

SEVERE HEPATITIS CAUSED BY EPSTEIN-BARR VIRUS WITHOUT INFECTION OF HEPATOCYTES

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Although hepatitis is a common feature of primary Epstein-Barr virus (EBV) infection, severe liver injury is rare and its pathogenesis is unclear. A previously healthy girl developed severe hepatitis with prolonged jaundice. Serologic examination showed that she had primary infection with EBV. An extremely high Epstein-Barr viral load was observed in her peripheral blood. The viral load decreased in parallel with symptomatic improvement. Histologic examinations showed spotty necrosis of the liver parenchyma and infiltration by CD8⁺ T cells. The CD8⁺ T cells, not

hepatocytes, were positive for EBV. Possible mechanisms of viral hepatitis without infection of hepatocytes are discussed. *HUM PATHOL* 32:757-762. Copyright © 2001 by W.B. Saunders Company
Key words: Epstein-Barr virus, hepatitis, primary infection, real-time quantitative PCR.

Abbreviations: EBV, Epstein-Barr virus; PBMC, peripheral blood mononuclear cell; PCR, polymerase chain reaction; EBER-1, Epstein-Barr–encoded small RNA 1; BCIP, 5-bromo-4-chloro-3-indoyl phosphate; CTL, cytotoxic T lymphocyte.

Epstein-Barr virus (EBV) is ubiquitous in humans, and most individuals become seropositive for EBV by the time they are young adults. The primary EBV infection is usually asymptomatic, but sometimes it causes infectious mononucleosis. In the acute phase of infectious mononucleosis, elevated transaminase levels are found in 80% of patients, but jaundice is only noted in 6.6%.¹ Hepatitis caused by primary EBV infection is usually mild and self-limited. Rarely, the hepatitis results in hepatic failure with severe jaundice.¹ The pathogenesis of the hepatitis seen in infectious mononucleosis is unclear. Here, we describe a previously healthy girl with primary EBV infection who developed severe hepatitis with prolonged jaundice. Virologic and histologic examinations were performed to clarify the mechanism of the hepatitis.

CASE REPORT

A previously healthy 1-year-old girl was admitted to University Hospital, Nagoya University School of Medicine, on January 9, 1999, because of fever, jaundice, and an enlarged liver and spleen. She had no significant family history or prior serious infections. Her parents had noted diarrhea and darkening of the conjunctiva 4 weeks before admission. She had been hospitalized in another hospital and treated for 3 weeks, but her jaundice had worsened and fever had developed. On the day of admission, she was jaundiced and febrile (39.9°C) and had a reddened throat and edematous face. Her liver and spleen were palpable 4.5 and 1.5 cm below the costal margin, respectively. Her white blood cell count was 15,500/ μ L, hemoglobin 10.3 g/dL, and platelet count 198,000/ μ L, and a blood smear showed 55% neutrophils and 42% lymphocytes. Serum total bilirubin was 15.5 mg/dL, direct bilirubin 11.0 mg/dL, aspartate aminotransferase 2,148 IU/L, alanine aminotransferase 857 IU/L, total protein 4.8 g/dL, and albumin 3.1 g/dL. Both serum immunoglobulin and complement levels were normal. Her lymphocyte subsets were not altered. Mitogen responses against phytohemagglutinin, concanavalin A, and staphylococcal cowan A were within the normal range. Her coagulation system was altered: prothrombin time, 40% (normal, 80% to 120%); activated partial thromboplastin time, 49.6% (normal, 80% to 120%); fibrinogen, 41 ng/dL (normal, 200 to 400 ng/dL); and fibrin-degraded products, 195 ng/dL (normal, <100 ng/dL).

MATERIALS AND METHODS

Samples

Whole blood was obtained from the patient. Plasma and peripheral blood mononuclear cells (PBMCs) separated by density gradient were used for quantitative polymerase chain reaction (PCR). The PBMCs were further fractionated into CD3⁺, CD19⁺, and CD16⁺ cells by an

TABLE 1. Localization of Epstein-Barr Virus DNA in Fractionated Peripheral Blood

	EBV DNA (copies/10 ⁵ cells) in Fraction of			
	CD3 ⁺ Cells	CD19 ⁺ Cells	CD16 ⁺ Cells	PBMCs
Patient	4,700	3,170	ND†	3,430
Controls*				
1	0	22	0	64
2	0	20	0	20
3	120	1760	180	110

*Control patients with infectious mononucleosis without severe hepatitis.

