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ORIGINAL ARTICLE

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Detection of DNA, mRNA and early antigen of the human cytomegalovirus using the immunomax technique in autopsy material of children with intrauterine infection

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Abstract The present study focuses on the immunomax technique in association with the avidin-biotin-peroxidase complex (ABC) technique and a non-isotopic variation of in situ hybridisation (ISH) for optimal microscopical detection of human cytomegalovirus (HCMV). The studies were performed on an archival paraffin material originating from five children deceased due to intrauterine infection. The results of immunocytochemical and hybridocytochemical studies, with or without amplification using biotinylated tyramine, were compared with the routine histopathological results and results obtained using the polymerase chain reaction (PCR). Early antigen (EA)-HCMV was demonstrated in approximately twice as many cells as detected in the routine staining and also in cells that seemed morphologically intact. The hybridocytochemical studies confirmed the presence of HCMV DNA in cells that were positive in the immunocytochemical tests and, in addition (using the ISH-immunomax technique), in cell nuclei of intact myocardial myocytes. In general, fewer cells manifested the presence of HCMV mRNA than the presence of HCMV DNA. The immunomax technique was found to be more sensitive than the techniques of classical immunocytochemistry or of ISH. The former technique permitted the documentation of a higher number of HCMV replication sites than could be detected using the latter techniques. However, the clinical course of HCMV infection or the cause of death of the children was not directly related to the intensity of HCMV expression in tissues.

Keywords Immunomax technique · In situ hybridisation · PCR · Cytomegalovirus · Congenital cytomegalic inclusion disease

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Introduction

The cytomegalovirus (CMV), a species-specific member of the *Herpesviridae* family, subfamily of *Betaherpesvirinae*, belongs to the most frequent class of human pathogens, which induce, as a rule, asymptomatic but persisting infection in humans with normal immunity. Signs and symptoms of the disease induced by human (H) CMV infection most frequently develop in cases of immunosuppressive treatment of various diseases following transplantation of organs or bone marrow and in the course of the acquired immunodeficiency syndrome (AIDS). The disease may also result from intrauterine infection with HCMV [1, 28]. According to World Health Organization (WHO) data, HCMV infection represents the most frequent intrauterine infection and affects 0.4– 2.3% of living newborns. Almost 90% of the newborns with the congenital HCMV infection exhibit no clinical symptoms. However, periodically they excrete the virus in urine and saliva. Later on, the children may be affected by serious neurological complications, including progressive deafness, visual disturbances and

mental retardation [1, 3]. In 5% of the children, the typical symptomatic inclusion body disease develops and another 5% of the children present atypical symptoms [3].

Pathogenetic mechanisms of the intrauterine infection with HCMV remain poorly recognised. Histopathological studies and analysis of HCMV gene expression in various organs and cell types permit accurate determination of the range of virus replication, causative involvement in the induction of injury to vitally important organs and cell death [5, 28, 29]. The data provide valuable supplementation to the not always uniform clinical pattern of HCMV infection and to laboratory results.

In recent decades, we have witnessed a development of rapid, economic and increasingly sensitive techniques for detection of the HCMV both at the stage of acute infection and in the latent form [2, 11, 21, 25]. For detection of the virus in tissue, multiple molecular biology techniques are being employed, including polymerase chain reaction (PCR) and in situ hybridisation (ISH), with the use of non-radioactively labelled probes particularly recommended in recent years [19, 31].

Since the 1990s immunocytochemical techniques have been employed, followed by in situ hybridisation (ISH) with or without simultaneous DNA detection in 1995 and for mRNA detection in 1997. The new technique of amplification with biotinylated tyramine (immunomax, catalysed reporter deposition, CARD, and tyramide signal amplification, TSA) is now in use [10, 14, 23, 32]. In the presence of H_2O_2 and peroxidase, the applied biotinylated tyramine becomes incorporated due to free radical formation and reaction with the electron-rich fragments of protein molecules (tyrosines, tryptophans, phenylalanines, etc.), which surround the sites of peroxidase. Therefore, addition of the streptavidin-peroxidase complex results in a more intense labelling [7, 10, 13, 14].

Using the ABC technique and classical ISH with simultaneous application of the immunomax technique and the routine histopathological diagnosis of the infection, we compare the sensitivity of the detection of active HCMV replication in autopsy material of children who died due to intrauterine HCMV infection. Results on HCMV DNA detected using ISH were compared to results of DNA detection using PCR, applied to the analogous, paraffin-embedded tissues.

