A Case of Severe Chronic Active Infection with Epstein-Barr Virus: Immunologic Deficiencies Associated with a Lytic Virus Strain

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Infectious mononucleosis (IM) is a self-limiting, lymphoproliferative disease induced by primary infection with the Epstein-Barr virus (EBV). Infection with EBV leads in general to lifelong asymptomatic persistence of the virus. We report the case of a woman who acquired IM at the age of 15 years and then suffered from recurrent high fever, fatigue, and signs of immunologic disorder for more than 12 years until she died of liver failure. In an attempt to describe and to define the course of chronic active infection with EBV, we performed immunologic and molecular assays that demonstrated lytic replication of EBV in the B and T cells of the peripheral blood. In addition to signs of humoral and cellular immune deficiency, we detected an EBV strain with an impaired capability to immortalize B cells and a tendency to lytic replication, thus contributing to the pathogenesis of this chronic active infection.

Infection with the Epstein-Barr virus (EBV), which belongs to the group of human pathogenic herpesviruses, results in lifelong persistence of the virus. Whereas in younger children the primary infection is in most cases asymptomatic, EBV is the causative agent of infectious mononucleosis in older children and adults [1]. Infectious mononucleosis is a self-limiting lymphoproliferative disease that lasts in general for 3–6 weeks, with the typical clinical symptoms of fever, cervical lymphadenopathy, and sore throat. Sometimes hepatosplenomegaly, hepatitis, and a number of other rare complications appear (reviewed in [2]). In the blood, the number of mononuclear lymphoid cells is massively elevated and atypical T cells are present.

Primary infection with EBV is characterized serologically by the appearance of IgM and IgG antibodies to viral gene products of the lytic replication cycle (early antigen [EA], viral capsid antigen [VCA]). During the acute phase of infectious mononucleosis, antibodies to the EBV-associated nuclear antigen (EBNA) proteins of the viral latency are absent. During convalescence, the titers of the EA-specific antibodies decline and finally disappear, indicating efficient control of lytic-virus replication in the blood [3]. The titers of VCA-specific antibodies also decline but stay detectable on a lower level throughout life, supposedly because of recurrent stimulation with viral particles shed in the saliva. Eventually, the appearance of IgG antibodies to the EBNAs correlates with convalescence.

In rare cases, infectious mononucleosis takes a chronic active course (chronic active EBV infection), and the symptoms of infectious mononucleosis then last for months to years (reviewed in [4]). The pathogenesis of chronic active EBV infection is unclear. Various immunologic disorders [5–9] have been described as occurring in individuals with chronic active infection, and some investigators believe that virus strains with a reduced capacity to immortalize B cells and an enhanced tendency to lytic replication have been involved in the pathogenesis [8, 10–12].

Recently, we isolated for the first time a virus strain with a lytic phenotype in a case of chronic active EBV infection and demonstrated that the reduced capacity to immortalize B cells correlated with an overexpression of viral regulatory genes of the lytic cycle [13–15].

Case Report

Clinical presentation of the case. A 27-year-old woman with a chronic disease was admitted to our hospital in April 1996. In 1984, 12 years earlier, infectious mononucleosis and primary infection with EBV were diagnosed on the basis of serological findings and clinical symptoms, including high fever and swollen cervical lymph nodes. From that time on, the woman was physically and psychologically severely impaired. In addition to chronic fatigue, with the need for 12–14 hours of sleep a day, up to five times a year the woman had recurrent high fever (temperatures to 39°C) and purulent infections of the sinuses and bronchi.

Reactivated mononucleosis was suspected for the first time in 1985. In addition, febrile septal panniculitis was diagnosed because of recurrent reddish-brown to violet discoloration of

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Table 1. Serological findings indicating severe reactivation of Epstein-Barr virus (EBV) infection in a woman with chronic active EBV infection.

