Case Report

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Morphological and Molecular Diagnosis of a Fatal Form of EBV Infectious Mononucleosis in a Child

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Mortal cases of acute Epstein-Barr virus (EBV) infection in the form of mononucleosis have been seldom described and used to be related to complications of the disease. In this report, the case of a 3-year-old girl is described, with severe form of infectious mononucleosis, deceased in the course of respiratory-circulatory insufficiency with a sudden cessation of heart action. Particular attention was given to histological lesions, phenotype of inflammatory cells and to expression of proteins and EBV RNAs (EBERs) in tissues, examined using immunocytochemical techniques and *in situ* hybridization. Histological patterns were dominated by massive lymphocyte infiltrates (mostly CD45RO+ and CD3+ cells), mainly in lungs and in liver and, less pronounced, in kidneys and in leptomeninx. Lymphocyte proliferation exhibited polyclonal character: both lambda and kappa chains were present. No myeloblastic differentiation could be demonstrated. The EBV proteins, as well as EBV RNAs (EBERs) were detected both in small lymphocytes B and in enlarged (blast) cells, frequently resembling Reed-Sternberg cells. In our tissue material co-expression of the two proteins (EBNA2+, LMP1+) and EBER has been demonstrated in every organ, in accordance to the latency III pattern described by other authors.

Introduction

Epstein-Barr virus (EBV; Human Herpesvirus 4), widely disseminated in the human population and included to the *Herpesviridae* family, *Gammaherpesvirinae* subfamily, carries a double-stranded DNA of, approximately, 172 kb in size [11, 12, 58]. EBV represents the first herpesvirus the genome of which has been fully cloned and sequenced [1, 10, 56]. The virus manifests tropism to B lymphocytes, some cells of epithelial origin and to fibroblasts [3, 7, 60, 66]. The virus binds to cells with surface receptors specific for glycoproteins of viral envelope, i.e. for gp350/220 (CD21 molecule) [13, 70, 72].

Etiological links between EBV and infectious mononucleosis (IM) were demonstrated in 1968 [21]. The infection starts in epithelium of nasopharynx, soon involving the basement membrane-contacting B lymphocytes, which become disseminated throughout the lymphoid system to most organs [32, 43, 52]. IM, in its essence, involves a self-restricting lymphoproliferation. It is characterized by a transient immunosuppression with overexpansion of atypical lymphocytes, first of all T lymphocytes, CD8+. EBV infects and stimulates mainly B lymphocytes, inducing polyclonal humoral and cell-mediated immune responses [43]. EBV may infect various subpopulations of B lymphocytes, those from germinal centers, memory B lymphocytes, but also some naive B-cells [22, 37]. In peripheral blood of IM patients up to 1% of EBV-infected B lymphocytes can be demonstrated [33]. IM manifests peak incidence in adolescents and in young adults. Physical examination demonstrates fever, pharyngitis, lymphadenopathy and splenomegaly [7, 52]. Severe signs of IM persist for 2–4 weeks and are followed by recuperation. In everyday medical practice, diagnosis of EBV infection is restricted to symptomatic cases and clinically difficult cases and the routinely applied tests detect heterophile antibodies or antibodies directed against selected antigenic components of the virus [26, 28, 42, 53]. PCR technique was found to be highly sensitive in demonstration of various strains of EBV and/or to detect mutations in viral genome [25, 64].

Over 90% of adults throughout the world manifest latent infection with Epstein-Barr virus [46]. Mortal cases of acute EBV infection in the form of mononucleosis have been seldom described and used to be related to complications of the disease [24, 62, 68]. In the reports age of the affected patients is worth attention: small children and persons of advanced age have dominated the group [24, 40, 69, 71]. A particularly severe, chronic and sometimes deadly EBV infection was noted in persons with familial, genetically conditioned disturbances in the lymphoid system, such as X-chromosome linked lymphoproliferative syndrome (Duncan's disease), various deficits in cell-mediated immunity (e.g., deficit of NK cells) and others [45, 55]. In hemopoietic organs, viscera and central nervous system, diffuse infiltrates were noted, consisting of lymphocytes, plasma cells and histiocytes (some with erythrocytes). Another variety of X-linked lymphoproliferative syndrome was described in a 10-month-old boy who died suffering from hepatic insufficiency, interstitial pneumonitis, meningoencephalitis and aplastic anemia [71]. Another case of the fulminant course of IM was described in a 20-month-old boy with severe hepatic failure [24]. Studies confirmed infiltrates of immunoblasts in multiple organs, including liver. The hepatic lesions seemed to result from abnormal killer cell activity of the suppressor/cytotoxic T cells and NK cells, and were not related to replication of the virus. The hemophagocytic syndrome represents a particularly grave complication of IM. Various clinico-pathological forms of the disease have been described. Its relationship with EBV infection was suggested by studies including *in situ* hybridization (ISH), conducted to detect viral genome or EBER [15, 62]. In blood of patients with the syndrome high loads of EBV DNA were detected [62]. In a large group of patients (52 persons) with fatal IM probable stages of bone marrow damage were described, secondary to the hemophagocytic syndrome in the course of IM and directly responsible for death of the patients [48]. The other description of the fatal course of IM pointed to an unexpected sudden death due to acute myocarditis, possibly caused by the EBV. In the autopsy material, myocardial necrosis was noted, with marked lymphocyte infiltration. Presence of EBV genome in myocardium could not be detected until the sensitive PCR technique was employed for the purpose [25]. Immunocytochemical study of lymphocytic infiltrates showed strong positivity for a T-cells marker (CD45RO) in the myocardium and pharyngeal mucosa. PCR technique, employed for testing of cerebrospinal fluid, confirmed EBV infection as a cause of death of an immunocompetent patient with necrotizing hemorrhagic encephalitis. EBV DNA was detected by qualitative PCR in autopsy brain material. In this case, serological tests were positive in IgG class demonstrating presence of antibodies reactive both with VCA and EBNA [14]. The other reported causes of death in IM include renal failure accompanied by respiratory insufficiency [9]. In an 11-month-old child pulmonary lymphoid hyperplasia was also noted, previously not encountered in immunocompetent individuals. Immunocytochemical techniques allowed demonstrate LMP in uninfected B lymphocytes, with no expression of EBNA [40].

A Case Description

Three-year-old child, JK, of female sex from the normal, first pregnancy, normal delivery at term (Hbd 38), body weight at birth: 3050 g, Apgar 10. The child was routinely vaccinated, never suffered from any diseases. Family anamnesis disclosed no chronic diseases; the child had contact with three persons suffering from mononucleosis. In the course of disease of the child her father was hospitalized due to mycoplasma-induced pneumonia. First signs of the disease developed approximately one month before death in the form of dermal lesions (fine-macular rash on extremities and buttocks), swelling of the face and infection of the upper respiratory pathways. The child was treated symptomatically in the outpatient clinic. Due to deterioration of clinical course and fever rising to 39°C, diarrhea and vomiting she was referred to the regional hospital. Infectious mononucleosis was diagnosed (anti-VCA of IgM class: 90.14 plus, of IgG class: 1342 AU/ml), accompanied by hepatitis (AspAT: 587, AlAT: 208 U/l) and pneumonia (diffuse bronchopneumonia lesions in parahilar and inframedial regions of both lungs). Laboratory data documented increasingly accentuated anemia, thrombocytopenia, leucopenia (Hb 5.2 mmol/l, E 3.21 T/l, L 2.5 G/l, Ht 0.27 l/l, MCV 82 fl, PLT 67 G/l); peripheral blood smear manifested numerous stimulated lymphocytes of variable maturity and 1%-blast reaction (blasts 1%, metamyelocytes 3%, clubbed forms 7%, segmented forms 5%, lymphocytes 77%, monocytes 6%, other 1%; 50% stimulated lymphocytes of variable maturity); bone marrow was not examined; high levels of inflammatory indices were disclosed (ESR three-number level, peak: 105/155 mm/h, CRP 48 mg/l), repeated blood culture demonstrated no pathogens, throat smears showed yeast-like fungi and *Pseudomonas aeruginosa*; urinalysis demonstrated traces of proteins and leukocyturia, parameters of renal function were normal (urea 18 mg%, creatinine 0.6 mg%). Symptomatic treatment was paralleled by administration of antibiotics, transiently of steroids (encorton) starting at the 9th day of treatment due to marked swelling of throat and tonsils. Due to gradual deterioration of general condition, lack of appetite, markedly reduced diuresis, dete-

rioration of the laboratory indices, the girl was referred to the Observation-Infectious Ward of the Voivodship Hospital. Upon admission, the child was in severe condition, she was weak, in supine position, with preserved logical verbal contact. Upon physical examination, a significant respiratory effort, pale skin with individual papular lesions on cheeks, evidently swollen eyelids and feet, large, softened palatine tonsils with chalk-white deposits drew attention of the doctor. Oral cavity contained large amount of mucus, with individual erosions on mucosa. Moreover, a generalized lymphadenopathy was noted (in particular, cervical lymph nodes were enlarged, forming solid, symmetrical packs). Tachycardia of 160/min order, respiration 40/min. Over lungs, exacerbated alveolar murmur was noted, with dry rale on the right side. Abdomen was large, distended, hepatosplenomegaly was noted (liver and spleen protruded 3 cm below costal arch). Ascites was noted. In the voivodship hospital diagnostic tests were extended. Laboratory data documented progressively aggravating anemia, leucopenia (Hb 8.1 g%, E 2.98, L 2.1G/l, Hct 0.25 l/l, MCV 84 μm^3), thrombocytopenia (34–52 G/l) associated with a normal clotting system; myelogram was difficult to evaluate due to 5 days of treatment with steroids in the regional hospital (the result: one marrow with few nucleated cells, with structures of both erythroblastic and granulocytic order of differentiation, with numerous atypical lymphoid cells). Upon estimation of blood group („A'' Rh plus) presence of warm type IgG class autoantibodies was detected and, in addition, accentuated inflammatory indices were recorded, including growing levels of C-reactive protein (CRP 189–266 mg/l), elevated levels of immunoglobulins of all classes (IgG: 24.8, IgA: 1.75, IgM: 8.01 g/l), of gamma-globulins in electrophoresis (total protein: 64 g/l, albumins: 22.28, globulins: alpha1 3.36, alpha2 9.12, beta1 2.52, beta2 3.21, gamma 23.52 g/l), accompanied by lowered levels of complement components C3 and C4 (C3: 1.0 g/l, C3c: 0.497 g/l, C4: 0.049 g/l) and hypoalbuminemia, elevated levels of bilirubin and of aminotransferases (total bilirubin: 3.68 mg%, AspAT: 233, AlAT: 152 IU/l); blood glucose: 62 mg%; uric acid: 3.89 mg%; preserved renal function (creatinine: 0.3 mg%, urea: 21 mg%). The urine contained protein (1.44 g/l), leukocytes (8–10), acetone, urobilinogen, hyaline and granular casts. Ultrasonography of abdomen demonstrated significantly enlarged, hyperechogenic liver of uniform structure, gallbladder with traits pointing to its inflammation: wall of 9 mm in thickness with traces of fluid in the site of gallbladder, markedly enlarged, uniform spleen, 14 cm in length, presence of accessory spleens (0.7 cm, 0.6 cm, and 2 cm in length), both kidneys manifested normal corticomedullary structure and normal systems of calyces, pancreas observed in fragments, of an irregular outline, non-homogeneous echo and structure. Moreover, abdominal cavity contained large volume of echo-less fluid, urinary bladder was poorly filled, hepatic hilus contained enlarged lymph nodes; chest X-ray documented the presence of inflammatory lesions in the left lower pulmonary lobe, slightly enlarged heart silhouette of an abnormal shape and widened pulmonary blood vessels in hilar regions with signs of moderate pulmonary stasis. Presence of a fluid in pericardial sac was confirmed by ultrasonography. Due to the demonstrated circulatory abnormalities the girl was consulted by a cardiologist. Electrocardiogram demonstrated a rhythmic sinus rhythm, 150/min, normogram, slightly marked disturbances in depolarization, blood troponin – absent. Due to the data of anamnesis and the presented signs/symptoms blood culture was performed (proved to be sterile) and virological tests were conducted searching for infections with cytomegaly virus (anti-CMV antibodies of IgM class: positive, 1.22, of IgG class: negative), HIV (immunoenzymatic test for HIV1 and HIV2 specific antibodies: negative), *Mycoplasma pneumoniae* (antibodies in IgM class: positive, 31.7, in IgG class: negative, 5.8 VE). Despite the intense symptomatic treatment, antibiotics, anti-viral drugs, hepatocyte-protecting drugs, vascular sealers, strict monitoring, fluid balance and daily checks of body weight (18 – 19 – 20.4 – 19.5 – 18.9 kg) the girl continued to be in fever. A gradual increase was observed in inflammatory indices, accompanied by increasingly severe anemia, leucopenia and deterioration of clinical condition of the child. At the fourth day a decrease in body weight was achieved and increase in diuresis (up to 700 ml/day). Despite the applied therapy, at the fifth day of hospitalization, in morning hours general condition of the patient abruptly deteriorated, with intensification of auscultatory pathology (numerous wheezes, fine and coarse rale over lungs, extended expiration with tachypnoe, 44/min), tachycardia 179/min, dull heart sounds, liver and spleen margins protruded 15 cm and 10 cm below costal arch, respectively. At 12.10 the child suddenly lost consciousness, which was followed by cardiac and respiratory arrest; despite immediate resuscitation attempts no spontaneous heart and respiratory function was achieved and at 13.15 p.m. the child was pronounced dead.