Material and methods

Tissue material

Archival paraffin blocks with autopsy material (brain, liver, pancreas, stomach, large intestine, kidneys, myocardium, adrenal glands, parotid, lungs and spleen) originated from five children, deceased due to intrauterine symptomatic infection with CMV from 1988 to 1995. The children were patients of the B. Krysiewicz Voivodship Children Hospital. Preliminary diagnosis of the congenital infection with CMV was based on serological tests and the main clinical symptoms soon after birth. The diagnosis was confirmed using histopathological testing of selected organs, in which giant cells were detected with typical intracellular inclusions. None of the children received any specific anti-viral therapy.

Table 1 Organs studied for human cytomegalovirus (HCMV) presence in children with intrauterine infection with the virus. + the organ was examined

Tissue/ autopsy no.	30/88	59/90	1/92	16/92	8/95
Pancreas	+		+	+	
Liver	+	+	+	+	+
Stomach			+		
Colon			+		+
Heart	+	+	+	+	+
Lungs	+	+	+		+
Adrenal glands			+		+
Kidneys		+	+	+	+
Brain		+	+	+	
Parotid		+			
Spleen	+				
Sum	5	6	9	5	6

As the negative control, fragments of liver, pancreas, lungs and kidneys were used. These were isolated from six adult Wistar strain rats, fixed in 10% formalin and embedded in paraffin.

The studies were conducted on serial, 5-µm paraffin sections and placed on SuperFrost/Plus microscope slides. The archival material was fixed in buffered 10% formalin and embedded in paraffin using the routine procedure. At first, the above-mentioned tissues were evaluated following classical staining with haematoxylin and eosin (H&E). The immunocytochemical and the ISH studies, associated with the immunomax technique, were performed on subsequent sections of at least four selected organs in each child and in selected tissues of the control rats (Table 1).

Immunocytochemical studies

For immunocytochemical studies, mouse monoclonal antibodies, CCH2 [immunoglobulin (Ig)G_1, kappa)], were employed, directed against CMV-early antigen (EA) protein (Dako). The studies followed the classical ABC technique [6], associated with the immunomax technique. In the latter technique, the key reaction involved a 10-min incubation with 10 µl biotinylated tyramine dissolved in 0.1% Tween 20 in the presence of 10 µl 3% H_2O_2 in 1 ml phosphate buffered saline (PBS; room temperature). This was followed by another application of streptavidin complex. The colour reaction was evoked with the horse radish peroxidase substrate, 0.05% diaminobenzidine (DAB) in 0.05 M Tris-HCl buffer, pH 7.6, supplemented with 0.001% H_2O_2. Positive reaction manifested, in at least three sequential sections, as a dark brown or black precipitate in the cell nucleus and cytoplasm of the morphologically altered cells (giant cells) or in the cells which manifested no lesions under the light microscope.

Control reactions were based on substituting specific antibodies with normal sera of the respective species in 0.05 M Tris-HCl, pH 7.6, supplemented with 0.1% bovine serum albumin (BSA) and 15 mM sodium azide (negative control).

In situ hybridisation

The following molecular probes were used for ISH:

1. CMV oligonucleotide probe NCL-CMV, fluorescein isothiocyanate (FITC) conjugated (Novocastra Laboratories) complementary to HCMV early gene transcript
2. CMV ingenius probe (CMV early phase), digoxigenin labelled (R&D Systems) – a mixture of three oligoprobes constructed for the published sequences of Towne and AD169 strains, complementary to regions of preserved early phase CMV DNA sequence

In this portion of the studies the following antibodies were used:

1. Sheep monoclonal anti-digoxygenin antibodies (Fab fragments), labelled with horseradish peroxidase (Boehringer Mannheim)
2. Rabbit antibodies against FITC, labelled with horse radish peroxidase (Dako)

Similar to the immunocytochemical studies, biotinylated tyramine (synthesized in the Department of Histology and Embryology, University of Medical Sciences in Wroclaw) was also applied in the ISH studies.

In the hybridocytochemical studies, the classical ones and those associated with the immunomax technique, sequential sections of the tissue material, which were tested earlier by immunocytochemical techniques, were applied. Several variants of the in situ nucleic acid hybridisation protocols were applied, following recommendations of the R&D systems and our own modifications of mRNA detection [8, 9].