Date	EA-IgM (ELISA)	EA-IgG (IFT)	EA-IgG (ELISA)	EA-IgA (IFT)	VCA-IgM (IFT)	VCA-IgG (IFT)	VCA-IgA (IFT)	EBNA (IFT)	EBNA-1 (ELISA)
Oct 1985	ND	1:128	ND	ND	−	1:8,192	ND	ND	ND
Dec 1986	ND	1:2,048	ND	ND	ND	1:16,384	ND	Weak +	ND
Jan 1987	ND	1:2,048	ND	ND	−	1:16,384	ND	Weak +	ND
Mar 1996	+	1:4,096	+	1:128	+	1:131,072	1:8,192	Weak +	−
Apr 1996	+	1:4,096	+	1:128	+	1:131,072	1:8,192	Weak +	−
May 1996	+	1:4,096	+	1:128	−	1:131,072	1:8,192	Weak +	−
Dec 1996	+	1:4,096	+	1:128	+	1:131,072	1:8,192	Weak +	+

NOTE. Data in table are titers or positive/negative findings of indicated assay. ELISA was based on recombinant antigens. EA = early antigen; EBNA = EBV-associated nuclear antigen; IFT = immunofluorescence test; ND = not determined; VCA = viral capsid antigen; + = positive; − = negative.

the skin and very pressure-sensitive subdermal nodes on both legs. After 6 years of disease, a long-lasting treatment with 2.5–5.0 mg of prednisolone a day caused the subdermal nodes to disappear. The recurrent fever, however, persisted.

Since the age of 9 years, the patient had had a large number of warts on both the hands and feet. In 1985, condylomata appeared in the genital region. As treatment for the condylomata, in 1994 and 1995 the woman twice received (for a period of 10 days each) 10^6 units of IFN-α a day, but no significant improvement occurred. In 1987, at the age of 18, the woman acquired tuberculosis, which was cured within 1 year by treatment with isoniazid and rifampin. Although during childhood no infectious or immunologic abnormalities were observed, the clinical symptoms described above were indicative of deficiencies in the humoral and cellular immunity of the patient, at least since 1985.

Serological and molecular data indicated strong reactivation of the EBV infection. Serological data from after the occurrence of the primary infection with EBV in 1984 were available since the year 1985 (table 1). Typical heterophilic antibodies were detectable over a period of ~1 year and then disappeared. In 1985, 1 year after the primary infection, the titers of IgG antibodies to VCA and EA were still very high, indicating recurrent viral reactivation. In 1996, 12 years after the infection, the virus-specific antibodies had reached dramatic titers: IgG to VCA, 1:131,072; IgA to VCA, 1:8,192; IgG to EA, 1:4,096; and IgA to EA, 1:128 (table 1). IgM antibodies to VCA were also detected.

EBNA-1- and EBNA-3A-specific antibodies, sought with ELISAs and western blot assays (both based on recombinant antigens), were undetectable until December 1996, 2 months before the woman died (table 1; figure 1, lanes 6 and 8). In comparison, an immunofluorescence test before December 1996 showed a weakly positive EBNA signal, which may have been due to weak expression of some of the other EBNAs, like EBNA-2, which we detected with a western blot assay (figure 1, lane 7).

The failure to detect EBNA-1-specific antibodies is indicative of infectious mononucleosis and is often observed in cases of chronic active EBV infection. The western blot assay further confirmed lytic replication of the virus in the blood by detecting antibodies to the viral thymidine kinase, p138, p54, p18, p23, and BZLF-1 (figure 1, lanes 1–5 and lane 9, respectively).

Following the detection of various EA-specific antibodies, in April 1996 we detected with PCR ~500 viral genomes in 1 mL of serum (table 2). (In an investigation of healthy seropositive persons, no viral DNA could be found in serum, even in cases of serologically confirmed transient reactivation of EBV [3].) Using a quantitative PCR with an internal standard [13], we found at that time ~6×10^3 viral particles in 30 mL of throat-washings, indicating lytic replication of EBV in the oropharyngeal tissues also and shedding of the virus into the saliva (figure 2).

In order to identify the cells that were the source of the lytic replication of the virus in the peripheral blood, the peripheral blood mononuclear cells (PBMCs) were fractionated by positive selection of the $CD19^+$ B cells and $CD3^+$ T cells. Total RNA was isolated, and in an aliquot of RNA corresponding to

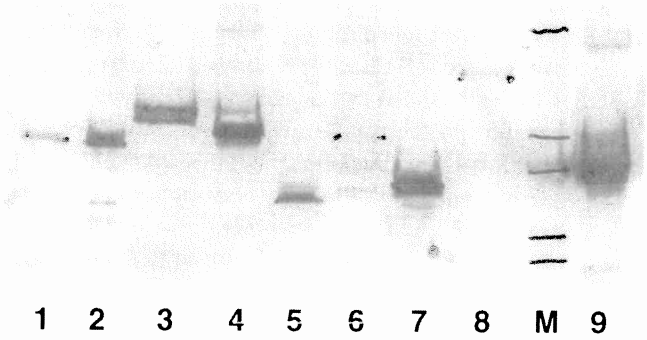

Figure 1. Analysis of the humoral immune response to Epstein-Barr virus (EBV) in April 1996. Recombinant antigens derived from viral proteins were transferred to a membrane (western blot) and used for immunochemical detection of antibodies in the serum of a woman with severe chronic active EBV infection. Lytic cycle proteins: thymidine kinase (*lane 1*), p138 (*lane 2*), p54 (*lane 3*), p18 (*lane 4*), and p23 (*lane 5*). Latent cycle proteins: EBV-associated nuclear antigen (EBNA)–1 (*lane 6*), EBNA-2 (*lane 7*), EBNA-3A (*lane 8*), and the immediate-early regulatory protein BZLF-1 (*lane 9*).

Table 2. PCR detection of Epstein-Barr virus (EBV) in serum and in biopsy specimens of solid organs in a case of chronic active EBV infection.

Date	Specimen	Viral genomes
Mar 1996	Serum	−
Apr 1996	Serum	500 eq/mL
	PBMCs	1×10^3 eq/10^6 PBMCs
May 1996	Serum	200 eq/mL
	PBMCs	1×10^3 eq/10^6 PBMCs
Dec 1996	Serum	100 eq/mL
	Whole blood	5×10^6 eq/10^6 PBMCs
Dec 1996*	Liver biopsy	+
	Placenta	+
Jan 1997*	Liver biopsy	+
	Spleen biopsy	+
	Ascites	+
	Bone marrow	+

NOTE. eq = Genome equivalents; PBMCs = peripheral blood mononuclear cells; + = positive; − = negative.

* No serum or whole blood available to determine the eq in the blood.

6×10^5 cells, the expression of viral genes of the latency cycle (EBNA-1) and the lytic replication cycle (BZLF-1, BcLF-1/VCA) was tested by reverse transcriptase (RT)–PCR with subsequent nested PCR and Southern blot hybridization [3] (figure 3). To control the quality of the RNA preparations, RT-PCR for histone 3.3 was performed (figure 3A) [16]. In the PBMCs prior to fractionation and in the $CD3^-/CD19^-$ population after removal of the B and T cells (figure 3A, lanes 1 and 4, respectively), messenger transcripts of both BZLF-1 and EBNA-1 were detected, indicating both latent and lytic infection with EBV in an uncharacterized cell population.

Expression of EBNA-1, however, was shown in neither the T-cell fraction nor the B-cell fraction (figure 3A, lanes 2 and 3, respectively). Instead, in the B cells, transcripts of BZLF-1 were found (figure 3A, lane 2), and in the T cells, transcripts of BcLF-1 (VCA) were found (figure 3B, lane 5), both indicative of lytic replication of EBV. The absence of expression of EBNA-1 in the T- and B-cell populations correlated with the lytic replication in these cells and strongly argued for the existence of a lytic, nonimmortalizing virus strain [13].

Virological analysis showed an immortalization-deficient lytic strain of EBV. In several reports in the literature, rare strains of EBV were believed to be involved in the pathogenesis of chronic active EBV infection. Therefore, we performed immortalization assays to screen for EBV strains impaired in their capability to immortalize B cells and a tendency to lytic replication. To confirm that the viral genomic DNA that we detected with PCR in the saliva was derived from shed infectious viral particles, we additionally performed superinfection assays of Raji cells, as described previously by Alfieri et al. [10, 11] (figure 4). The latently infected, EBV-positive Raji cells were incubated with throat-washings from the patient, and expression of EA was assayed after 48 and 72 hours postinfection by means of an immunofluorescence test with a polyclonal rabbit serum. Whereas the mock-infected Raji cells only showed background fluorescence (first row of panels, figure 4), incubation with the throat-washings resulted in distinct EA-specific fluorescence 48 hours postinfection and 72 hours postinfection due to superinfection with virus in the saliva (second row of panels, figure 4).

Although we thus showed that viral particles were present in the saliva and the immortalization assays were performed in the presence of cyclosporin to inhibit any cytotoxic T-lymphocyte activity, no EBV-immortalized cell lines, neither spontaneous nor infected with virus from saliva, could be established (none of 10 assays were positive). This was in contrast to cases of infectious mononucleosis, in which lymphoblastoid cell lines are always obtained, and which were comparable to our case with respect to the virus load in the peripheral blood. Therefore, immortalization could be expected and the failure to establish EBV-positive cell lines was indicative of an EBV strain that was impaired in its ability to immortalize B cells. Alternatively, the B cells of the patient might have been permissive only of lytic replication of EBV. Since the patient's PBMCs were infected in culture with the immortalizing EBV strain B95-8 and yielded EBV-positive, immortalized cell lines in four of four assays, however, the second possibility was precluded.

In order to characterize this strain of EBV, we tried to isolate the virus by infection of cord blood lymphocytes (CBLs). Neither cocultivation of the CBLs with the patient's (EBV-positive) PBMCs, to allow infection by cell-cell fusion, nor infection of CBLs in cell culture with virus preparations derived from the patient's saliva yielded immortalized (CBL-derived) cell lines, thus supporting the idea of a lytic, nonimmortalizing strain of EBV. In an effort to determine the type of EBV involved in this case of chronic active EBV infection, viral DNA was purified from infectious particles in the saliva.

On the basis of multiple sequence variations in several genes, two major types of EBV (type A and B) have been

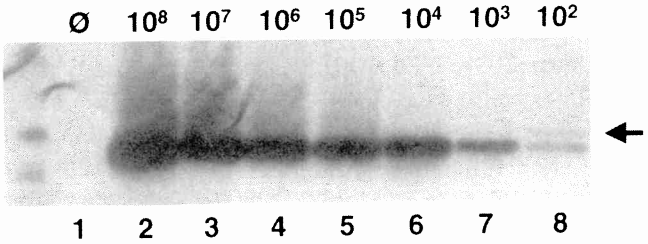

Figure 2. Quantification of EBV in the saliva. Genomic DNA of viral particles was purified from throat-washings and used for quantitative PCR. An internal competitor with a 30-bp deletion was added in decreasing amounts (*lanes 2–8*) as an internal standard [13]. In the reaction, which yielded two equally intense bands of the competitor (*lower*) and of the viral DNA (*upper*), the numbers of competitor molecules and viral genomes in the reaction tube (here, 10^2 genome equivalents [eq], equal to 6×10^3 eq per mL of throat-washing) are identical (*lane 8*). *Lane 1* (∅) shows the negative control without DNA.