Material and Methods

The tissue material included organs (lymph nodes, liver, spleen, lungs, kidneys, heart, brain) obtained on autopsy. The studies were conducted on serial, 5 μm paraffin sections, placed on the SuperFrost/Plus microscopical slides. The material was fixed in buffered 10% formalin and embedded in paraffin using the routine procedure.

Immunocytochemical studies

Mouse anti-human monoclonal antibodies were employed (Tables 1 and 2). Then, the sections were treated with primary MAb at night at 4°C, then secondary biotinylated link anti- mouse and anti-rabbit IgG (DAKO) and with the streptavidin-biotin-peroxidase complex (ABC) (DAKO). The studies followed the classical ABC technique [23], associated with the ImmunoMax technique [29, 30] (for detecting CD21 and ZEBRA). In both techniques microwave-oven pretreatment for antigen retrieval was used. In ImmunoMax technique, the key reaction involved 3 min incubation with biotinylated tyramine (PerkinElmer Life Sciences, Inc.) in dilution 1:50 at RT. This was followed by another application of streptavidin complex. The color reaction was evoked with the HRP substrate, 0.05% 3,3-diaminobenzidine tetrahydrochloride (DAB; DAKO) in 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 0.001% H_2O_2. In tissue sections, endogenous peroxidase was blocked with 1% H_2O_2. Control reactions employed control sera of the respective species in 0.05 M Tris-HCl, pH 7.6, supplemented with 0.1% BSA and 15 mM sodium azide (negative control) (DAKO). Most of the histological preparations were subjected to double immunocytochemical reactions, including lymphocyte B (CD20, CD21), T (CD45RO), NK-like cells (CD57), macrophage marker (CD68) or marker of endothelial cells (Von Willebrand Factor) and one of the EBV proteins. The terminal reaction product was visualized using DAB (brownish-black signal) or Vector VIP substrate kit for peroxidase (Vector Labs, Burlingame, CA, USA) (purple reaction product). Some preparations were counterstained with hematoxylin. Control reactions were based on substituting specific antibodies with normal sera of the respective species in 0.05 mM. Tris-HCl, pH 7.6, supplemented with 0.1% BSA and 15 mM sodium azide (negative control).

In situ hybridization (ISH)

For this purpose the fluorescein-conjugated Epstein-Barr Virus Probe ISH Kit was used (Novocastra Labs, Newcastle upon Tyne, UK) for demonstrating Epstein-Barr virus-encoded RNA (EBER) transcripts. The ISH reaction protocol followed Novocastra procedures with modifications of our own, published previously [30]. Following preliminary stages of the ISH protocol, a probe was incubated on paraffin sections for 2 h at 37°C, and then the sections were washed 3×3 min in TBS, containing 0.1% Triton X-100, and incubated again for 30 min with a blocking solution (normal rabbit serum dissolved in TBS, containing 3% bovine albumin and 0.1% Triton X-100) at room temperature and for next 30 min with rabbit anti-FITC/AP diluted 1:100 in TBS, 3% w/v BSA, 0.1% v/v Triton X-100. The sections were then washed in TBS and in alkaline phosphatase substrate buffer, pH 9.0, for 5 min. The subsequent stage involved 14–16 h incubation in a solution of 5-brom-4-chlor-3-indolylphosphate (BCIP) and nitro blue tetrazolium (NBT) in dimethylformamide, dissolved at 1:50 in a buffer (100 mM Tris/HCl, 100 mM NaCl, 50 Mm $MgCl_2$). One µl Levamisole hydrochloride was added per each ml of the diluted enzyme substrate. The final stage involved rinsing of the preparations in distilled water (2×5 min) and mounting them in the aqueous balsam (DAKO). Positive control for the hybridocytochemical reaction included paraffin preparations of cells infected with EBV *in vitro*, attached to the kit (Novocastra).

Semiquantitative evaluation of immunoreactive cells was applied in detection of EBV proteins and EBER, taking into account mean score for 5 visual fields under 400× magnification according to the following scale: + = 1–10% scattered or focally arranged cells; ++ = 11–29% immunopositive cells; +++ = >30% of immunopositive cells. A cytoplasmic, membranous and nuclear reaction was evaluated.

Results

Histopathology

Upon autopsy, enlarged cervical, mediastinal, and abdominal lymph nodes were detected. Liver and spleen were augmented. Histological patterns were dominated by massive lymphocyte infiltrates, mainly in lungs and in liver and, less pronounced, in kidneys and in leptomeninx.

Microscopically, all the lymphoid organs examined (lymph nodes, spleen, tonsils, thymus) manifested blurred structural patterns due to extensive inflammatory infiltrates. Lymph nodes were markedly enlarged and uniformly infiltrated with polymorphic cells, i.e. with small lymphocytes, large blasts, occasionally resembling Reed-Sternberg cells (large, bilobar cell nuclei with visible nucleoli) and individual plasma cells (Fig. 1A). In some lymph nodes individual lymphoid follicles were preserved. Blood vessels with traits of blood congestion contained individual lymphoid cells. Sporadically, large atypical lymphoid cells were observed, containing cell nucleus typical for EBV-infected cells (Fig. 1B). Some lymph nodes contained numerous lipid-laden macrophages. In the thymus vast infiltrates were noted of polymorphic lymphoid cells as well as erythrorrhagia, traits of hyperemia and only few Hassal's corpuscles.

Histopathological lesions in other selected organs, sampled on autopsy

1. Lungs

All samples of the lungs manifested exponents of moderate or extensive atelectasis. In numerous alveoli their lumen was fully filled with transudate/exudate liquid with individual macrophages/monocytes and lymphoblasts. In

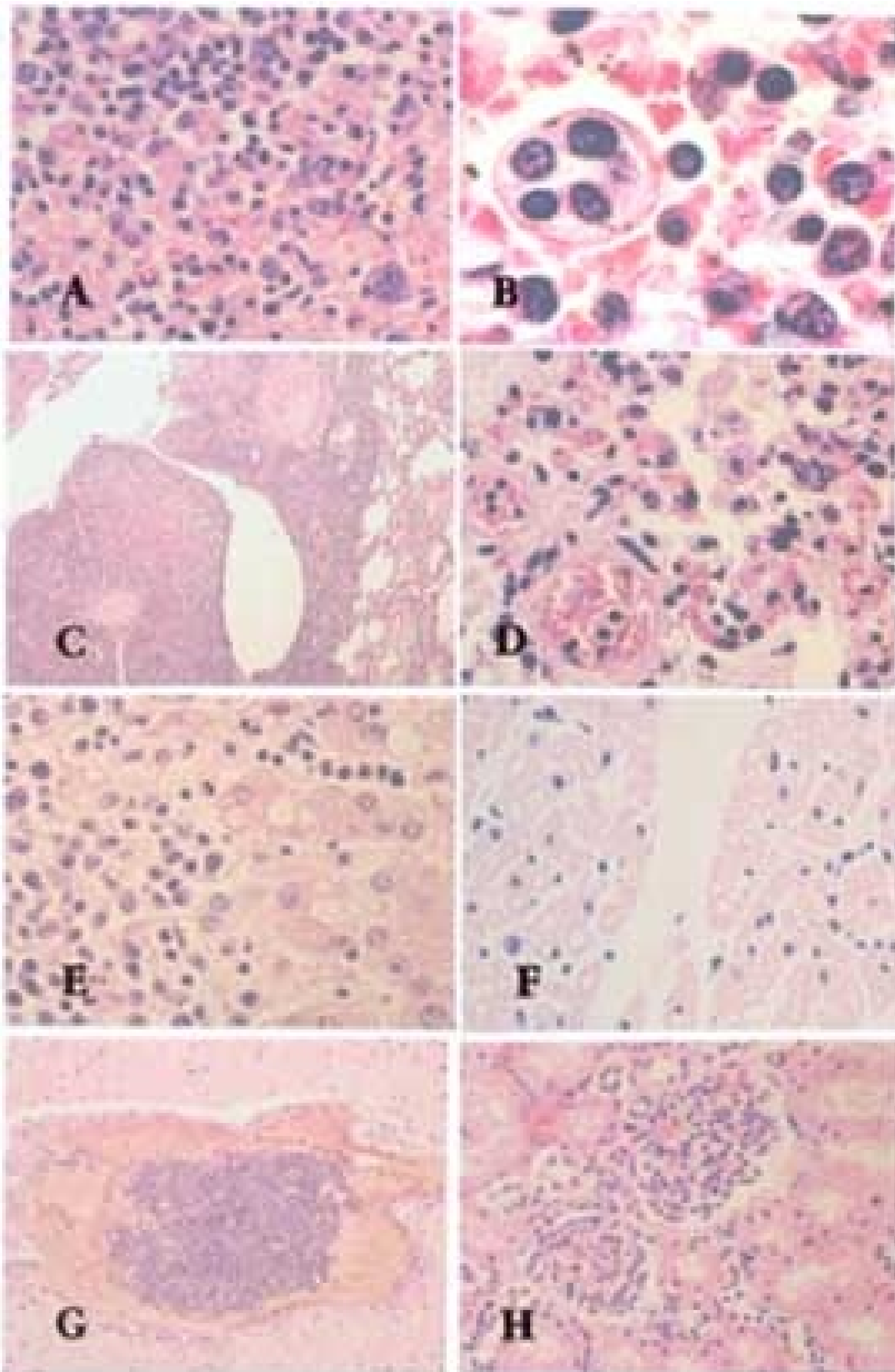

Fig. 1. Histological patterns with massive lymphocyte infiltrates in the organs infected by EBV. (A) lymph node with polymorphic cells, i.e. with small lymphocytes and large blasts; (B) high power magnification of one large, atypical, multinucleated cell resembling Reed-Sternberg cells in the lymph node; (C) vast infiltrates could be noted in regions adherent to bronchi, bronchioli, and to blood vessel adventitia; (D) vast proportion of pulmonary parenchyma occupied by vast infiltrates of lymphoid cells, individual plasma cells, atypical transformed lymphocytes and lymphoblasts; (E) vast accumulation of polymorphic lymphoid cells (including large atypical lymphocytes) in the liver, which also separated individual lobules; (F) the myocardium with individual lymphoid cells; (G) the accumulation of the lymphoid cells in the lumen of some veins, occasionally occupying even more than 50% of vascular lumen in central nervous system; (H) the infiltrates of lymphoid cells in kidney, traits of immaturity of renal glomeruli, tubular degeneration and a slight proliferative reaction in renal glomeruli. All figures: HE. Original magn. 100× (C, G); 200× (F, H); 400× (A, D, E); 1000× (B).

these regions of lungs septa were evidently thickened, sinusoids showed traits of congestion, with relatively numerous erythrocytes and lymphoid cells in their lumen. A proportion of pulmonary alveoli demonstrated traits of acute distension, with ruptured septa and protruding emphysematous spicules. Vast proportion of pulmonary parenchyma was occupied by vast infiltrates of lymphoid cells, individual plasma cells, atypical transformed lymphocytes and lymphoblasts (Figs. 1C and 1D). Particularly vast infiltrates could be noted in regions adherent to bronchi, bronchioli, and to blood vessel adventitia. Multiple bronchi were denuded of epithelial lining while their lumen contained remnants of the epithelial cells, numerous lymphoid cells of variable size and lipid-laden macrophages. Some sets of ciliated epithelium were present in the lumen of alveolar ducts and in distended pulmonary alveoli. Many blood vessels of pulmonary circulation showed traits of hyperemia.

2. Liver

Structure of the organ manifested a preserved lobular pattern, with clearly dilated (in some parts very significantly) portal spaces. The dilation reflected vast accumulation of polymorphic lymphoid cells (including large atypical lymphocytes), which also separated individual lobules (Fig. 1E). Sporadically, the cells presented mitotic figures. Close to central veins lipid vacuoles of variable size were seen. Few hepatocytes, mainly those localized at the periphery of lobules, presented traits of fatty change. In individual hepatocytes traits of cholestasis could be noted. Hepatic sinusoids and spaces of Disse were moderately widened, with visible Ito's cells and focally present individual lymphoid cells. Lining of the sinusoids was normal, with erythrocytes and very numerous leucocytes in the lumen, reaching up to the widened portal spaces. The infiltrate cells included polymorphic lymphoid cells and individual plasma cells.

3. Heart

In myocardium relatively numerous lymphoid cells were observed (mainly small lymphocytes) as well as individual plasma cells and large lymphoblasts, localized in perivascular spaces or in the vessels themselves (Fig. 1F). A proportion of cells manifested traits of myocardiocytolysis of a variable advancement.

4. Central nervous system

The most pronounced accumulation of the typical for IM lymphoid cells was noted in the lumen of some veins, occasionally occupying even more than 50% of vascular lumen (Fig. 1G). In cerebral hemispheres the regions contained individual lymphocytes. Moreover, widening of perivascular spaces was observed, in particular those of capillaries. Within cerebellar hemispheres few, mostly small lymphocytes were seen. No traits of edema were encountered in the central nervous system.

5. Kidney

In the organ, infiltrates of lymphoid cells were detected, located under the capsule and around blood vessels. Moreover, traits of immaturity of renal glomeruli, tubular degeneration and a slight proliferative reaction in renal glomeruli were seen (Fig. 1H).

Immunocytochemical studies

1. Phenotype of cells in inflammatory infiltrates

Inflammatory infiltrates contained a mixed cell population, including T lymphocytes (CD45RO+, CD3+), B lymphocytes (CD20+, CD79a+) as well as numerous scattered monocytes and macrophages (CD68+) (Table 1). Thus, it seemed that slightly more cells in inflammatory infiltrates showed phenotype of T lymphocytes (CD45RO+, CD3+) (Fig. 2A). Lymphocyte proliferation exhibited polyclonal character: both lambda and kappa chains were present. No myeloblastic differentiation could be demonstrated (the cells were CD117 negative, CD15 negative).

2. Expression of EBV proteins of lytic phase (BZFL1) and latent phase (EBNA2, LMP1)

In each of the organs examined expression of all three proteins manifested a variably intense expression (Table 2). Proteins of the latent phase were present mainly in B lymphocytes, follicular dendritic cells (CD20+, CD21+), few T lymphocytes (CD45RO), and in cells of CD57+ phenotype (Figs. 2C and 2D). The antigens were detected both in small B lymphocytes and in enlarged (blast) cells, frequently resembling Reed-Sternberg cells, and the enlarged cells frequently showed the CD68+ phenotype. EBNA2 was located mainly in the cell nuclei, mainly in small lymphoid cells, LMP1 was observed both on cell membranes and in the cytoplasm of infected cells and the protein more frequently was noted in the enlarged blast cells (Figs. 2B and 2E). The immunopositive cells were present mainly in perifollicular and interfollicular regions of lymph nodes, in pulp cords in the spleen, and in infiltrates of lymphoid cells in the remaining organs. Cells immunopositive for the BZLF1 (ZEBRA) protein were less frequent as compared to cells with expression of the remaining proteins but they were demonstrated in every organ, being most frequent in lungs and lymph nodes (Fig. 2F). The protein was present in lower number of cells, small lymphocytes and/or plasma cells (no parallel staining with anti-CD30 was performed) and in blast cells (including CD68+ blasts). Expression of the protein manifested a more diffuse pattern. As a rule, the protein expression was not seen in cells of CD20+ phenotype.

TABLE 1
Studies on immunophenotype of inflammatory infiltrate cells in selected organs obtained upon autopsy from 3-year-old girl with infectious mononucleosis

Antigen	Antibody source (dilution)	Organ					
		lymph node	spleen	liver	lungs	kidney	heart
CD45RO	Dako (2)	+	+	+	+	+	negative
CD3	Novocastra (50)	+	+	+	+	+	negative
CD20	Dako (20)	+	+	+	+	+	negative
CD21	Novocastra (100)	+	+	+	+	+	negative
CD79a	Dako(1)	+	+	+	+	+	negative
CD68	Dako (1)	+	+	+	+	+	negative
CD57	Dako (100)	+	+	+	+	+	negative
CD117	Dako (400)	Negative					
CD15	Dako (50)	Negative					
CD30	Dako (1)	Negative					
kappa and lambda chains	Dako (1)	+	+	nt	nt	nt	nt

nt – not tested

TABLE 2
Immunocytochemical and hybridocytochemical detection of CMV and EBV proteins in autopsy material of a child deceased in the course of diffuse form of EVB-induced infectious mononucleosis

Antigen	Antibody source (dilution)	Organ					
		lymph node	spleen	liver	lungs	kidney	heart
CCH2&DDG9 (EA-HCMV)	Dako (1)	Negative					
EBNA2	Dako (50)	+	+	+	+	+	nt
LMP1	Dako (50)	++	++	++	++	++	nt
ZEBRA (BZFL1)	Dako (20)	++	++	+	+	+	nt
EBERs	Novocastra	+++	++	+	+	+	nt

+ – 1–10% scattered or focally arranged cells; ++ – 11–29% immunopositive cells; +++ – over 30% immunopositive cells; nt – not tested

3. Expression of HCMV proteins

In none of the organs examined expression of the early HCMV proteins (76 kD and 43 kD) could be detected.

In situ hybridization

Small non-polyadenylated EBV RNAs (EBERs)

Expression of EBERs was evident and affected numerous EBV-infected cell nuclei of transformed lymphocytes in all the organs studied but it was most intense in lymph nodes and in spleen. It was seen mainly in lymphocytes of small and average size but also in the enlarged cells, which resembled Reed-Sternberg cells (Fig. 2G). In this case no double stainings were conducted. In the control material, supplied with the kit for detection of EBER, expression of EBER was particularly intense within numerous cell nuclei, except of nucleoli (Fig. 2H).

Results obtained using ABC, ImmunoMax techniques and *in situ* hybridization are presented in Tables 1 and 2.

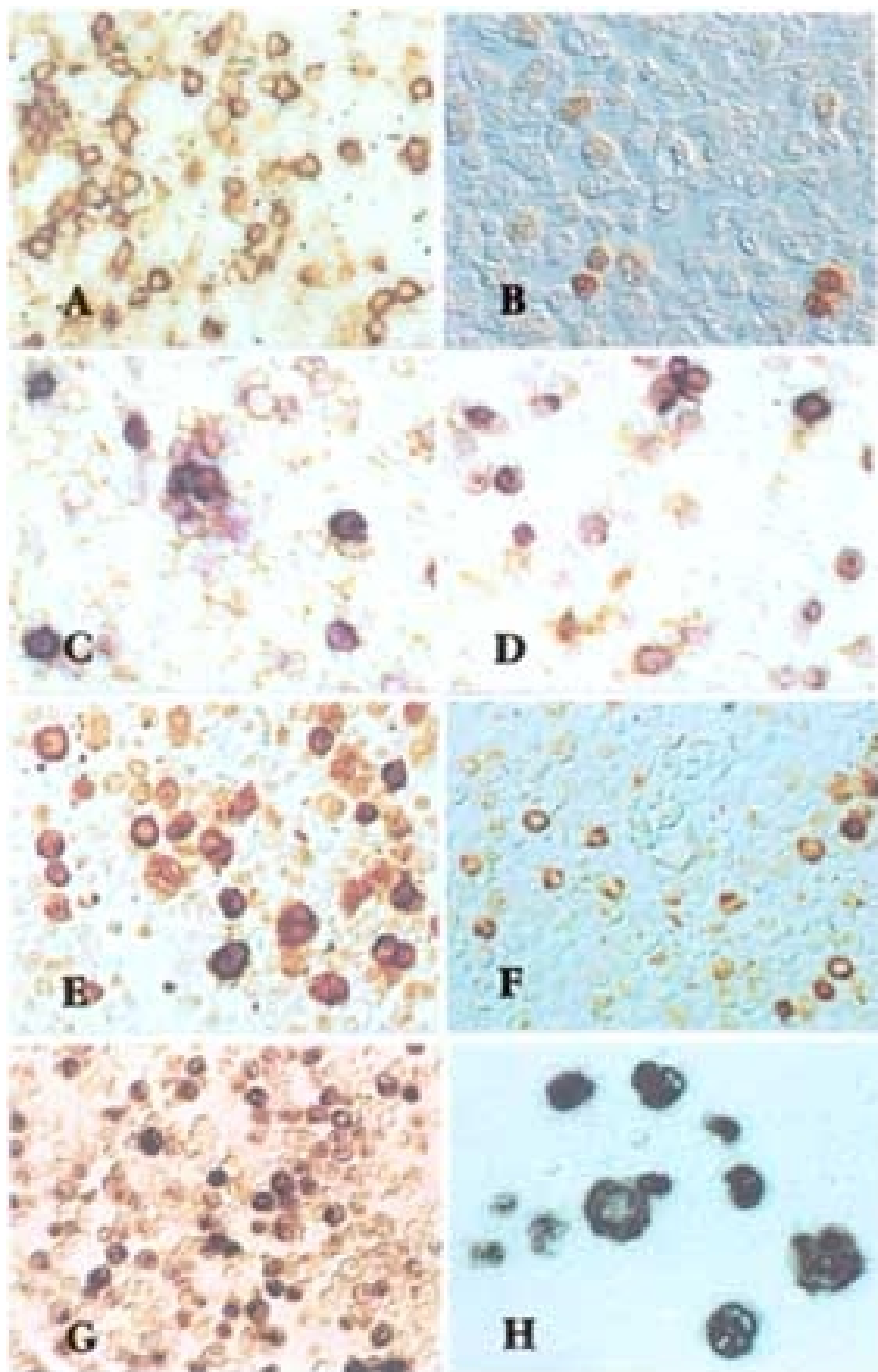

Fig. 2. Immunocytochemical analysis of EBV infection: (A) localization of CD45RO+ cells in the inflammatory infiltrates in spleen; (B) EBNA2 expression in individual cell nuclei in the lymph node; (C) double staining for LMP1 (purple) and CD20 (brown) in the lymph node; (D) double staining for LMP1 (purple) and EBV-receptor (CD21) (brown) in the lymph node; (E) LMP1 localization in spleen; (F) immunocytochemical localization of BZLF (ZEBRA) in lymphoid, sometimes atypical cells. ABC-Immunomax technique. Original magn. 400× (G) Hybridocytochemical reaction for EBV-RNA (EBER) in many cells of lymph node. Original magn. 400×. (H) Intense hybridocytochemical reaction for EBV-RNA (EBER) in many cells of positive control material infected with EBV. Original magn. 1000×.

TABLE 3
Most frequent histological features in autopsy material of IM victim

Organ	Histological features	Cit. author
Lymph nodes	lymph node enlargement; lymphadenitis numerous large pyroninophilic cells (immunoblasts) in paracortical zone and extending throughout the node abundant B-cell follicles; large hyperplastic GC dilated sinuses with abundant densely packed monocytoid B cells perivascular accumulations of normal and abnormal lymphocytes increased infiltrates in paracortical zone expansion of nonfollicular areas by a polymorphous population of lymphocytes augmented response of T lymphocytes giant cells, resembling Reed-Sternberg cells; massive intravascular and perivascular infiltration by lymphocytes and atypical mononuclear cells transformed cells of noncleaved cell type plasma cells infiltration scattered immunoblasts containing prominent nucleoli brisk mitotic activity focal perinodal infiltration large necrotic areas in the subcapsular regions marked infiltration of immunoblasts abundance of postcapillary venules; necrosis	4; 6; 8; 16; 24; 44; 49; 54; 59; 67; 69
Tonsils	tonsilar enlargement massive intravascular and perivascular infiltration by lymphocytes and atypical mononuclear cells marked follicular hyperplasia well-demarcated or geographic areas of necrosis focal areas with residual reactive GCs obliteration of normal histological pattern of the organ due to excessive proliferation of EBV infected B lymphocytes and cytotoxic T lymphocytes	38; 54; 59
Spleen	enlargement of the red pulp with engorged sinuses; massive intravascular and perivascular infiltration by lymphocytes and atypical mononuclear cells broad pulp cords, rich in macrophages small to medium-sized inconspicuous follicles, mostly lacking GCs in the white pulp scanty blasts and epithelioid cells no necrosis; marked infiltration of immunoblasts	24; 54
Thymus	marked infiltration of immunoblasts	24
Lungs	interstitial pneumonitis pulmonary lymphoid hyperplasia	4; 8; 40; 71
Heart muscle	myocardial necrosis with marked lymphocytic infiltration myocarditis	4; 8; 24
Kidney	glomerulonephritis marked infiltration of immunoblasts	4; 8; 24
Brain	meningoencephalitis necrotizing hemorrhagic encephalitis traits of edema perivascular infiltrates of mononuclear cells	4; 8; 14; 71
Liver	in portal spaces infiltrates of mononuclear cells invasion of sinusoids focal necrosis of parenchyma with infiltrates of mononuclear cells marked infiltration of immunoblasts diffuse feathery degeneration of the hepatocytes infiltrates of Ts, Tc, and NK lymphocytes in portal spaces and sinusoids	4; 8; 24

Discussion

Fatal cases of IM, even if described in the literature of the subject, are rare. Spleen rupture, complications in the central nervous system, renal insufficiency, fulminant hepatic failure, virus associated hemophagocytic syndrome, severe hemolytic anemia, severe acute myocarditis or lymphoproliferative diseases [9, 14, 17, 24, 25, 34, 39, 40, 48, 62, 68] used to cited as a cause of death at the acute stage of the disease. Particular attention is given to cases of IM, in the course of which malignant lymphoproliferative diseases developed, including lymphomas of B and/or T lymphocytes [21, 27, 34, 62, 63, 68, 73, 76]. In such complications, virally transformed B or T lymphocytes may undergo progression from polyclonal to monoclonal proliferation [2, 32, 65, 76]. In differential diagnosis additional tools of molecular biology are used to demonstrate EBV in tumor cells as well as to demonstrate lymphoid-cell gene rearrangements [27, 59]. Detection of EBV DNA at a tissue level is linked to poor prognosis, particularly in hemophagocytic syndrome linked with the infection and in malignant lymphoma [62]. Application of *in situ* hybridization permitted to distinguish differences of EBV DNA distribution pattern in IM, Hodgkin's disease and non-Hodgkin's lymphomas. In general, in neoplastic proliferation the signal is much more uniform from cell to cell than in IM, and the labeling is found in much greater percentage of cells. In the case of IM, 1–10% of the lymphoid populations manifest intense staining and immunoblasts prevail among the stained cells [59]. Other descriptions of non-Hodgkin's lymphoma manifestation in the course of IM reported two children, deceased in the course of acute liver insufficiency with generalized hemorrhagic diathesis and complications in other organs. Upon autopsy, abdominal mesenteric lymph node-originating lymphoma was diagnosed in the first child, while the other was shown to suffer from lymphoblastic lymphoma, originating from T lymphocytes [68]. In each of the cases the correct diagnosis could not be established *in vivo* despite the insightful and extensive diagnosis. A similar case involved an adult person with no evident immune deficit, who developed severe, fatal form of B cell lymphoma in the course of IM [34]. Apart from lymphomas, etiological role of EBV is stressed in cases of tumors like Hodgkin's disease, nasopharyngeal carcinoma and gastric cancer [36, 75]. Sporadically, EBV infection was linked to development of mammary and lung carcinomas [18].

The IM case described in this report refers to a three-year-old girl in whom a severe clinical course of IM resulted in death in the fourth week of the disease. The acute EBV infection was established of the basis of typical clinical signs/symptoms, laboratory tests and serological results (high IgM and IgG antibody titers against VCA EBV). In addition, antibodies of IgM and IgG class reactive with CMV and antibodies of IgM class directed to *Mycoplasma pneumoniae* were disclosed. The implemented therapy proved ineffective and the direct cause of death of the child was cardiac arrest. Upon autopsy, the typical for the disease pathological lesions were detected, dominated by enlargement of all lymphatic organs and the liver. Outside of the lymphatic system, routine histological staining demonstrated lymphoid infiltrates, most extensive in lungs and in liver. The least pronounced inflammatory lesions were detected in kidneys, central nervous system, alimentary tract. In the heart the lesions were insignificant. Despite the presence of respective anti-CMV immunoglobulins of IgM class, no lesions typical for HCMV infection (giant cells with inclusion bodies) could be detected. Histological lesions detected in our material have been compared to those noted most frequently in other reports of the subject (Table 3). Application of immunocytochemical techniques and of *in situ* hybridization permitted to broaden the morphological diagnosis as well as to evaluate intensity of lesions induced by replication of EBV and/or by secondary inflammation. Results of the studied allowed, first of all, exclude in our patient a neoplastic proliferation in the course of EBV infection. In blast cells, which resembled Reed- Sternberg cells, no CD15 or CD30 expression was noted. Studies performed by Reynolds et al. on phenotype of immunoblasts resembling Reed-Sternberg/Hodgkin cells have demonstrated that in IM the cells were CD30+ but CD15 negative and the absence of a collarette of T cells around the RS/H-like cells distinguished the latter from the cells present in Hodgkin's disease [57]. Moreover, the absence of CD117 expression in the cells argued against neoplastic expansion of lymphocytes. Proliferation of B lymphocytes was of a polyclonal character since the cells contained immunoglobulin molecules with both lambda and kappa chains. Cellular infiltrates in lymphoid and other organs (liver, kidneys, lungs) were composed mainly of T lymphocytes of CD45RO+ and CD3+ phenotype (around 60% cells), B lymphocytes (CD20+, CD21+, CD79a+) and numerous, scattered macrophages/monocytes/dendritic cells (CD68+). Many of the lymphocytes manifested atypical, transformed (blast) character. Frequently the large mononuclear cells carried also CD68+ antigen (macrophages, dendritic cells, monocytes). This confirmed data of other authors, who reported that in IM expansion of T CD8+lymphocytes, and activation of T CD4+ lymphocytes, and of CD45RO-positive lymphocytes take place [25, 47]. The highest proportions of B and T lymphocytes in IM at the tissue level was reported by Niedobitek et al. [49]. Moreover, the authors noted that as many as up to 40% of the cells with EBV expression were plasma cells [49]. In the case of our patient we have not been able to confirm it: plasma cells did not

dominate inflammatory infiltrates in the organs studied. Immunocytochemical studies on EBV expression demonstrated presence of ZEBRA (BZLF1) protein, typical for the lytic phase of the infection or for a transition from the latent to the lytic phase, at least in a proportion of the inflammatory cells. In addition, expression of the two latent proteins, EBNA2 and LMP1 was documented. The proteins were detected in a majority of cells with the CD20+ phenotype, in line with observations of other authors [49, 50]. The synchronous detection of proteins of lytic phase and of the so called latent proteins is possible due to the biology of EBV [32]: in non-permissive cells, such as in humans are normal B lymphocytes, EBV infection used to acquire the latent form. On the other hand, expression of the latent genes represents a consecutive stage in the cascade of events in the course of lytic infection. The latent genes may undergo expression also under effect of a superinfection with another pathogen [32]. On the other hand, the nasopharyngeal epithelial cells represent, both *in vivo* and *in vitro* the so called permissive cells for EBV infection [60, 61]. In the cells, complete replication of the virus and formation of its progeny take place. In our tissue material of nasopharynx, in which structural and functional damage could be expected, no such lesions could be noted due to extensive destruction of the tissue. The organs with particularly high expression of the studied EBV proteins included lymph nodes and lungs, followed by liver and spleen. Expression of EBV antigens was demonstrated mainly in the enlarged, atypical B lymphocytes (CD20+, CD21+), but also in the other cell populations (CD45RO+, CD57+ and CD68+). This corroborated observations of other authors who demonstrated EBV also in T lymphocytes in the paracortical T-zone and some macrophages [54]. However, we could not confirm observations of Niedobitek et al. on expression of the lytic phase protein in plasma cells since no double staining was conducted in this case. Application of *in situ* hybridization permitted to detect RNAs of EBV (EBERs) in EBV-infected cells, practically in all the organs studied. It should be stressed that particularly high numbers of EBER-positive cells were noted in lymph nodes and in spleen. Role of EBERs has not been fully clarified. They are thought to be most abundant in EBV latent infection but their role also in the lytic event has not been excluded [32]. According to some authors, EBERs are not indispensable for transformation of primary lymphocytes B by EBV, according to the other the *in vivo* binding site for oncoprotein c-myc in the promoter for EBV encoding EBER1 suggests a specific role for EBV in lymphomagenesis [2, 51]. In cases of IM, various authors have described one of two latency patterns: some documented IV latency program [37, 49], the other type III of EBV latency [19]. In the latency pattern IV, expression of EBNA2 takes place in the absence of LMP1 expression and expression of EBER remains to be fully clarified [36]. Viral genes expressed in type III latency include all of the EBNAs, the LMPs, and EBERs [19]. The demonstrated by us coexistence of both latent proteins and of EBERs in the majority of the organs studied argues rather for the III latency program [36, 37]: first of all, we have detected expression of EBERs, in line with results of other authors [20]. The authors have described expression of EBERs mainly in numerous medium size and large (blastic) interfollicular lymphocytes, as well as in follicle mantles, and germinal centers in tonsils, lymph nodes, and in appendix [20]. In tonsils, EBER-positive lymphocytes were noted adjacent to the epithelium but not in the epithelium itself [49]. In a single, fatal case of IM, diffuse infiltrates of atypical blast cells were seen in multiple organs, many of which showed strong EBER expression. EBER expression could still be detected in phagocytized cells but was not seen in the nuclei of the phagocytosing histiocytes [20]. In our tissue material coexpression of the two proteins (EBNA2, LMP1) has been demonstrated in every organ, in disaccord to the previously described latency IV program [36, 38]. The literature reports on detection of LMP in infected lymphocytes in the absence of EBNA expression in a fatal form of IM in a small child exist also [40]. Effects of superinfection with other pathogens (CMV, *Mycoplasma pneumoniae*) cannot be excluded in the so high expression of EBV in multiple vitally important organs in our patient, which could secondarily lead to intensification of the inflammatory lesions and death of our patient. Moreover, in small children immune system is known to be immature and EBV infection of B lymphocytes could accentuate the immunosuppression in IM, extending the potential for complications, including neoplastic proliferation, in the age group [35]. Although, using immunocytochemical techniques, neoplasia has been excluded as a cause of death, determination of EBV genome clonality would require additional studies: patients with nonfatal IM may lack monoclonal EBV and monoclonal gene rearrangements [74].

Our results have confirmed that *in situ* hybridization is particularly sensitive and relatively easy to perform when used to detect EBERs. It could be performed on routinely fixed and paraffin-embedded tissue material, and confirmed the observation of the other authors [5, 20, 24, 31, 41].

Summary and Conclusions

It should be stressed that cases of IM, particularly involving the youngest and the oldest patients, require high diagnostic alertness. A widened laboratory effort is important, aimed to evaluate, first of all in cases of an atypical course, either EBV load in serum or expression of EBV *in situ*. Patients with high viral load in blood and with particularly in-

tense expression of EBV in vitally crucial organs seem to require more intense clinical supervision. Due to variable suggestions of investigators as to the cause of death in the fatal situations, evaluation of direct mechanisms which lead to death, particularly in cases of immunocompetent patients, require further studies.

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