For detection of HCMV DNA, a digoxygenin-labelled oligonucleotide probe was used. This probe was complementary to conservative regions of CMV DNA early phase. Detection of CMV DNA in paraffin sections was performed according to recommendations of Boehringer Mannheim and the appropriate literature data [31]. Optimum conditions of ISH were tested for paraffin sections and for detection of both mRNA and DNA of HCMV. In addition, the optimum parameters of the immunomax technique were established. For this reason, various concentrations of biotinylated tyramine, times of reaction, and antibody and genetic probe levels were examined.

The contents of EA-HCMV antigenic protein and of HCMV nucleic acids (mRNA and DNA) were calculated using the semiquantitative technique of scoring mean number of positive cells with the scale of 0 to ++++, where + denotes up to 10 positive cells, ++ 11 to 30 positive cells, +++ 31 to 100 positive cells and ++++ more than 100 positive cells for 10 fields of the section, examined at a 100× magnification under a light microscope. The calculations were performed on the mean of three consecutive sections of each organ in a given patient and resulted in a mean number (Table 2). The results obtained were compared with the numbers of giant cells noted in H&E-stained sections and examined in the same manner.

PCR technique

Tissue fragments were extracted twice with xylene and then washed twice in ethanol. After drying them, DNA was isolated from the samples. For this purpose, DNA isolation buffer, which was devoted to isolation of the acids from paraffin-embedded tissues, was prepared. The buffer contained 50 mM Tris, pH 8.5; 1 mM ethylene diamine tetraacetic acid (EDTA); 0.5% Tween 20 and 200 µg/ml proteinase K. Buffer (200 µl) was added to each sample. After 3 h incubation at 55°C, the tubes were centrifuged for 30 s, placed in 95°C for 8–10 min and centrifuged again for 30 s. The tubes were stored at –20°C. In order to increase effectiveness of amplification of the DNA isolated from paraffin embedded tissues, the thermic profile of the PCR reaction was modified, and the number of reaction cycles was increased. The thermic profile run was as follows:

1. Preliminary denaturation: 94°C, 7 min
2. Annealing: 42°C, 30 s
3. Elongation: 72°C, 2 min
4. Denaturation: 95°C, 50 s
5. Final annealing: 42°C, 1 min
6. Terminal elongation: 72°C, 7 min

The cycles 2 to 4 were repeated 50 times. Primers for amplification of the HCMV genome fragment were designed on the basis of literature data [27]:

- CMV IE1 upstream 5’ CCA CCC GTG GTG CCA GCT CC 3’
- CMV IE2 downstream 5’ CCC GCT CCT CCT GAG CAC CC 3’

Table 2 Number of giant cells with detectable intracellular inclusion bodies [haematoxylin and eosin (H&E)], cells immunopositive for early antigen (EA) and cells with detectable mRNA or DNA (immunomax technique) of human cytomegalovirus in the selected autopsy material. *0* no positive cells detected, + up to 10 positive cells, ++ 11 to 30 positive cells, +++ 31 to 100 positive cells, ++++ over 100 positive cells, *nt* not tested

Autopsy no.	Liver				Kidney				Pancreas				Lungs				Heart				Brain			
	H&E	EA	RNA	DNA	H&E	EA	RNA	DNA	H&E	EA	RNA	DNA	H&E	EA	RNA	DNA	H&E	EA	RNA	DNA	H&E	EA	RNA	DNA
30/88	++	+++	+	++	nt				++	+++	nt	+++	+	++	0	+	0	nt			nt			
59/90	0	+	0	+	0	0	nt	0	nt				0	0	nt	0	0	nt			+	+	0	+
1/92	+	+	+	+	++++	++++	+++	++++	+++	++++	+++	++++	+	++	+	++	0	0	+	+	0	0	0	0
16/92	+	+	+	+	0	+	+	+	+	+	0	+	nt				0	nt			0	0	nt	0
8/95	+	++	+	+	++++	++++	+++	++++	nt				0	0	nt	nt	0	0	0	+	nt			

The amplified fragment originateed from the region of the immediate early gene exon 4 and was 159 bp in length. The amplification reaction was conducted in a 10-µl reactive mixture, containing 100 ng template, 0.25 U Taq polymerase (Qiagen), 1 µM of each of the primers, buffer containing 10 mM Tris-HCl (pH 8.7 at 20°C), $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 50 mM KCl, 1XQ solution and 200 µl of each dNTP (dATP, dCTP, dGTP, dTTP). The products were separated using agarose (2%) gel electrophoresis.

