamHI* (lane 2), *DpnI* (lane 7, *3 U/5 μg;* lane 8, *12 U/5 μg) or MboI* (lane 11, *3 U/5 μg;* lane 12, *12 U/5 μg).* Lane 1 *(negative control) contains 5 μg PstI digested human placenta DNA. After electrophoresis through a 1.4% agarose gel, the DNA was blotted onto a nitrocellulose filter and probed with the ^{32}P-labeled 3.0-kb EBV BamHI-W fragment purified from pEBV-W.*

tion with recombinant plasmid DNA, we used the restriction enzymes *Mbo*I and *Dpn*I, which recognize the same DNA sequence (5′GATC3′). *Dpn*I only cleaves this sequence when the A-residue is methylated, whereas *Mbo*I is inhibited by this modification.[21] Because methylation of A-residues is common in prokaryotes (eg, in the bacterial host for pEBV-W), but does not occur in mammalian DNA,[22] the combination of these enzymes in Southern blot analysis can be helpful in determining the source of DNA. Marker DNA consisted of human placental DNA (digested with *Pst*I) to which *Bam*HI-digested pEBV DNA was added. *Mbo*I did not cleave the methylated restriction sites of pEBV DNA; as a result, only *Bam*HI fragments are present in lanes 11 and 12. Patient EBV DNA (lanes 9 and 10) is not methylated at the *Mbo*I restriction sites, which explains the *Mbo*I fragments in lanes 9 and 10. From the data presented above it can be concluded that the *Bam*HI-W fragment was present in lymphoma tissue at approximately 50 copies per cell. Because the *Bam*HI-W fragment is present at approximately 6 to 11 copies per viral genome,[17] the estimated mean virus load per cell ranged from 5 to 10.

Discussion

The present report of a T-cell lymphoma supplements the recent data on three patients with EBV-related T-cell lymphoma.[11] Additionally, Kikuta et al[23] recently localized EBV within CD4$^+$ T-cells of a patient with chronic EBV infection associated with Kawasaki-like disease. Thus, five cases indicate that EBV may be implicated in the pathogenesis of T cell disorders. In the present case, it was excluded that residual B cells and not the T cells harbored the virus. Using a two-color immunofluorescence test comparable with that used by Kikuta et al,[23] we demonstrated that the EBNA was present in individually identified CD8$^+$ immunoblastic lymphoma cells. Epstein–Barr virus was further demonstrated by detection of EBV genome using Southern blot analysis. Notably, the sample analyzed contained almost no residual B cells and consisted for more than 90% of CD8$^+$ tumor cells. The CD8$^+$ phenotype is in contrast to two out of three reported cases with a CD4$^+$ phenotype.[11]

Epstein–Barr virus has been related with fatal but oligoclonal B-cell proliferative disorders.[9] Clonality of EBV-associated lymphoid proliferations can be assessed now by Southern blot analysis of immunoglobulin gene, or T-cell receptor gene rearrangements, or of genomic termini of viral DNA within the infected cells.[24] We could not perform these analyses because of a lack of further frozen material. However, the clonality and the malignant character of the tumor is strongly supported by the recurrent 6q− chromosomal abnormality and the lack of CD5 reactivity as assessed in the final blast cells.

Before the development of the final T-immunoblastic lymphoma, our patient had a 1-year history of lymphoproliferative disease. This was interpreted as a chronic EBV infection from a clinical point of view, in particular because of highly elevated titers to EBV antigenic determinants (IgG anti-VCA, 1:10,000; anti-EA, 1:640) but low titers to EBNA.[4] From a pathologic point of view, all biopsies were considered to be compatible with AILD. On revision, a diagnosis of AILD-like T-cell lymphoma was thought to be more appropriate, indicating a neoplastic rather than a reactive disease. We note that most cases of AILD are now considered to represent neoplastic disorders, in particular because of the finding of clonal T-cell receptor rearrangements and the high frequency of development into overt T-cell lymphoma.[25] In the present case, the neoplastic nature of the initial disease is strongly supported by the finding of the 6q− chromosomal abnormality.

The issue raised pathogenetically is how the patient's initial AILD-like T-cell lymphoma, and later transformation

to immunoblastic T-cell lymphoma, were related to the EBV. Interestingly, EBV has been associated with AILD previously.[26,27] Detection of EBNA in the lymph node with AILD-like lymphoma, taken in October 1984 and the high EA and VCA-antibody titers from the onset of disease, suggest a direct and primary causal relationship. Conversely, EBNA detection in the earliest lymph node material (October 1983) was unsuccessful. Moreover, only a part of the finally $CD8^+$ immunoblasts contained detectable EBNA. These data might suggest that EBV has infected neoplastic T cells during the course of disease and was not a primary oncogenic event. Possibly EBV can infect T cells in an early phase of differentiation, when T cells are positive for the C3d receptor (CD21).[28] Epstein–Barr virus infection of neoplastic T cells might have provided an additional stimulus to proliferation, resulting in a high-grade T-immunoblastic lymphoma. Thus, we can not exclude that EBV was only an additional oncogenic factor in the multistep process of tumor progression to the immunoblastic lymphoma. Although highly speculative, this might parallel the role of EBV in the pathogenesis of endemic Burkitt's lymphoma to some extent. Recently, it was suggested that in the multistep scenario of the development of the lymphoma, EBV is not a primary, but a secondary event, providing a complementary signal to allow outgrowth of cells with a rare chromosomal aberration[29]

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