FETAL BRAIN DAMAGE IN HUMAN FETUSES WITH CONGENITAL CYTOMEGALOVIRUS INFECTION: HISTOLOGICAL FEATURES AND VIRAL TROPISM

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Abstract

Human cytomegalovirus (HCMV) causes congenital neurological lifelong disabilities. To date, the neuropathogenesis of brain injury related to congenital HCMV (cCMV) infection is poorly understood. This study evaluates the characteristics and pathogenetic mechanisms of encephalic damage in cCMV infection.

Ten HCMV-infected human fetuses at 21 weeks of gestation were examined. Specifically, tissues from different brain areas were analyzed by: *i*) immunohistochemistry (IHC) to detect HCMV-infected cell distribution, *ii*) hematoxylin-eosin staining to evaluate histological damage and *iii*) real-time PCR to quantify tissue viral load (HCMV-DNA). The differentiation stage of HCMV-infected neural/neuronal cells was assessed by double IHC to detect simultaneously HCMV-antigens and neural/neuronal markers: nestin (expressed in early differentiation stage), doublecortin (DCX, identifying neural cells with determined lineage) and neuronal nuclei (NeuN, identifying mature neurons).

HCMV-positive cells and viral DNA were found in the brain of 8/10 (80%) fetuses. For these cases, brain damage was classified as mild (n=4, 50%), moderate (n=3, 37.5%) and severe (n=1, 12.5%) based on presence and frequency of pathological findings (necrosis, microglial nodules, microglial activation, astrocytosis and vascular changes). The highest median HCMV-DNA level was found in the hippocampus (212 copies/5ng of humanDNA [hDNA], range: 10-7,505) as well as the highest mean HCMV-infected cell value (2.9 cells, range: 0-23), followed by that detected in subventricular zone (1.8 cells, range: 0-19). This suggested a preferential viral tropism for neural stem/progenitor cells (NSPCs), residing in these regions, confirmed by the expression of DCX and nestin in 94% and 63.3% of HCMV-positive cells, respectively. NeuN was not found among HCMV-positive cells and was nearly absent in the brain with severe damage, suggesting HCMV does not infect mature neurons and NSPCs do not differentiate into neurons. This could lead to known structural and functional brain defects from cCMV infection.

Introduction

Human cytomegalovirus (HCMV) is the most frequent congenital virus infection worldwide, with an incidence of approximately 0.64% live births (Mack et al. 2017; Rawlinson et al. 2017). Although congenital HCMV (cCMV) infection may involve many fetal organs, the most significant injuries are those related to lesions in the nervous system that are irreversible and persist for life (Fowler et al. 2003; Gabrielli et al. 2009; Kenneson and Cannon 2007). Permanent neurological damage occurs in up to 60-70% of newborns symptomatic at birth (10% of congenitally infected cases) and in the 10-15% of those asymptomatic (Cheeran et al. 2009; Gaytant et al. 2002). Neurological outcomes may include sensorineural hearing loss, visual impairments, microcephaly, encephalic palsy, mental retardation, epilepsy and seizure (Boppana et al. 2013; Cheeran et al. 2009; Gaytant et al. 2002; Schleiss 2008; Zhang and Fang 2019). Clinical conditions involving the central nervous system (CNS) are mainly the result of intracranial calcifications, periventricular cysts, white matter abnormalities, intraventricular adhesions, ventriculomegaly, altered sulcation/gyrus patterns and cerebellar abnormalities (Fink et al. 2010; Kwak et

al. 2018; Malinger et al. 2003). To date, the pathogenesis of injury in the developing fetal brain related to cCMV infection is poorly understood (Gaytan et al. 2002; Schleiss 2008). Results obtained by previous studies, involving HCMV infected human fetuses, showed that it could be due to a multifactorial process including the effects in the brain of direct viral replication, inflammatory and immune response to infection as well as the encephalic hypoxic ischemia secondary to HCMV-related placental damage (Gabielli et al. 2009, 2012). The development of the CNS is a complex process that includes proliferation and differentiation of neural stem/progenitor cell (NSPCs), neuronal migration, neurite outgrowth, and synapse formation (Zhang and Fang 2019). Viral replication could have a key role in neuropathogenesis of HCMV-induced brain injury interfering with these developmental stages. Studies, mainly performed on cultured murine and human brain cells, indicate that CNS infected cells have altered intercellular communication, deficient response to neurotransmitters and cell cycle changes (Cheeran et al. 2009; Ho and van den Pol 2007; Lokensgard et al. 1999; Schleiss 2008). In addition, Tsutsui Y et al. showed a preferential tropism of murine cytomegalovirus (MCMV) for the immature and proliferating neural cells (Tsutsui et al. 2002, 2005). These findings could also implicate a specific brain localization of viral infection, as well as those described for other herpes viruses, such as the herpes simplex virus-1 (HSV-1), for which temporal lobe abnormalities are commonly reported in patients with herpes simplex encephalitis (Levitz 1998; Mook-Kanamori et al. 2009). All these findings were poorly investigated in human cases of cCMV infection.