Results

Selected clinical data on children deceased due to congenital infection with HCMV are shown in Table 3.

Histopathological findings

Eleven types of organs were examined (at least five organs in each patient) (Table 1). In the routine H&E staining, the presence of giant cells with intracellular inclusions was demonstrated in the pancreas, colon, liver, lungs, kidneys and brain (Table 2). Particularly extensive cytopathic lesions were seen in cells of renal parenchyma and in the pancreas. In the kidneys, the affected cells included mostly altered cells of the proximal tubule epithelium (Fig. 1). In the pancreas, the cytopathic lesions were seen both in the exocrine portion and in the Langerhans islets. In the liver, the numbers of giant cells were relatively low. All of the children presented signs of interstitial pneumonia (Table 3). Detection of giant cells in the organ presented particular difficulties. Individual giant cells were found in the interalveolar septa in only two of four examined patients (case 1 and case 3) (Table 2). In a single case, *Pneumocystis carinii* was demonstrated in the lungs (case 3). Four of five examined children manifested traits of myocardial ischaemia, myocardial necrosis and/or myocarditis (cases 1, 2, 3 and 5; Table 3). Upon routine staining of the cardiac sections, the presence of giant cells could not be demonstrated in any of the children. Nonspecific inflammation with infiltrates of mononuclear (mainly lymphocytes) and multinuclear cells were particularly intense in the lungs, kidneys and liver. Haemorrhage to the central nervous system and to the lungs was seen in one child (case 1). Inborn malformations (microcephaly, internal hydrocephalus, cerebral atrophy, microgyria and intracranial calcification) were observed in two of the children (case 4 and case 5; Table 3).

Detection of EA using ABC-immunomax technique

Results obtained using the ABC technique with the simultaneous application of the immunomax technique are presented in Table 2. Application of the biotinylated tyramine increased sensitivity of the reaction by up to three orders of magnitude compared with the classical ABC technique. The technique permitted the visualisation of pathological cells much more clearly by duplicating the numbers of detected sites of CMV replication and permitting detection of EA HCMV in morphologically unaltered cells. The EA HCMV was detected in the studied material mainly in giant cells but also in morphologically intact cells, including blood leucocytes and cells of the vascular endothelium. The most numerous immunopositive, pathologically altered cells could be seen in the renal epithelium of proximal tubules, with the strongest reaction in cell nuclei when compared with the cytoplasm (Fig. 2). On average, twice as many immunopositive cells were seen in the pancreas and the lungs compared with giant cells seen in routinely stained preparations (Fig. 3). In routinely stained preparation, the numbers of immunopositive cells in the liver were higher than the numbers of giant cells in three of the five examined children (Table 2). Intensity of the reaction varied, with the presence of the signal both in the cytoplasm and in cell nuclei of the enlarged cells (Fig. 4). In one of three examined patients (case 2), a focus of individual immunopositive cells in the brain was demonstrated. The numbers of immunopositive cells in the liver, pancreas and kidneys varied between individual patients (Table 2). The immunocytochemical reaction for EA was negative in selected organs of rats.

Detection of HCMV DNA and HCMV mRNA using ISH immunomax technique

Application of the immunomax technique permitted significant (up to a few hundred-fold) augmentation of

Fig. 1 Fragment of renal parenchyma in human cytomegalovirus (HCMV) infection with giant cells in tubular epithelium. Note the presence of nuclear inclusion bodies surrounded by the halo ("owl eyes"). H&E staining ▶

Fig. 2 Immunocytochemical localisation of early antigen (EA) human cytomegalovirus (HCMV) in cytomegalic cells of renal tubular epithelium. Note the stronger reaction in the cell nuclei than in the cytoplasm. ABC-immunomax technique

Fig. 3 Immunocytochemical detection of early antigen (EA) human cytomegalovirus (HCMV) in pancreatic acinar cells. The positive reaction is noted mainly in cell nuclei of cytomegalic cells. ABC-immunomax technique

Fig. 4 Fragment of liver lobule with cytomegalic cells. Note the immunopositivity of cell nuclei and the weak cytoplasmic reaction for the presence of human cytomegalovirus (HCMV) early protein. ABC-immunomax technique

Fig. 5 Hybridocytochemical detection of HCMV DNA in cytomegalic cells of the altered epithelium of renal tubuli. Immunomax technique.

Fig. 6 Hybridocytochemical reaction for human cytomegalovirus (HCMV) DNA in an individual hepatocyte. Immunomax technique

Fig. 7 Hybridocytochemical detection of human cytomegalovirus (HCMV) mRNA in cytomegalic cells of renal tubular epithelium. Note that the weak reaction localised mainly in the cell nuclei. Immunomax technique

Fig. 8 Hybridocytochemical detection of human cytomegalovirus (HCMV) DNA in cell nuclei of myocardium. Immunomax technique. All *bar markers* represent 20 µm

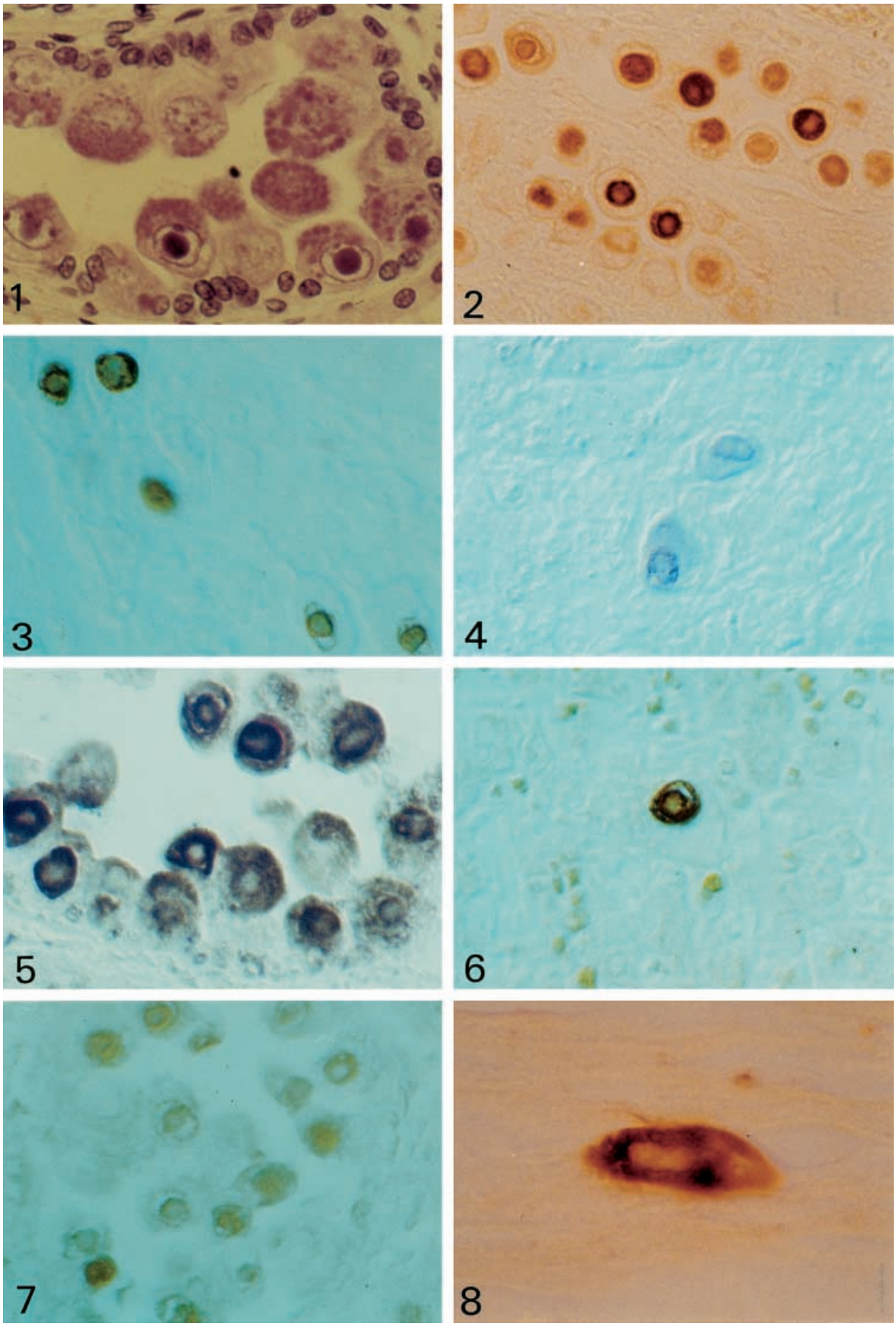

Fig. 1–8 Legend see page 485

Table 3 Synopsis of clinical and morphological data. *CNS* central nervous system, *HCMV* human cytomegalovirus. *Ig* immunoglobulin

Case, autopsy no.	Age at death (days)	Gender	Histopathological findings	Clinical outcome	Serology in the children
1, 30/88	1	f	Hyaline membranes and massive haemorrhage to pulmonary parenchyma. Internal haematocephalus I-IV and diffuse bilateral subpial haemorrhage. Interstitial pneumonia, myocarditis, cytomegalic nephritis, pancreatitis	Intrauterine sepsis in the foetus. Hypertrophic cardiomyopathy, pneumonia, hepatitis, hepato-splenomegaly, bleeding to CNS Cause of death: respiratory/circulatory insufficiency	Anti-CMV: non-tested
2, 59/90	80	f	Interstitial cytomegalic pneumonia, bilateral lobular pneumonia incipient. Cytomegalic sialadenitis, nonspecific interstitial nephritis, nonspecific chronic hepatitis, myocardial ischaemia, hyperplasia of adrenal glands, inanitio	Intrauterine sepsis in the foetus, Dystrophia, microcephaly, petechiae, hepatosplenomegaly, hepatitis, pneumonia, infection of urinary system Cause of death: respiratory/circulatory insufficiency	Anti-CMV:IgM+IgG–
3, 1/92	100	m	Generalised massive cytomegalic infection of lungs, liver, pancreas and kidneys. Bilateral pneumocystic interstitial pneumonia, myocardial ischaemia	Intrauterine sepsis, interstitial pneumonia, hepatosplenomegaly, petechiae, hypotrepsy, secondary anaemia, jaundice, cardiomyopathy Cause of death: Respiratory/circulatory insufficiency, sudden cessation of circulation,	Anti-CMV: IgM+IgG–<?1>
4, 16/92	69	f	Congenital malformation of skull, microcephaly and internal hydrocephalus I–III, external hydrocephalus, simple total cerebral atrophy, diffuse bilateral lobular pneumonia	congenital HCMV syndrome, microcephaly, internal hydrocephalus, pneumonia, obturative bronchitis, spastic paresis, seizures Cause of death: respiratory/circulatory insufficiency	Anti-CMV: IgM–IgG+<?1>
5 8/95	33	f	Microcephaly, internal hydrocephalus I-IV, microgyria, intracranial calcification, encephalomalacia, generalised cytomegalic infection of liver, pancreas, colon and kidneys, interstitial pneumonia, myocardial necrosis	Microcephaly, internal hydrocephalus, sepsis, encephalomalacia, encephalitis, pneumonia bilateralis, myocarditis Cause of death: respiratory/circulatory insufficiency and sudden cessation of circulation	Anti-CMV:IgM–IgG non-tested<?1>

sensitivity of the hybridocytochemical reaction when compared with classical ISH. In the studied sections, the distribution of cells with the presence of HCMV DNA in the renal tubular epithelium overlapped with the detected distribution of the viral early protein (compare Fig. 2 and Fig. 5). In the lungs and the pancreas, the hybridocytochemical localisation of HCMV DNA was consistent with that detected using immunocytochemistry and pertained an almost identical number of cells (Table 2). In the liver, HCMV DNA was localised in cell nuclei of widely spread hepatocytes. The cells were enlarged or of a normal size, frequently without the pale halo around the intranuclear inclusions (Fig. 6). Moreover, HCMV DNA was demonstrated in the endothelial cells of liver sinuses, in leucocytes in their lumen and in blood leucocytes of pulmonary and adrenal vessels.

The combination of hybridocytochemical and the immunomax techniques in the studied autopsy material permitted localisation of viral nucleic acid in the cells in which no EA HCMV was detected. In our material, this pertained to cardiac myocytes in which nuclear localisation of HCMV DNA was noted in morphologically unaltered cells in two patients examined for this parameter (Fig. 7, Table 2).

In the studied material, detection of HCMV mRNA could be achieved only when using immunomax amplification of the signal. The reaction product was less evident than that for DNA of the virus, which was detected in the same tissues. Compared with the detection of DNA or EA, the reaction product was either detected in a lower number of cells, or it was not detected at all (Fig. 8). Hepatic localisation of the transcript of the early HCMV gene corresponded to the localisation documented using the immunocytochemical techniques. No HCMV mRNA could be demonstrated in endothelial cells or in leucocytes in the lumen of vessels, even when the immunomax amplification was employed.