†CD16⁺ cells were not measured because enough mononuclear cells were not obtained.

immunobead method (DynaBeads; Dynal AS, Oslo, Norway). The beads were detached, and purity was estimated by flow cytometry. The purity was between 90% and 95%. As controls, PBMCs were obtained from 3 patients with infectious mononucleosis without severe hepatitis, fractionated, and analyzed by quantitative PCR. These control patients had mildly elevated transaminase levels (<200 IU/L) but no jaundice. All materials were obtained after informed consent from the parents.

Real-Time Quantitative PCR Assay With a Fluorogenic Probe

DNA was extracted from either 2×10^6 cells or 200 μ L of plasma and was eluted in distilled water. For the real-time PCR assay, 250 ng of DNA from PBMCs or 10 μ L of DNA solution from plasma was used. The real-time quantitative PCR assay was performed, as previously described.² To normalize the amount of EBV DNA in PBMCs or fractionated samples, human β -actin DNA was quantified by real-time PCR, and then the number of EBV genomes were corrected and expressed per 10^5 cells.³

Histology

Liver tissue was fixed in 10% formalin, dehydrated in alcohol, and then embedded in paraffin. Samples were sectioned at 4- μ m thicknesses. Routine hematoxylin and eosin-stained sections were made for histologic examination. Immunostaining was performed by a streptavidin-biotin peroxidase complex method (Histofine SAB kit, Nichirei, Tokyo, Japan) using monoclonal antibodies against CD45RO (UCHL-1; Dako A/S, Glostrup, Denmark), CD4 (IF6; Nichirei), CD8 (C8; Nichirei), CD20 (L26; Dako A/S), CD56 (123C3; Monosan, Am Arden, Netherlands), and Fas antigen (CD95/APO1; Dako A/S). Diaminobenzidine or acetyl ethyl carbazol was used for visualization. Before the staining for CD4, CD8, and CD56, microwave antigen retrieval was performed.

The in situ hybridization assay was performed using the Epstein-Barr (EB)-encoded small RNA 1 (EBER-1; Dako A/S) as previously described.² Briefly, paraffin sections of the liver tissue were cut, applied to glass slides, fixed, and dipped in phosphate-buffered saline. Each slide was hybridized with the alkaline phosphatase labeled EBER-1 probe, washed, and reacted with 5-bromo-4-chloro-3-indoyle phosphate (BCIP) for visualization.

For double labeling with immunostaining and in situ hybridization, the immunohistochemical studies were performed before in situ hybridization using a previously published technique with the monoclonal antibodies against

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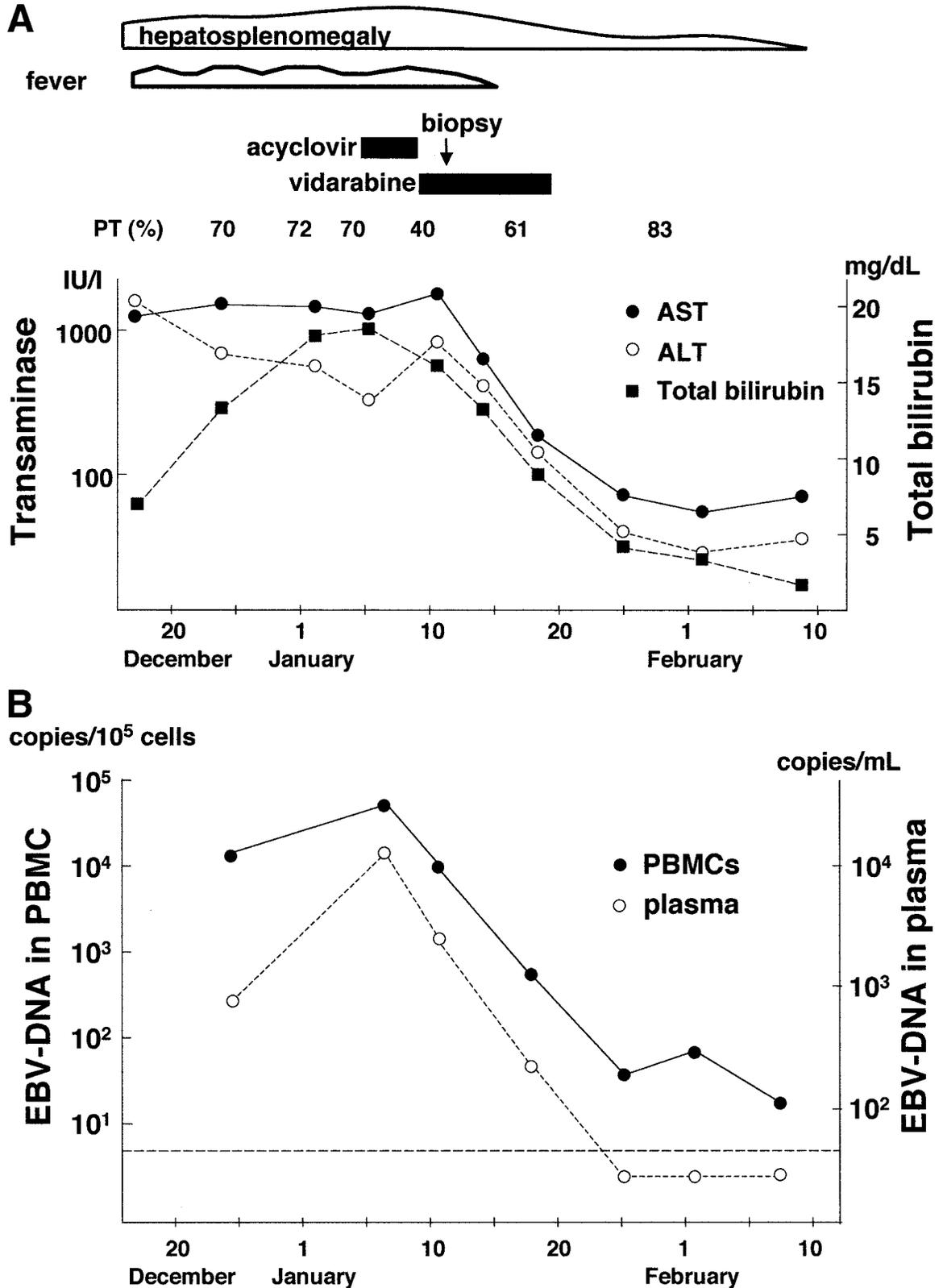


FIGURE 1. (A) Clinical course and change in laboratory findings. PT, prothrombin time; AST, aspartate aminotransferase; ALT, alanine aminotransferase. (B) Change in the viral load in peripheral blood. The EBV DNA copy number was quantified by the real-time PCR assay. The dotted line indicates the limit of detection.

