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Determination of CMV placentitis

Diagnostic application of the polymerase chain reaction

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Abstract Cytomegalovirus (CMV) infection constitutes an important cause of intrauterine death. In the present study CMV infection of placentas resulting from intrauterine deaths was assessed by immunohistochemistry and by the polymerase chain reaction (PCR). Among 32 cases of chronic villitis examined, 7 were found by PCR to be associated with CMV infection, although light microscopic examination revealed only 3 of them, while 4 had shown positive immunohistochemical staining. In conclusion, CMV may be considered to be a relatively common cause of placentitis, and PCR is a helpful tool in confirming the nature of the disease.

Key words CMV · Placentitis · PCR

Introduction

Although cytomegalovirus (CMV) infection is usually asymptomatic in healthy individuals, certain groups, such as neonates and immunocompromised patients, are at higher risk of developing severe infections [2, 3]. CMV is one of the most frequent causes of intrauterine infection in humans [1], and it is estimated that 150 symptomatic infants are born in Greece every year [7]. Intrauterine transmission occurs transplacentally during maternal viraemia [14]. Infection of the placenta by CMV is a common cause of chronic villitis, but characteristic viral inclusions may be difficult to demonstrate and many cases of CMV placentitis remain undiagnosed [11].

Several approaches have been tried in attempts to overcome the difficulties of diagnosis by conventional methods. These include immunocytochemistry, in situ hybridization and the polymerase chain reaction (PCR) [5, 6, 10, 13]. The results of previous studies are in agreement on the higher sensitivity of these methods than of histology, but remain controversial, as the frequency of CMV infection detected ranges between 9% and 80%. This discrepancy may be explained by epidemiological observations according to which the frequency of infection for particular microbial agents may vary between different populations. Moreover, statistical conclusions cannot be drawn from the quite small numbers of cases examined.

We used the PCR method to detect CMV infection in placental tissues. The present retrospective study aimed to confirm the association of CMV infection with nonspecific chronic villitis and its relation to the intrauterine deaths and to determine the frequency of infection in the Greek population.

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Materials and methods

We examined 856 placentas in a 6-year period from 1990 to 1995. The gestational age of the fetuses ranged from 16 to 40 weeks. Routine macroscopic and microscopic examination was performed according to the method of Kloos and Vogel in all placentas. Briefly, tissue was fixed after autopsy in 10% phosphate-buffered formalin. After fixation, tissue was embedded in paraffin and 5-μm tissue sections were stained with eosin-haematoxylin. On light microscopy we diagnosed chronic villitis if chronic inflammation composed predominantly of lymphocytes was detected in the stroma of several villi. A small number of plasma cells was found in some placentas, including cases of CMV infection. Immunohistochemistry was performed in all cases by the streptavidin–biotin complex method using monoclonal anti-CMV antibody (Dako).

Briefly, thick sections from each block were obtained and DNA was extracted by treatment with Proteinase K (200 μg/ml) in lysis buffer (50 mM Tris, 1 mM EDTA, 0.5% SDS) after removal of paraffin in xylene and precipitation in ethanol [15]. The DNA was purified by extraction with phenol/chloroform and precipitated in ice-cold ethanol. The precipitated DNA was then redissolved in dH_2O and quantitated by spectrophotometry.

For DNA amplification by PCR 1 mg of total extracted DNA was amplified by PCR in a 100-μl or 50-μl total volume reaction. The amplification mixture contained buffer, deoxynucleotide triphosphates, primers for the late antigen *gp64* gene, and Taq polymerase and was overlaid with mineral oil. The amplification took

place in a thermal cycler using conditions specified elsewhere [12]. Briefly the thermal profile used was: denaturation at 95° C for 25 s, primer annealing at 42° C for 15 s, primer extension at 72° C for 60 s. Fifty cycles of amplification were performed. An aliquot of the PCR product was subjected to horizontal gel electrophoresis in a 2% agarose gel, stained with ethidium bromide and photographed under UV light. The length of the amplification product was determined using molecular weight markers [pGEM, ΦX174/*Hae*III (Promegal)]. Infection by CMV was determined by the presence of an amplification fragment 139 bp in length.

The primers chosen amplify conserved regions of the CMV genome and minimize the possibility of a false-negative result, which could be caused by strain variation. Each set of reactions included at least one positive and one negative control. Parallel reactions with K-ras primers producing amplification fragments 157 bp in length were run to ensure that the DNA templated efficiently.

The amplification efficiency may have varied between the different clinical samples. In some cases, primers amplifying a genomic sequence – a 157-bp fragment of the human K-*ras* gene – were included in the same tube to check the quality of the DNA isolated from the paraffin-embedded tissue, to define the efficiency of the amplification reaction, and to exclude false negatives. After optimization of the reaction conditions, the reactions were repeated at least three times to check the results. The primers used do not amplify other members of the Herpesviridae family or other viruses, such as HSV, HZV, Raji (EBV), adenovirus, enterovirus [12].

Several measures were taken to avoid contamination leading to false-positive results: physical separation of rooms for pre-PCR and post-PCR work, use of different pipetting devices, work under laminar flow and performance of several controls.

The primers used for CMV late antigen pg64 [8] were LA1 CCGCAACCTGGTGCCCATGG (upstream primer) and LA2 CGTTTGGGTTGCGCAGCGGG (downstream primer).

Results

Chronic villitis was found in 32 (3.73%) of the 856 placentas examined. All cases of chronic villitis observed adjacent to placental infarction were excluded from our study. Intrauterine growth retardation (IUGR) was found in 95 cases. Chronic villitis was found in 12 cases associated with IUGR (18.18%). This is a significantly higher rate than in the non-IUGR group, in which only 20 of the 790 cases were diagnosed as chronic villitis (2.53%).

Table 1 presents the clinicopathological data of all the cases of chronic villitis, including those with CMV placentitis. In 3 cases cytomegalic inclusion bodies were detected by light microscopic examination in stromal cells of the chorionic villi (Fig. 1). In 2 of them, generalized CMV infection was manifested. Autopsy showed CMV inclusions in various organs, including brain, lung, liver, spleen and kidneys. Four cases were immunohistochemically positive for the presence of CMV (Fig. 2). Positive results were obtained in 7 of the 32 cases studied by PCR (Fig. 3). The results, either positive or negative, were checked by repeated reactions. CMV infection was detected only in cases of severe or even moderate chronic villitis. In particular, 5 out of 7 positive cases were classified as severe and the remaining 2 as cases of moderate villitis. None of the cases with mild chronic villitis was determined as CMV placentitis.

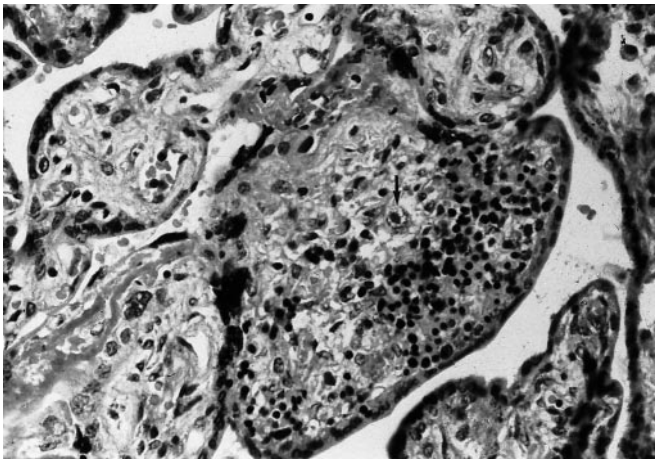

Fig. 1 Cytomegalovirus (CMV) placentitis with the characteristic viral inclusion bodies (*arrow*). Table 1, case 9. H&E, ×225

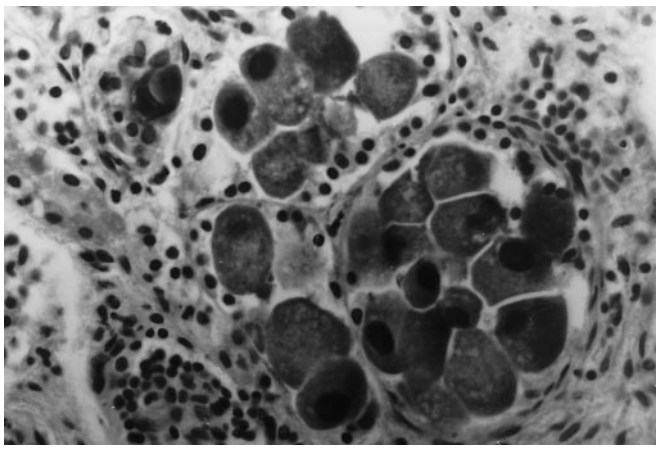

Fig. 2 CMV intracellular inclusion bodies in epithelial cells of renal tubules. Table 1, case 9. PAP, ×400

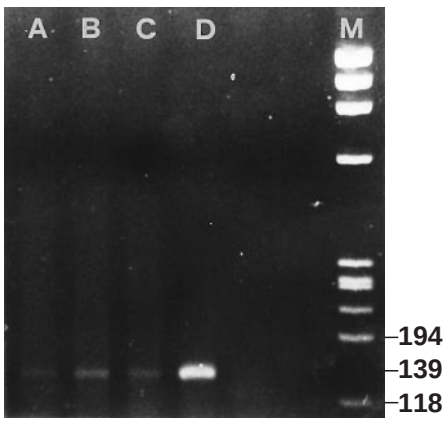

Fig. 3 Amplification of CMV DNA sequences by PCR. The PCR product is electrophoresed in a 2% agarose gel and visualized by ethidium bromide staining. The primers used amplify a region 139 bp in length. *Lanes A, B, C, D* positive samples, *M* molecular weight marker. ΦX174/*Hae*III

Table 1 Detection of placental cytomegalovirus (*CMV*) infection by histology and polymerase chain reaction (*PCR*) in patients with intrauterine infection

Case no.	Maternal age (years)	Gestational age/weeks	Birth	Birth weight	Placental weight	Histopathology of the placenta	CMV PCR
1	27, primigravida	16	Spontaneous abortion	A twin 150 g B twin 75 g	220 g	Moderate chronic villitis-placental haematoma	–
2	24, primigravida	32	Stillborn	1300 g	300 g	Mild chronic villitis	–
3	28, multigravida	24	Spontaneous abortion	385 g	165 g	Moderate chronic villitis	–
4	26, primigravida	36	Stillborn – omphalocele	2100 g	370 g	Mild chronic villitis	–
5	37, primigravida	40	Live-short arm of 5th chromosome	3250 g	400 g	Mild chronic villitis – subchorionic thrombosis	–
6	18, primigravida	39	Stillborn	3400 g	530 g	Severe villitis	–
7	36, primigravida	29	Termination of pregrancy – 47xx	1000 g	285 g	Moderate chronic villitis – accelerated maturation	–
8	23, primigravida	30	Stillborn – placental abruption	1300 g	260 g	Moderate chronic placentitis – accelerated maturation	–
9	26, primigravida	32	Live (7 h)	1650 g	280 g	Severe CMV chronic villitis-viral inclusions CMV infection of many organs	+
10	32, multigravida	39	Stillborn – Rh incompatibility	3500 g	1050 g	Severe CMV chronic villitis – viral inclusions	+
11	29, primigravida	31	Live (25 min)	1400 g	245 g	Moderate chronic villitis – accelerated maturation	+
12	29, primigravida	34	Stillborn – placental abruption; hypospadias – absence of one umbilical artery	1900 g	225 g	Mild chronic villitis – accelerated maturation – placental haematoma	–
13	29, primigravida	38	Stillborn	3350 g	590 g	Severe chronic villitis	–
14	22, primigravida	31	Stillborn	1300 g	250 g	Severe chronic villitis	–
15	30, multigravida	24	Stillborn	150 g	55 g	Moderate chronic villitis – accelerated maturation	–
16	32, multigravida	36	Stillborn – diabetes melitus of pregnancy	3800 g	530 g	Mild chronic villitis	–
17	27, multigravida	36	Stillborn	1800 g	245 g	Severe chronic villitis – accelerated maturation – intervillous thrombosis	–
18	40, multigravida	22	Termination of pregnancy – trisomy 21	120 g	75 g	Moderate chronic villitis	–
19	23, primigravida	25	Stillborn	440 g	270 g	Severe chronic villitis	–
20	24, primigravida	39	Stillborn – long cord – umbilical vessels, thrombosis	3150 g	410 g	Mild chronic villitis	–
21	27, primigravida	29	Termination pregnancy – multiple congenital anomalies	1400 g	235 g	Mild chronic villitis	–
22	30, multigravida	32	Live (31 h) – premature delivery	1050 g	180 g	Severe chronic villitis	+
23	25, primigravida	22	Stillborn	450 g	130 g	Moderate chronic villitis – accelerated maturation	–
24	31, multigravida	29	Stillborn – essential hypertension	650 g	110 g	Moderate chronic villitis	–
25	24, primigravida	21	Stillborn	275 g	50 g	Mild chronic villitis	–
26	30, multigravida	24	Stillborn	60 g	180 g	Mild chronic villitis – accelerated maturation	–
27	38, multigravida	32	Stillborn	1800 g	225 g	Moderate chronic villitis	+
28	29, multigravida	19	Stillborn	150 g	75 g	Severe CMV chronic villitis; viral inclusions – CMV infection of many organs	+
29	32, multigravida	36	Stillborn	2650 g	340 g	Moderate chronic villitis	–
30	22, primigravida	24	Stillborn	320 g	135 g	Moderate chronic villitis – accelerated maturation	–
31	26, primigravida	32	Stillborn	1100 g	200 g	Severe chronic villitis	+
32	29, multigravida	24	Stillborn	275 g	85 g	Moderate chronic villitis – accelerated maturation	–

Discussion

The frequency of CMV placentitis has not been clearly determined. It has been estimated, by conventional methods, that 1–2% of cases of chronic villitis are related to CMV infection [9]. Viral inclusion particles diagnostic for CMV are reported in about one third of cases with CMV villitis. Indeed, the detection of the typical of CMV cytoplasmic inclusions, which are often not apparent on histological examination, is considered to be a rare finding. In a previous study using PCR, CMV DNA was detected in 4 of the 44 placentas examined [6]. In this study placental tissues from 32 cases of intrauterine deaths attributed to chronic villitis were examined by immunohistochemistry and PCR for the detection of CMV DNA sequences. We found a higher percentage of CMV placentitis than previously reported (about 21%). We speculate that this difference may reflect an elevated rate of infection in the Greek population. Moreover, CMV placentitis in 5 out of 7 cases was related to intrauterine growth retardation. On the basis of these results, the present study identifies a close association between IUGR and chronic villitis, which may reflect an important but hitherto underestimated role of CMV in the pathogenesis of these disorders.

We would like to underline that with conventional histological methods we were able to identify CMV placentitis in 3 cases among the 32 studied. Immunohistochemistry appeared to improve the sensitivity of CMV detection slightly, revealing 4 positive cases including those determined microscopically. Finally, by PCR we were able to detect 4 new cases of CMV placentitis as well as verifying the 3 already determined.

We conclude that according to the findings of the present study there is a strong indication that an important proportion of the cases diagnosed as chronic villitis of uncertain origin are in fact due to CMV infection.

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