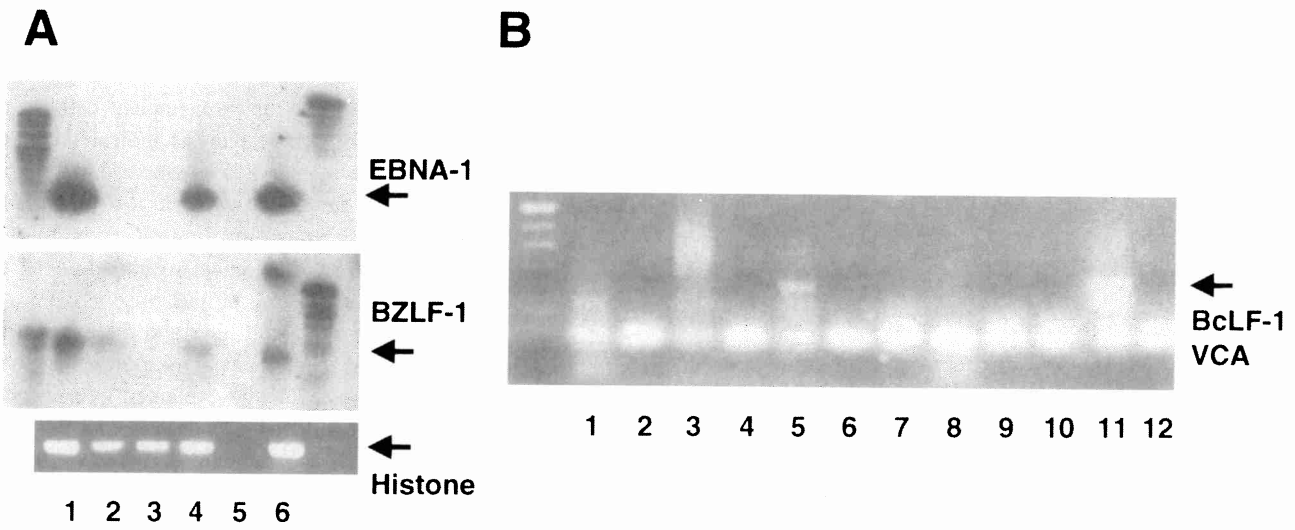

Figure 3. Expression of viral genes of the lytic replication in the peripheral blood. Mononuclear cells of the peripheral blood were purified by ficoll-gradient centrifugation, and the $CD19^+$ and $CD3^+$ cells were positively purified with use of magnetic Dynabeads (Dynal, Hamburg, Germany). With use of reverse transcriptase (RT)-PCR, expression of the latent-cycle gene EBNA-1 and of the lytic-cycle genes BZLF-1 and BcLF-1/VCA was tested for [3]. The quality of the RNA was checked with histone-specific RT-PCR [16]. *A*, the RT-PCR results for EBNA-1 and BZLF-1 are visualized by hybridization of a Southern blot and the digoxigenin detection system (Boehringer Mannheim, Mannheim, Germany). *Lane 1*, mononuclear cells prior to purification (peripheral blood mononuclear cells [PBMCs]); *lane 2*, $CD19^+$ B cells; *lane 3*, $CD3^+$ T cells; *lane 4*, $CD19^-/CD3^-$ cells after removal of T and B cells; *lane 5*, negative control; *lane 6*, positive control. *B*, for BcLF-1/VCA and histone, the products of PCR were stained with ethidium bromide. *Lanes 1 and 2* are mononuclear cells prior to purification (PBMCs); *lanes 3 and 4*, $CD19^+$ B cells; *lanes 5 and 6*, $CD3^+$ T cells; *lanes 7 and 8*, $CD19^-/CD3^-$ cells after removal of T and B cells; *lanes 9 and 10*, negative controls; and *lanes 11 and 12*, positive controls. Lanes 2, 4, 6, 8, 10, and 12 show RNA controls (with RNase treatment).

described previously. The capacity of the type B strain to immortalize B cells was shown to be less than that of the type A strain. PCR for the type A and type B alleles of the EBNA-2 gene [17], as well as PCR with a type-independent primer pair, failed to give any signal for EBNA-2. However, analysis of the antibody response in the patient with chronic active EBV infection by means of western blots with reading-frame-specific recombinant proteins demonstrated EBNA-2-specific antibodies (figure 1).

Immunologic and cytological data obtained in 1996. Over the years, neither persisting lymphocytosis nor lymphadenopathy was observed. In accordance with the clinical symptoms indicating insufficient cellular immunity against viral and bacterial infections, only very limited lymphocyte reactions were measured in stimulation assays with mitogens (concanavalin A, phytohemagglutinin, and pokeweed mitogen [PWM]) and after incubation with typical recall antigens (tuberculin; herpes simplex virus-1; tetanus and diphtheria toxin; *Candida*; cytomegalovirus; and rubella, varicella, mumps, measles, influenza A, and influenza B viruses).

Stimulation of 10^5 PBMCs with PWM for 24 hours resulted in significantly reduced synthesis of TNF-α (78.4 pg/mL; normal, >3,000 pg/mL). This unresponsiveness of the T cells to classic antigens was first observed in 1985. The analysis of the blood cell count indicated weak pancytopenia (leukocytes, 3.7/nL; normal, 4.8–10.8/nL). A striking finding was the nearly complete absence of $CD19^+$ and $CD10^+/CALLA^+$ B cells (0/μL each; normal, 100/μL and 20/μL, respectively) and $CD20^+$ B cells (11/μL; normal, 100/μL). Nevertheless, the IgG concentration was slightly enhanced (2.63 g/dL; normal, 0.7–1.6 g/dL), whereas IgA, IgM, and IgE concentrations were normal. However, this was not surprising since the concentration of immunoglobulins does not always correlate with the number of B cells in the peripheral blood. It is supposed that in such cases the antibody-secreting B cell clones may reside in the secondary peripheral lymphoid organs.