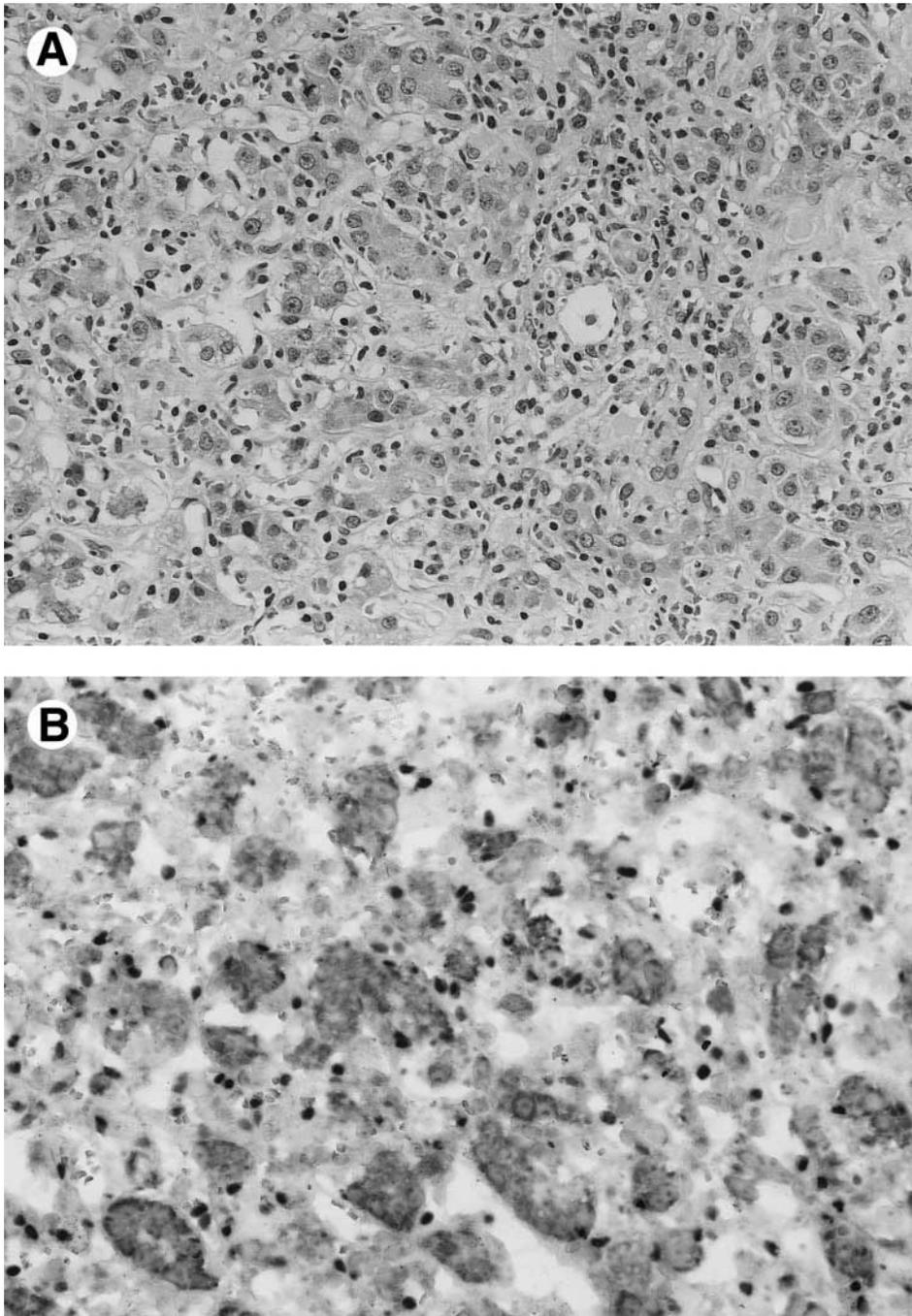


FIGURE 2. Histologic examination of the liver. (A) Lobular and portal infiltration of lymphocytes. A lymphocyte is present within a small bile duct to the right and center, and bile pigment is not so obvious in the liver parenchyma. (Hematoxylin and eosin; original magnification $\times 200$.) (B) In situ hybridization assay using the EBV-1 probe. Positive signals are identified in the nucleus of the lymphocytes. (BCIP; original magnification $\times 400$.)

CD4, CD8, and CD56.⁴ The in situ hybridization assay followed using the procedure outlined above.

RESULTS

Anti-EB early antigen immunoglobulin (Ig) M was positive, and anti-EB nuclear antigen IgG was negative, indicating that she recently had primary infection with EBV. Infections by hepatitis A, B, and C virus, cytomegalovirus, herpes simplex virus, and human immunodeficiency virus were ruled out by negative results for either viral antigens or specific antibodies. A real-time quantitative PCR assay detected large amounts of EBV DNA in both PBMCs and plasma. Combined with the serologic findings, hepatitis induced by primary infection

with EBV was strongly suspected. To determine which cells in the peripheral blood were infected, PBMCs were fractionated, and EBV DNA was quantified in each fraction (Table 1). The copy number of EBV DNA was high in both the CD3⁺ and CD19⁺ fractions, indicating that T and B cells were infected in the patient. On the other hand, B cells were mainly infected in control patients with infectious mononucleosis without severe hepatitis (Table 1).

The clinical course of the patient is summarized in Fig 1A. Acyclovir was administered intravenously without clinical improvement, so treatment was changed to vidarabine. Because jaundice and coagulopathy worsened, a liver biopsy was performed on the 3rd day of admission. After the biopsy, the fever

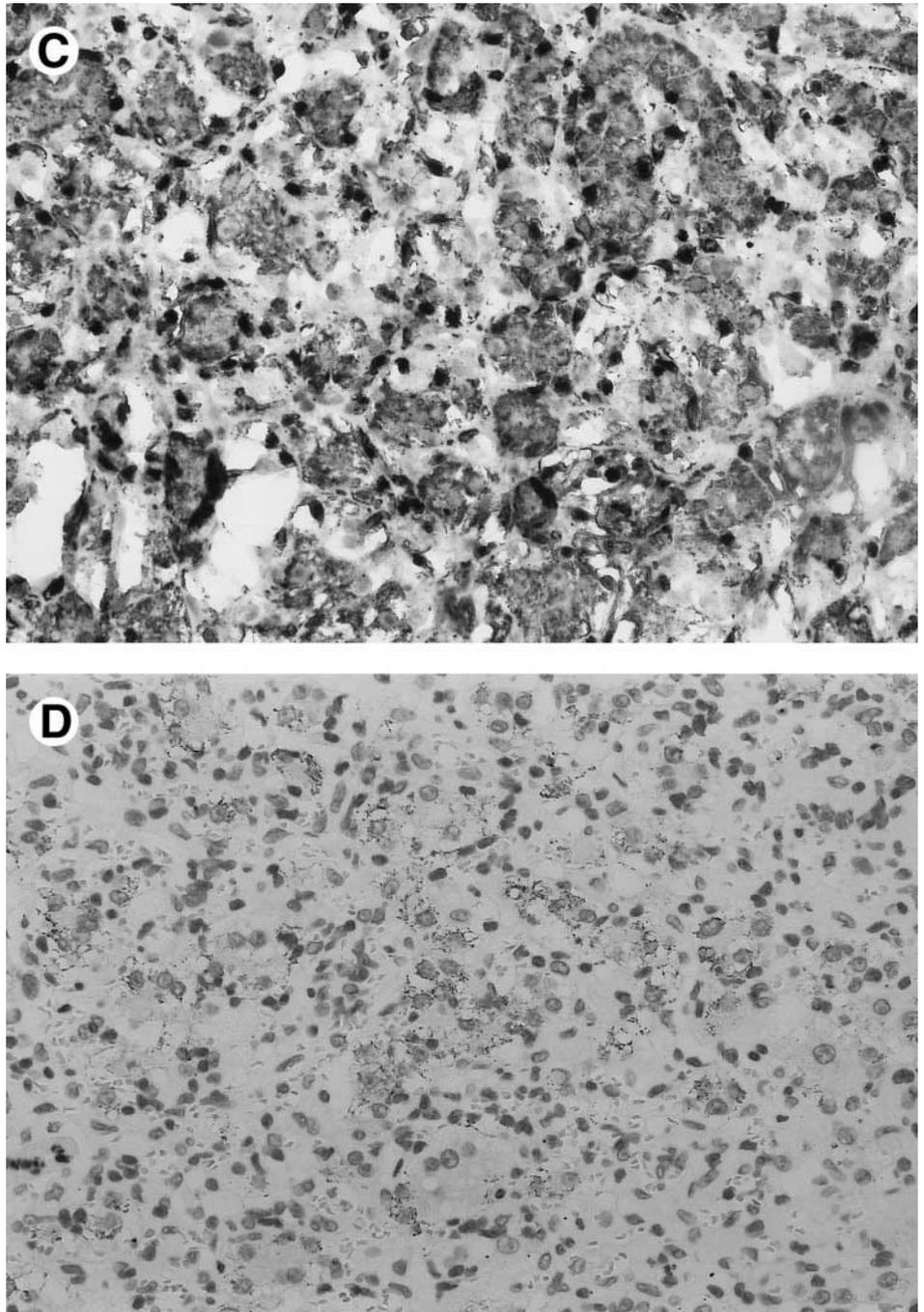


FIGURE 2. (Continued) (C) Double labeling with in situ hybridization using the EBER-1 probe and immunostaining with anti-CD8 antibody. Small lymphocytes are double labeled for EBER-1 and CD8 (BCIP for EBER-1 and diaminobenzidine for CD8; original magnification $\times 400$.) (D) Immunostaining with anti-Fas antibody. Membranous staining is seen in many hepatocytes. (Acetyl ethyl carbazole; original magnification $\times 200$.)

disappeared and serum transaminase levels decreased, followed by improvement of the jaundice and coagulopathy. She was discharged 1 month later, and has been healthy for 18 months. Viral load was serially monitored using the real-time quantitative PCR assay (Fig 1B). At the peak of hepatitis, the amount of EBV DNA reached 3.6×10^4 copies/ 10^5 cells in PBMCs and 1.7×10^4 copies/mL in plasma. These numbers are extremely high compared with those of patients with uncomplicated infectious mononucleosis.² The amount of EBV DNA started to decrease before there was clinical improvement. After 18 months, the EBV DNA disappeared from her blood.

To clarify the mechanism of hepatitis, the liver specimen was examined. Histologic examination showed spotty necrosis

of the liver parenchyma with cholestasis and infiltration by mononuclear lymphocytes in lobular and portal areas (Fig 2A). These lymphocytes were mostly positive for CD45RO and CD8, but negative for CD20 and CD56 (figures not shown), indicating that they were mainly memory CD8⁺ T cells. Infiltrating CD8⁺ T cells were positive for EBER-1, but hepatocytes were negative for EBER-1 RNA (Fig 2B, 2C). These hepatocytes expressed Fas antigens (Fig 2D). Recent reports have noted that soluble products of the immune response, such as soluble Fas ligand, interferon γ , and tumor necrosis factor α , induce hepatitis.⁵⁻⁷ In the acute phase of hepatitis, the concentrations of soluble Fas ligand and interferon γ were elevated in the patient's plasma (soluble Fas ligand, 7.8 ng/mL; interferon γ , 46 pg/mL).

DISCUSSION

Although severe liver injury is rare in primary EBV infection, fulminant hepatic failure is the main cause of death in patients with fatal infectious mononucleosis.^{1,8,9} Most reported fatal EBV infections are associated with immunodeficiency syndromes, including human immunodeficiency virus, complement deficiency, and X-linked lymphoproliferative disease.⁸ X-linked lymphoproliferative disease was first described by Purtilo et al and is found exclusively in boys.⁸ We describe here a previously healthy girl with primary EBV infection who developed severe hepatitis with prolonged jaundice. It is unlikely that she had either congenital or acquired immunodeficiency because she had no previous serious infections and did not have any abnormal results on immunologic tests, including mitogen responses. Such severe hepatic injury in otherwise healthy females with primary EBV infection has been reported sporadically.⁹

The patient had a huge viral burden compared with other infectious mononucleosis patients without complications² (Table 1). The high EBV load might be caused by the proliferation of EBV-infected T cells. Recent studies indicate that T cells harbor EBV in viral-associated hemophagocytic, chronic active EBV infection, and T-cell lymphoproliferative disorder after EBV infection.¹⁰ Baumgarten et al¹¹ reported that T cells in peripheral blood contained EBV in a case of life-threatening infectious mononucleosis. It is possible that these EBV-infected T cells escape the host's immunity and thereby proliferate because they express fewer and less antigenic viral proteins than B lymphocytes.¹⁰

Few reports have studied the mechanism of EBV-induced hepatitis. It is generally thought that hepatitis viruses, including hepatitis B and C virus, are not directly cytopathic for hepatocytes, but that the associated liver diseases are caused by the immune responses to viral antigens expressed by infected hepatocytes.¹² Cytotoxic T lymphocytes (CTLs) destroy virus-infected hepatocytes through either the Fas/Fas ligand or perforin/granzyme B pathways.¹³ However, an experimental hepatitis B virus model showed that most of the hepatocytes were killed by inflammatory cells that CTLs recruit or activate and not by CTLs themselves.¹⁴ In a recent series of experiments, certain soluble products of the immune response, especially interferon γ ,⁵ tumor necrosis factor α ,⁶ and Fas ligand, induced hepatitis.⁷ In EBV-associated hepatitis, these products, which are produced by either EBV-infected CD8⁺ T-cells or infiltrating CTLs, may induce hepatocyte injury. Our histological observations of the patient's liver and high concentrations of soluble Fas-ligand and interferon γ in plasma support this hypothesis. The major difference between the mechanisms of EBV and hepatitis viruses is that

EBV unlikely infects hepatocytes, biliary epithelium, or vascular endothelium.⁸ In situ hybridization studies show that EBV-1 RNA exists in lymphocytes in patients with fatal EBV hepatitis,⁸ as seen in our patient. It is unclear why these EBV-infected T cells infiltrated in the liver. A recent animal model showed that activated CD8⁺ T cells are selectively trapped in the liver, primarily by intracellular adhesion molecule 1, which is constitutively expressed on sinusoidal endothelial cells and Kupffer cells.¹⁵ In EBV-associated hepatitis, EBV-infected CD8⁺ T cells, presumably activated CD8⁺ T cells, might accumulate in the liver and cause the hepatocyte injury, possibly through indirect effects. Although further studies are needed to clarify the precise mechanism of hepatitis caused by primary EBV infection, our observations shed light on the pathogenesis of viral hepatitis without infection of hepatocytes.

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