The present study aimed to implement the knowledge on histological characteristics of HCMV-induced encephalic injury and the mechanism by which the direct viral replication lead to neurological disorders. To our knowledge, this is the first study that analyzed human brain tissues from fetuses with cCMV infection to define in different encephalic areas the distribution of; *i*) histological features, *ii*) HCMV-infected cells and *iii*) tissue viral load. In addition, the differentiation stage of infected neural/neuronal cells was evaluated to clarify how HCMV infection could interfere with fetal brain development.

Material And Methods

Study design and patients

All congenitally HCMV infected fetuses submitted for histological examination at Pathology Unit, IRCCS S. Orsola Polyclinic from 2010 to 2019 were revised. We selected all fetuses with the same features: mothers with primary HCMV infection occurring before twelve weeks of gestation (WG), high viral load in amniotic fluid, elective termination of pregnancy at 21 WG, all brain regions with available paraffin-embedded blocks. Ten fetuses met these inclusion criteria. In table 1, maternal clinical information at the time of primary HCMV infection are summarized.

Table 1 Maternal clinical information at the time of serological diagnosis of primary HCMV infection.

Case No.	Age (years)	Serological diagnosis of primary CMV infection	WG at diagnosis	Symptoms	Laboratory findings
9	33	IgG seroconversion	11	-	Lymphocytosis
1	29	IgM antibodies and low IgG avidity	9	-	-
2	25	IgM antibodies and low IgG avidity	12	Influenza-like syndrome	Lymphocytosis and abnormal liver enzyme levels
7	35	IgM antibodies and low IgG avidity	10	Fever	Abnormal liver enzyme levels
3	37	IgM antibodies and low IgG avidity	8	-	-
4	19	IgM antibodies and low IgG avidity	10	Asthenia	Abnormal liver enzyme levels
5	22	IgG seroconversion	11	-	-
8	33	IgM antibodies and low IgG avidity	11	Fever	-
6	32	IgG seroconversion	9	-	-
10	39	IgM antibodies and low IgG avidity	10	-	-

WG: weeks of gestation; -: none

As control cases, seven fetuses at 21-weeks of gestation from HCMV seronegative women were also studied. Three were from a termination of pregnancy due to fetal cardiac malformation and the other four were spontaneous miscarriages caused by cervical incompetence. A total of 17 fetuses were analyzed and for each one, 10 brain regions were selected. In particular, tissues from the cortical areas (frontal, temporal, occipital, parietal) and underlying white matter, subventricular zone, thalamus, hypothalamus, hippocampus, basal ganglia and cerebellum were analyzed. All encephalic areas were examined by *i*) immunohistochemical staining to detect the presence and distribution of brain cells expressing HCMV-antigens; *ii*) hematoxylin-eosin staining to evaluate the histological damage in HCMV positive brain; and *iii*) real-time PCR to detect and quantify the tissue viral load. Double immunohistochemical staining for simultaneous detection of HCMV-antigens with markers identifying the differentiation stage of infected neural/neuronal cells, was performed. All analyses were carried out on sections obtained from paraffin-embedded blocks of tissue previously fixed in 4% formaldehyde.

Regarding histological analysis, a positive and a negative standardized sample were included for each immunohistochemical session in order to confirm the required sensibility and specificity of the antibodies used. In particular, tissues that did not express the target protein were used to confirm antibody specificity to the protein of interest.

All the histological slides were analyzed by two independent observers blind to virological results. Discordant results were resolved by a third observer.

The study was approved by the Ethical Committee of St. Orsola Polyclinic, University Hospital, Bologna, Italy (approval numbers: 14/2017/U/tess and 8/2010/O/Sper). The fetal tissues were analyzed after receiving informed consent from the parents prior to inclusion, according to the policies of the Ethical Committee and to the regulation of the Italian Ministry of Health.

Histological brain damage evaluation in HCMV-positive brain

One section of 3 microns/brain area was analyzed by hematoxylin-eosin staining, performed by standard method. The presence of necrosis, microglial nodules, microglial activation, astrogliosis, and vascular changes was evaluated. Microglial nodules consisted in clusters of activated microglial cells involved in immune response to HCMV-infected cells. Microglial activation was proved by microglial body cells with various morphologies: small rod-shape, amoeboid-like and spherical cells. Astrogliosis was characterized by cell body expansion (hypertrophy) and cells with clear nuclei (named Alzheimer type II cells). Vascular changes consisted in increased number of vessels with hypertrophy of endothelial cells (that showed plump, cuboidal form and protruded into the lumen).

Considering the severity and the frequency of pathological findings, the brain damage was classified as *i)* severe, in presence of tissue necrosis and multiple microglial nodules (≥ 3 /brain region); *ii)* moderate, in presence of multiple microglial nodules without necrosis and *iii)* mild, in presence of occasional microglial nodules (<3 /brain region) without necrosis. In all brain regions, 5 fields at 20 high-power field (HPF), were evaluated (Gabielli et al. 2012).

Detection and quantification of brain tissue viral load

Each selected brain area was anatomically identified, dissected from 2 sections of 8 microns and placed in a 1.5 mL tube for the deparaffinization procedure using 160 μ L of the Deparaffinization Solution (product code: 19093; Qiagen, Germany). DNA extraction was performed by the QIAasympy®SP instrument with the QIAasympy DSP DNA Mini Kit (product code: 937236; Qiagen, Germany). Purified DNA was eluted in 50 μ L and the contained human DNA (hDNA) was quantified using a real-time PCR assay, Quantifiler® