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Demonstration of Epstein-Barr virus DNA in a previously healthy boy with fulminant hepatic failure*

J. Deutsch[1], H. Wolf[2], H. Becker[3], B. Fuchs[3], U. Goriup[1], H.-M. Grubbauer[1], W. Muntean[1], Th. Popow-Kraupp[4], and D. Stünzner[5]

[1] Universitäts-Kinderklinik Graz, A-8036 Graz, Austria
[2] Max von Pettenkofer Institut der Universität München, D-8000 München, Federal Republic of Germany
[3] Pathologisch-Anatomisches Institut der Universität Graz, Graz, Austria
[4] Institut für Virologie der Universität Wien, Wien, Austria
[5] Hygiene-Institut der Universität Graz, Graz, Austria

Abstract. A previously healthy 9-year-old boy died from acute liver failure during an acute Epstein-Barr virus infection. Epstein-Barr virus DNA could be demonstrated in the liver by Southern blot – and by in situ hybridization techniques. The identification of the virus in the liver suggests a causal relation between the Epstein-Barr virus and the acute massive liver cell necrosis.

Key words: Epstein-Barr virus – Liver failure – Infectious mononucleosis – Hepatitis

Introduction

Infectious mononucleosis is characterized by a clinical syndrome with fever, tonsilllopharyngitis, and enlarged cervical lymph nodes [12, 17, 22]. The diagnosis has usually been based on the demonstration of the typical triad, (1) clinical findings compatible with the disease, (2) more than 50% mononuclear cells in peripheral blood smears, and (3) the occurrence of heterophilic antibodies [17]. Many children with Epstein-Barr virus (EBV) infection do not present with these classical features. Therefore, the demonstration of specific antibodies of the IgM class in the serum seems necessary to confirm an acute infection with EVB [11].

The liver is usually involved in the disease but fulminant hepatic failure is an unusual finding during the course of infectious mononucleosis [1, 2, 9, 10]. It seems to occur more frequently in patients with X-linked recessive lymphoproliferative syndrome [15, 16]. In the past, demonstration of a causal relation between EBV and liver cell necrosis was not possible. Recently developed techniques of nucleic acid hybridization now allow a correlation between EBV and specific cell types to be shown [24].

We describe the case of a previously healthy boy with infectious mononucleosis who developed an acute hepatic failure. In this patient EBV was demonstrated in liver tissue taken immediately after death using two different nucleic acid hybridization techniques.

Case report

A previously healthy 8-year-11-month-old boy presented with a history of fatigue, discomfort, upper abdominal pain and fever (38°C) of 9 days duration [6]. His parents had noted a darkening of the skin and a dark urine. His physician had noted a swelling of cervical glands, sinusitis and pleural effusions. On the day prior to admission total serum bilirubin was 46 µmol/l, the conjugated fraction 39.2 µmol/l, alanine aminotransferase (ALT) 306 U/l, and gammaglutamyltransferase 174 U/l. The family history did not reveal any signs of immunodeficiency.

On admission the patient was jaundiced, had enlarged cervical glands, inflamed tonsils with white-yellow stipples, and the liver and spleen were palpable 1.5 cm below the costal margin [6]. The peripheral blood smear showed 72% (= 8.7/nl) atypical mononuclear cells (Rieder-Glanzmann). The patient was febrile and treatment was started with erythromycin-succinate and noramidopyriniummethane sulphonate-sodium (Novalgin).

The boy developed clinical and laboratory signs of liver failure (ALT 1372 U/l, asparate aminotransferase 3640 U/l, bilirubin 145 µmol/l) with severe alterations of the coagulation system: thromboplastin time 18% (normal >70%), partial thromboplastin time 128 s (normal 35–55 s), thrombin time 128 s (normal 17–24 s), fibrinogen < 50 mg/dl (normal >150 mg/dl), Factor II 13% (normal >80%), factor V 38% (normal >80%), factor VII 17% (normal >80%), factor VIII 120% (normal >80%) and 39% respectively, antithrombin III 40% (normal >80%), thrombocytes 56/nl, on the 6th day after admission [6]. Erythromycin and antipyretics were withdrawn, and the patient was treated by daily exchange transfusions with fresh blood. Lactulose and neomycin administration was started orally, and he had a short course of silymarin and penicillin. Substitutions with coagulation factor concentrates, vitamin K, and antithrombin III could not improve the coagulation disorder nor prevent further bleeding. After resus-

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Offprint requests to: J. Deutsch, Universitäts-Kinderklinik Graz, Auenbruggerplatz 30, A-8036 Graz, Austria

Abbreviations: EBV = Epstein-Barr virus; ALT = alanine amniotransferase; KBR = complement binding reactions

citation from a cardiac arrest on the 9th day he developed a terminal renal insufficiency [6] and died on the 15th day of his illness.

Heterophilic antibodies against horse erythrocytes were detected by Monospot (Ortho Diagnostic Systems, Heidelberg, FRG) and IM-Quicktest (Human Corporation, Taunusstein-Neuhof, FRG), and the reaction of Paul-Bunnell (against sheep erythrocytes) was positive at a titre of 1:16. Specific antibodies (EBV-VCA, immune fluorescence) of the IgM class were shown to be positive at a titre of 1:64, and antibodies of the IgG class were shown to be positive at a titre of 1:256 [6]. Infections with ornithosis, influenza A and B, mycoplasma pneumoniae, parainfluenza 1,2,3, Q-fever, mumps, adenovirus, cytomegalovirus, echoviruses or coxsackieviruses were excluded by negative complement binding reactions (KBR), infections with measles or rubella viruses were excluded by negative haemagglutination, toxoplasmosis by negative immune fluorescence and hepatitis A and B were excluded by negative radioimmunoassay.

Antibodies to respiratory syncytial virus, herpes simplex and varicella viruses were shown to be positive at a titre of 1:10 (KBR). The serum levels of caeruloplasmin, copper, and alpha$_1$-antitrypsin were within the reference ranges of our laboratory. The total IgM level in serum was 4.02 g/l, total IgG and total levels IgA were normal.

Methods

A complete postmortem examination was done. Histologic examinations were performed on liver, gall bladder, gastrointestinal tract, spleen, lymph nodes, lung, kidney, skin and brain sections (routine HE, PAS, trichrome staining, additional staining of liver sections with Giemsa, diastase-PAS, iron-staining and reticulin-staining). Portions of liver tissue were frozen at −70°C and were stored for 1 month, until nucleic acid hybridization tests were performed. These tests were based either on DNA extracted from tissue (Southern blot, [19]) or were directly performed on sections of frozen tissue (in situ hybridization). The tissue-derived DNA was denatured to yield single-stranded DNA, incubated with equally single-stranded radioactively labelled viral DNA, thoroughly washed and exposed to autoradiography. Positive hybridization signals were regarded as markers for the presence of viral DNA.

1) Procedure of Southern blot hybridization [18]. Liver cell DNA was extracted, digested with *Bam*H1 endonuclease, separated on a 0.8% agarose gel denatured with 0.5 N NaOH, neutralized and transferred to a nitrocellulose filter by blotting. To determine the sensitivity, Raji-cell DNA was included as a control. According to reassociation kinetic experiments, each Raji cell contains 50 copies of EBV-DNA [25], slot 1: 20 µg, slot 2: 2 µg, slot 3: 0.4 µg, slot 4: 0.2 µg, and was processed in the same way. The blots were incubated at 68°C in 6 × SSC, 0.5% SDS, 5 × Denhardt's solution [5] with the addition of 100 µg/ml denatured calf tyhmus DNA. After 4 h the excess prehybridization mix was removed, I^{125}-labelled EBV-specific single-stranded hybridization probe MEBWS7 added and the incubation continued for another 16 h. After hybridization the filter was washed at 68°C in several changes of 2 × SSC, dried and exposed for 12 h.

The hybridization probe was derived by subcloning an internal repeat of EBV-DNA (W-Band after digestion with *Bam*HI) after *Sau*IIIa digestion into the double-stranded replicative form of m13mp8 bacteriophage. The infected bacteria release recombinant m13 phage containing single-stranded recombinant DNA. This DNA can be labelled efficiently (specific activity 5×10^8 cpm/µg) in the presence of Tl^{3+} with I^{125} in a chemical reaction [8].

2) Procedure of in situ hybridization [23]. Frozen sections of liver tissue 8 µm thick were fixed in a solution containing 3 parts methanol and 1 part concentrated acetic acid, incubated in 2 × SSC (1 × SSC = 0.15 *M* sodium chloride, 0.015 *M* sodium citrate) at 70°C for 30 min and treated with proteinase-K at 37°C for 15 min. Immediately before hybridization the preparations were denatured in 0.1 × SSC at 98°C for 3 s, chilled in the same buffer at 0°C and dried.

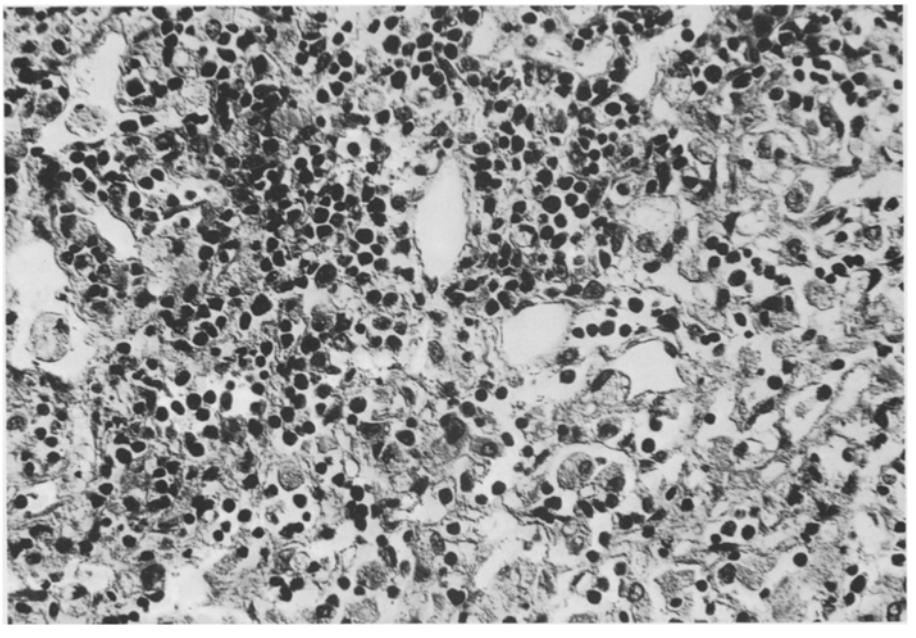

Fig. 1. Postmortem punctate of the liver, trichrom, 324 ×. Mononuclear cell infiltrates, necrosis of Kupffer cells and of liver cells

The hybridization probe contained 10^5 cpm ^{3}H-labelled EBV-DNA with a specific activity of about 5×10^6 cpm/µg in 50% formamide, 0.6 *M* sodium chloride, 10 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 100 µg/ml calf thymus DNA, 1 mg/ml bovine serum albumin, 1 mg/ml tRNA, 100 µg/ml Poly A, 0.02% wt/vol polyvinylpyrrolidone, 0.02% wt/vol Ficoll. The preparations were covered by 10 µl of this solution enclosed under a cover slip and sealed with Fixogum (Marabuwerke, Tamm, FRG). The hybridization was continued at 40°C for 4 h. The covering was then removed and the sections washed in five changes of formamide buffer (identical to hybridization solution, but without added polyanions) for a total of 15 h, rinsed with 2 × SSC, and dried. Then the preparations were dipped into Kodak-NTB emulsion (diluted 1:1 in 6 m*M* ammonium acetate, 40°C), dried and exposed at 4°C for 3 weeks. After development (Kodak-D-19 X-ray-film developer) they were stained with Giemsa.

Results

The postmortem examination revealed a necrosis of 95% of liver parenchyma with little regeneration in the periphery of liver lobules (Fig. 1). A few proliferating bile ductules were seen. Portal triads and sinusoids were massively infiltrated by monocytes and histiocytes. Kupffer cells were enlarged and showed single necroses [6]. There was no cholestasis, no fat depositions or infiltration by eosinophilic leucocytes.

The skin was jaundiced and exhibited petecchial bleeding. Massive bleeding was also seen into the peritoneum and peritoneal cavity, into gall bladder wall and cavity, into gastrointestinal tract, lymph nodes, lungs, myocardium, subepicardially and subendocardially, and into the kidneys. The kidneys showed tubular epithelial necroses [6]. A severe cerebral oedema was noted.

Hybridization studies

The autoradiographic pictures of "Southern blot" (Fig. 2) as well as of in situ hybridization (Fig. 3) showed a positive result. Because 0.5 µg Raji DNA (50 viral genomes per cell) shows a hybridization signal similar to 10 µg patient liver DNA the EBV genome content per average liver cell is 2.5. The black granules in Fig. 3 were caused by the radiation generated by the decay of radioactively labelled viral test DNA (EBV-DNA). This single-stranded test DNA was specifically bound to the complementary strands of "resident" EBV-DNA and formed a double-stranded EBV-DNA sequence. The granules could not be allocated to a certain cell type due to damaged cell structures caused by tissue necrosis and preparation artefacts.

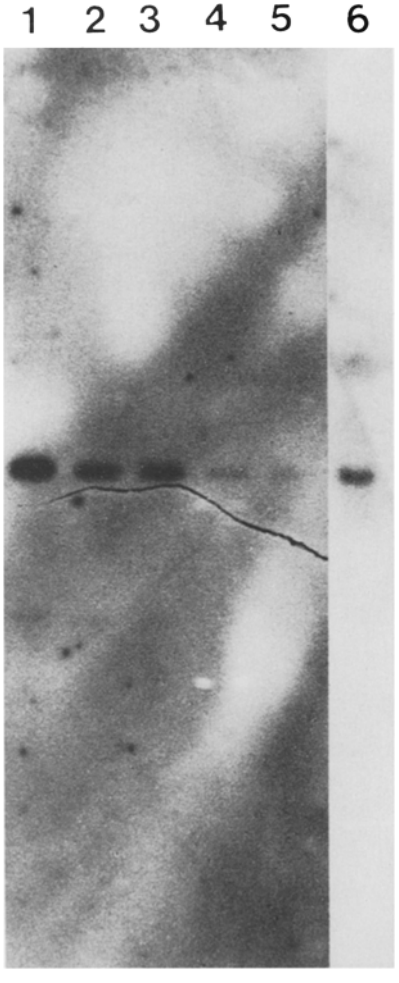

Fig. 2. Southern blot hybridization (W S7ss DNA 125 J): positions 1–5: Raji/*Bam*HI 2 µg, 1 µg, 0.5 µg, 0.2 µg, 0.1 µg/slot. Position 6: patient H.T., liver tissue DNA/*Bam*HI 10 µg/slot

Discussion

Liver involvement in acute EBV infections occurs in up to 95% of patients between the 6th and 15th day of illness and is usually mild [17].

Harries and Ferguson reported two instances of fulminant hepatic failure in infants [9]. Their diagnosis was based on clinical and laboratory features and on the demonstration of heterophilic antibodies in the older girl. Both children died on the 26th and 28th days, respectively. Recently, another girl aged 14 years had been reported to have died from fulminant hepatic failure during the acute stage of an EBV infection [10]. The diagnosis was confirmed serologically by an EBV VCA-IgM titre of 1:640, but a postmortem examination was not performed. There is no doubt that our patient suffered from an acute EBV infection. The clinical picture (tonsillitis, enlargement of cervical glands), peripheral blood smears, the presence of heterophilic antibodies in two different tests and specific antibodies of the IgM type confirm this infection [6, 11, 17, 22].

In male patients with X-linked lymphoproliferative syndrome a high incidence of massive hepatic necrosis during EBV infections was encountered [15, 16]. Purtilo suggested that immunodeficiency states are necessary for development of an acute liver failure [16]. In our patient, family history, clinical and laboratory data made the diagnosis of pre-existing immunodeficiency very unlikely.

Histologically, the liver involvement is usually characterized by a dense infiltration of portal triads and sinusoids by monoclear cells, by an activation of Kupffer cells and by focal liver cell necrosis [20]. Massive hepatic necroses have rarely been seen in adults [1, 2] and in children without immunodeficiencies [10, 15]. Histological examination of the liver in our patient suggested EBV as a cause of the liver disease, there were no indications of drug-induced liver disease [7, 14, 21, 27].

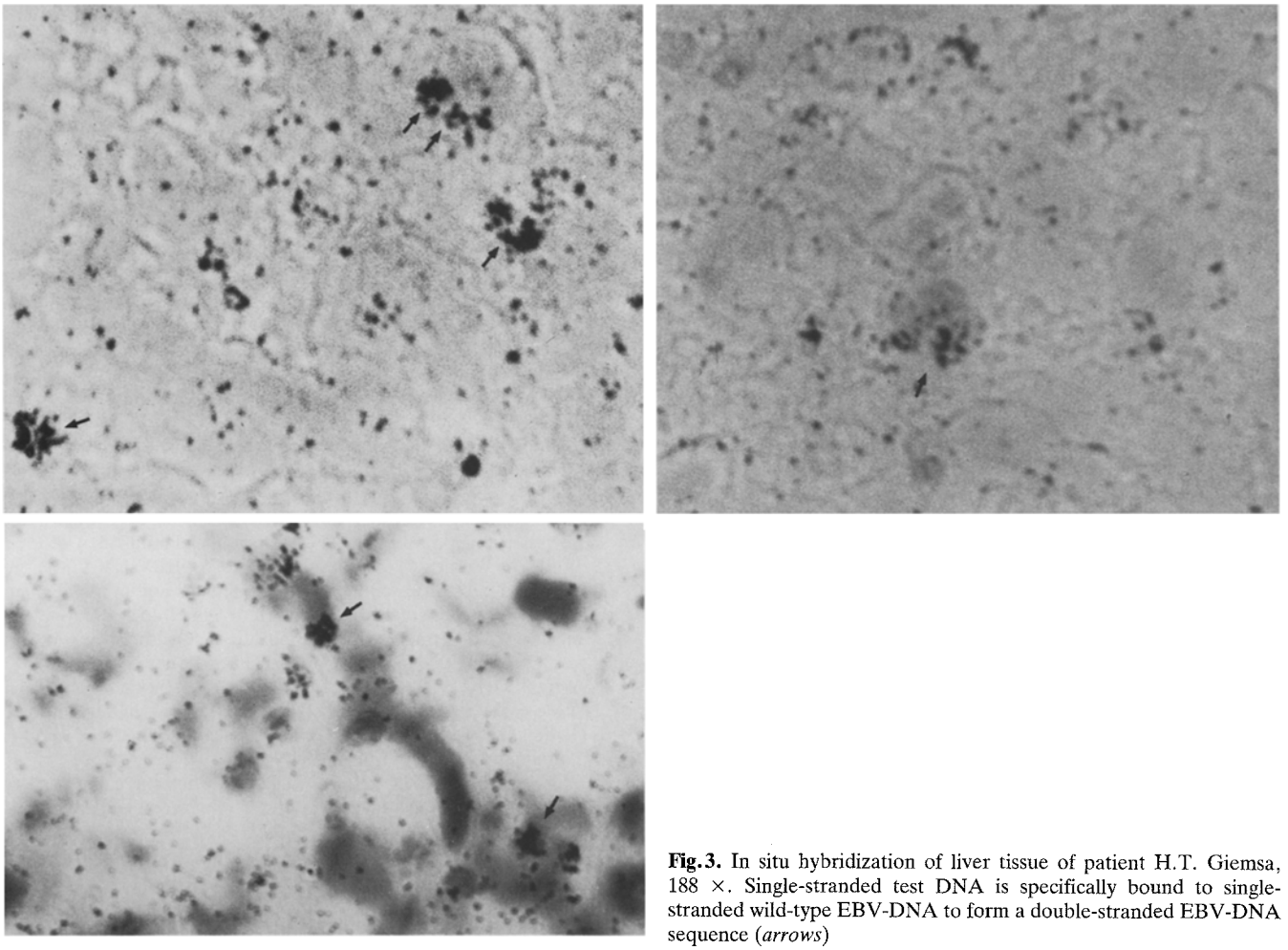

Fig. 3. In situ hybridization of liver tissue of patient H.T. Giemsa, 188 ×. Single-stranded test DNA is specifically bound to single-stranded wild-type EBV-DNA to form a double-stranded EBV-DNA sequence (*arrows*)

Since massive hepatic necroses, as mentioned above, have only rarely been reported, the identification of EBV in liver tissue seemed necessary to support the diagnosis. Nucleic acid hybridization techniques have been used successfully to demonstrate EBV-DNA in tumour cells of nasopharyngeal carcinoma and in poorly differentiated carcinomas of the tonsils [23]. These methods are based on the ability of single-stranded viral test DNA to form stable double strands with other nucleic acids with complementary sequences of at least five successive nucleotides [24]. Highly specific hybridization probes are obtained by cloning of viral DNA in plasmid vectors; cross reactions with closely related viruses can then be excluded by selecting appropriate fragments [24]. The sensitivity of the test system is determined by the specific radioactivity of the tracer DNA. In the case of in situ hybridizations the multiplying effect of the transcription of active viral genes can be used to achieve higher signals. In these preparations artefacts produced by DNA-binding proteins or local desiccations cannot completely be excluded [24]; therefore, it is preferable to use another independent technique to confirm the results. In our case we used purified DNA digested with restriction enzymes for the detection of viral sequences. The use of purified DNA excludes protein-mediated artefacts and restriction enzymes upon gelelectrophoresis create highly specific bands detectable with specific hybridization.

Southern blot hybridization and in situ hybridization were used for the first time to demonstrate EBV in the liver tissue of a patient. The demonstration of highly concentrated EBV-specific DNA in liver tissue confirms the presence of replicative Epstein-Barr viruses and suggests a causal relation between EBV and the acute hepatic necrosis. However, the pathologic mechanism of the massive necrosis of liver cells remains unclear. Immunologic mechanisms may play a role. The virus seems to enter the body in the nasopharynx and rapidly colonizes B-lymphocytes. This causes a T-cell and antibody response to terminate the infection [26]. Some T-cell defects apparently induced by EBV [4, 13] and the frequency of massive hepatic necrosis in patients with X-linked lymphoproliferative syndrome and EBV infection [15, 16] support this theory.

Unfortunately, the virus could not be allocated to a certain cell type. It possibly infiltrates the liver within lymphocytes. Whether it infiltrates the parenchymal cells of the liver in a similar way to epithelial cells of carcinomas [3, 23] and whether it exhibits a direct toxic effect on the liver cell remains to be elucidated.

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