Immunofixation assays indicated biclonal gammopathy (IgG-κ and IgG-λ, with a concentration of <2.0 g/dL each), which might have been in part responsible for the elevated IgG concentration. In addition, it has been reported that monoclonal gammopathy often resulted in reduction of $CD19^+$ B cells, probably because of the secretion of cytokines with antiproliferative effects by the corresponding B cell clone. Several autoantibodies were detected: antinuclear antibodies (titer, 1:320; normal, <1:80), anticardiolipin IgG (20.9 U; normal, <15 U), and IgG bound to platelets. A direct Coombs test also was weakly positive.

Cytogenetic analysis by Giemsa staining of chromosomes and analysis of band pattern and fluorescent in situ hybridization indicated a trisomy of chromosome 8 in 88% of the cells of the bone marrow, in contrast to 12% of anomalous cells in the peripheral blood, which may be indicative of a mosaic constellation or a monoclonal proliferation in the bone marrow. In summary, however, until December 1996 the cytological and histo-

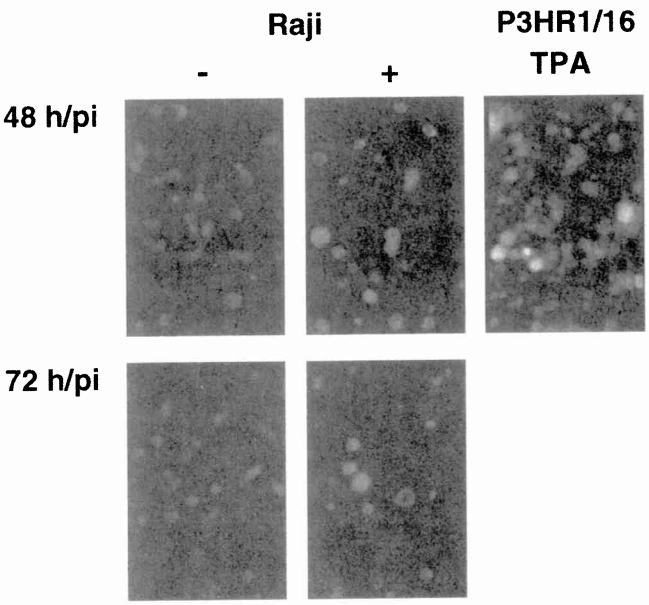

Figure 4. Demonstration of infectious viral particles in the saliva by superinfection of Raji cells. The Raji cells (4×10^5) were incubated for 2 hours in 1 mL of sixfold-concentrated throat-washings and afterward cultivated in RPMI 1640. At 48 and 72 hours postinfection (*h/pi*), the cells were analyzed by means of an immunofluorescence test for expression of early antigen (EA) with use of an EA-specific polyclonal rabbit serum. *Left panels*: Raji cells incubated with PBS; *middle panels*: Raji cells incubated with concentrated throat-washings; *right panel*: positive control, EBV-positive P3HR1/16 cells treated with phorbol 12-myristate 13-acetate to induce the lytic replication of the virus.

logic analysis of peripheral blood and bone marrow gave no signs of the existence of any malignant hematologic disease.

Final course of the disease. In June 1996 the patient became pregnant. In December 1996, during the 26th week of pregnancy, the transaminase and bilirubin levels began to rise and liver function parameters decreased. An analysis of the viral load with semiquantitative PCR demonstrated significant viremia with EBV in the peripheral blood; there were 100 viral particles in 1 mL of serum. With the same technique, 5×10^5 viral particles were detected in 1 mL of whole blood, including serum and all cells, indicating a very high cell-bound virus load. Since the decrease in liver functions was attributed to enhanced viral replication, the woman was treated with ganciclovir (2×300 mg/d) for ~1 month, without any profound improvement. In this situation it was decided the woman's baby should be delivered as soon as possible. Betamethasone was given on 2 successive days (8 mg/d) to enhance the development of the baby's lungs.

The woman gave birth to her (healthy) child in December (28th week of pregnancy), and the symptoms of liver failure transiently improved. Two weeks later the liver functions finally failed, and an organ transplant was performed. The explanted liver showed significant infiltration with T lymphocytes and deposition of eosinophilic material. Furthermore, moderate inflammation in the portal field and widespread multifocal necrosis were found. The reason for the severe dystrophy of the liver was unclear.