All of the control reactions for detection of HCMV nucleic acids proved negative. Similarly, no DNA or mRNA of the virus was detected in selected tissues of the control rats.

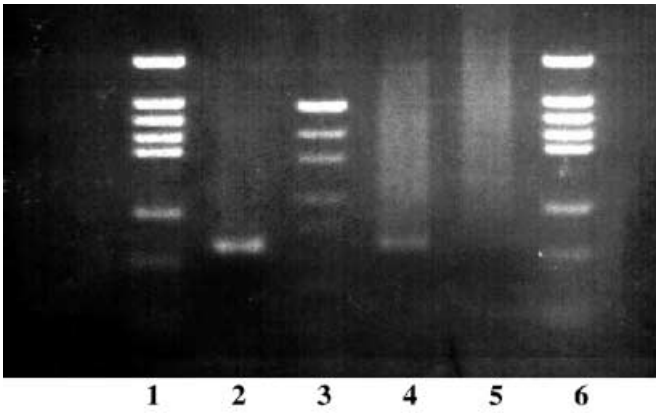

Fig. 9 Electrophoresis in 2% agarose of polymerase chain reaction (PCR)-amplified human cytomegalovirus (HCMV) DNA fragment, 159 bp in length, from the kidney (autopsy no. 8 of 95). *Lanes*: *1* and *6* contain the pBluescript size marker, digested with *Ava*II/*Hin*fI restriction enzymes. *Lane 2* contains the PCR-amplified product of a fragment of exon 4 of HCMV DNA (159 bp) using oligonucleotide primers CMV IE1 and CMV IE2. *Lane 3* contains the pUC19 marker digested with *Msp*I restriction enzyme, and *lanes 4* and *5* are the same as *lane 2* but employ a lower concentration of the deparaffinised material template

PCR technique

The presence of CMV DNA in the studied paraffin-embedded tissues was confirmed also by means of PCR (Fig. 9). One of the amplified fragments (autopsy no. 8 of 95) was inserted into pGEM – T easy vector (Promega) and sequenced using an automatic sequencing apparatus (Beckman). The obtained sequence was compared with sequences of the Entrez database by means of the Internet and employing the BLASTIN program. The analysis confirmed the compatibility of the amplified sequence with the HCMV sequence (exon 4, the immediate-early region, 159 bp in length).

Discussion

Pathogenetic mechanisms of intrauterine infection with HCMV and of its transmission to the foetus, both resulting in serious clinical consequences, remain under investigation [1, 29]. Effectiveness of the complex diagnosis of congenital infections with HCMV and the time at which the diagnosis is established frequently decide on the fate of the affected child. This stresses the need for new techniques, which exhibit high sensitivity and specificity, in order to detect the virus. The immunomax technique, used in association with immunocytochemical techniques and with ISH, represents such a novel approach [14, 23]. High sensitivity and specificity of the technique was documented previously by our group in the detection of mRNA for polypeptide hormones in animal tissues and in cultured cells of human thyroid medullary carcinoma [8, 9].