At that time, examination of the mononuclear cells in the peripheral blood by Southern blot analysis indicated a small population of cells with a clonal rearrangement of the T-cell receptor, which was in agreement with a low-malignancy T-cell lymphoma. A T-cell lymphoma, however, could not be confirmed by histologic and cytological examination of the bone marrow, the explanted liver, or the spleen. EBV was detected with PCR in the explanted liver and the spleen, and this finding was attributed to the heavy infiltration with lymphocytes. In addition, biopsies of the explanted liver, ascites, and the placenta were positive for EBV DNA. PCR excluded infection with cytomegalovirus, and serology showed no signs of infection with hepatitis B or C virus.

Finally, in January 1997, about 2 weeks after the transplantation, the function of the foreign liver also deteriorated very rapidly, and the patient died. A postmortem biopsy of the liver showed complete dystrophy but no signs of lymphoid infiltration. Vascular or cellular rejection of the organ was excluded. Immunohistochemical staining of the liver for viral LMP-1 (latent membrane protein) was negative.

Discussion

When the patient was admitted to our hospital, molecular and immunologic assays indicated a strong reactivation of lytic replication of EBV. Recently, we have shown that in individuals with serologically confirmed transient reactivation of EBV who are otherwise healthy, no messenger RNAs encoding VCAs could be detected, most probably because of an efficient cellular immune response to lytically infected cells [3]. In this case, however, the presence of VCA-specific mRNA demonstrated that lytic replication of EBV happened in the mononuclear cells of the peripheral blood. Latent infection was detected in an unidentified population of $CD19^-/CD3^-$ cells, probably T cells of the natural killer phenotype. This viral gene activity indicated that in the present case the control of lytic replication was quite insufficient and that probably the site of latent persistence of EBV was different from that in most other cases with virus reactivation. On the basis of the long period of time for which the infectious mononucleosis–like symptoms and serological parameters lasted after the primary infection with EBV, a chronic active infection was diagnosed.

The pathogenesis of chronic active infections with EBV is believed to be a multifactorial process. Immunologic disorders that cause insufficient elimination and result in an increase of EBV-infected cells may be a cofactor. This is true during infectious mononucleosis, when the organism comes into contact with the virus for the first time and the absence of virus-specific immune responses leads to the transient but massive proliferation of EBV-infected mononuclear cells in the peripheral blood. Virus strains with a reduced capacity to immortalize

B cells and a tendency toward lytic replication are also believed to be involved in chronic active EBV infection.

The failure to typify the present virus strain with PCR in the region of the EBNA-2 gene or even to amplify this region of the genome, which was shown to be of particular importance for the immortalization of B cells, indicated major sequence variations in this region. Several cases involving EBV strains with deletions in the EBNA-2 region, isolated from lesions with lytic viral replication, have been described [18, 19]. Recently, we reported the characterization of the virus strain SM, isolated in another case of chronic active EBV infection [13]. In this case the reduced capability to immortalize B cells and the enhanced tendency to lytic replication correlated with a significant overexpression of the lytic cycle gene BZLF-1.

Both an immunologic disorder and a particularly lytic virus strain seem to have contributed to the pathogenesis of chronic active EBV infection. Although an insufficient immune surveillance per se could also lead to an increase in the number of EBV-infected cells and thus could indirectly increase the number of cells with reactivating virus, this was not the case here. No lymphocytosis of B or T cell origin was observed over the years. Even a nearly complete absence of B cells was detected, probably also due to destruction of infected cells by the lytic replicative cycle of the virus or by immunologic elimination. Under conditions of impaired immune surveillance, lytic replication occurred in the B and T cells, progeny virus was shed into the blood, and infection of new B cells continued. The underlying immunologic defect leading to insufficient control of the permissively infected cells could not be identified.

The failure of liver functions due to heavy lymphoid infiltration and the rapid deterioration of the transplanted organ finally caused the death of the woman. The reason for the dramatic worsening of the chronic disease 12 years after the infection with EBV could not be established. Probably the additional suppression of the immune surveillance during pregnancy led finally to the observed lymphoid proliferation and infiltration of organs. The causative role of EBV in the chronic disease is now quite obvious, but the role of the virus in the fatal course of the disease could not be defined.

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