In this study, the immunocytochemical technique for detection of the early protein of HCMV has confirmed, in most cases, the localisation of giant cells, demonstrated by means of H&E staining. Moreover, the reaction product was much more evident and present in higher numbers of cells than was observed when using the routine staining. Application of the immunomax technique has allowed the identification of additional cells producing the viral protein. These cells were not identified with routine staining. They included hepatocytes, endothelial cells and blood leucocytes. The additional advantage of biotinylated tyramine amplification includes the potential to apply antibodies in thousand-fold lower concentrations, preserving the most evident signal of the reaction. This confirms economic advantages of applying the technique in pathological diagnosis. In this study, the applied mouse monoclonal antibodies, CCH2, detect a non-structural DNA-binding protein (the so-called polymerase processivity subunit; pp52 protein or ppUL44 protein, corresponding to the UL44 reading frame). The protein at the preliminary stage of HCMV infection is present in the cell nucleus and is transplaced at later stages of the infection toward the nuclear envelope and the cell cytoplasm [22, 33]. The prevalent nuclear localisation of the immunocytochemical reaction product in the majority of the sections studied by us points to the current production of the early protein, but its location in giant cells mainly indicates a late stage of the infection, accompanied by the typical cytopathic effect. The results are consistent with the study on dynamics of the inborn and the postnatally acquired HCMV infection. The latter studies have demonstrated prevalence of the early protein in the inborn infection with HCMV relative to the immediate early protein (IEA), which has dominated in the postnatally acquired infection [17]. In this study, the results on the detection of HCMV nucleic acids have confirmed the much higher sensitivity of the immunomax technique relative to classical hybridocytochemistry and the advantages of using ISH compared with immunocytochemistry. In the tissues studied by us, the number and distribution of cells with a positive signal for HCMV DNA have mostly coincided with the results obtained using imunocytochemistry. The immunomax technique has permitted the detection of viral nucleic acids in the cells. Upon routine histological staining, these cells have been free of lesions and contained no early protein. This can be exemplified by detection of viral DNA in cell nuclei of myocardial myocytes. Detection of HCMV DNA in the myocardium, accompanied by myocarditis or cardiomyopathy, corroborates observations of other authors in children with the inborn infection or in adult patients [5, 20]. HCMV DNA was detected in myocardium at all stages of myocarditis and in hypertrophic cardiomyopathy even if in such cases immunocytochemical studies failed to detect the early proteins or late proteins of HCMV [26].

Since the classical ISH has detected no gene transcript in any of the studied organs, detection of HCMV mRNA in the studied tissues has been possible only due to the amplification with biotinylated tyramine. Intensity of the reaction for HCMV mRNA was lower than the re-

action for HCMV DNA, but cellular localisation of mRNA was consistent with the detected HCMV DNA and the early protein within cell nuclei and cytoplasm of the infected cells. In general, HCMV mRNA has been detected in a somewhat lower number of infected cells (including giant cells) and has not been detected in endothelial cells or blood leucocytes. This corroborates observations of other authors who could not detect HCMV mRNA until they used PCR, which pointed to a low level of viral expression in the cells [12, 28].

The application of PCR for detection of DNA in paraffin-embedded tissues has proven successful after modification of the parameters of the technique. The technique has confirmed compatibility of the amplified sequence with the sequence of exon 4 (the immediate early region) of HCMV and, in our studies, has supplemented the complex diagnosis of active HCMV infection on the basis of the archival material.

The nonspecific inflammatory lesions noted in the histopathological examination have been consistent with results of the earlier studies pertaining to morphological alterations in autopsied tissues in cases of the congenital HCMV syndrome [1, 5, 16, 17]. Despite the symptoms of a diffuse intrauterine viral infection in all studied patients, death of vitally important organs (lungs, heart, brain) has not seemed directly connected with either the numbers of cells exhibiting evident cytopathic lesions (giant cells) or the expression of nucleic acids and early protein of HCMV at the tissue level. In our material, the highest numbers of giant cells have been encountered in the kidneys and the pancreas even if no clinically severe symptoms of renal insufficiency or pancreatitis have been described. The primary cause of death (respiratory–circulatory insufficiency) has reflected most probably on the release of inflammatory mediators under the effects of active replication of HCMV, which has resulted in a systemic inflammatory response (sepsis). Highly pronounced dysregulation of central immune mediators in the course of the viraemia has induced immunosuppression. This favoured viral replication in line with natural immaturity of foetal and newborn immune systems. The role of cytokines such as tumour necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 has been proven in sepsis. These lead to a diffuse vasculitis and secondary anoxia in the organs and result in multiple organ dysfunction syndrome [15, 18].

The present study has demonstrated that HCMV viral expression can be optimally detected using the immunomax technique. This has resulted in detection of a higher number of cells containing the viral protein and in localisation of cells, including the morphologically intact ones, which carry nucleic acids of HCMV. In the case of HCMV mRNA, detection of the gene transcript has been possible only when the immunomax technique was applied. Any further tissue-level explanation of cell injury pathomechanism in HCMV infection requires that the participation of cells that mediate virus transmission to distant organs is taken into account. In addition, the tissue expression of cytokines and TNF-α, in particular, should be considered. HCMV is supposed to participate in the control of TNF-α expression and in the resulting inflammatory response [4, 15, 30]. As found in in vitro studies, TNF-α may potentially serve as a stimulator of HCMV major immediate-early promoter in undifferentiated haematopoietic progenitors [24]. Application of the immunomax technique may prove useful also in such studies.

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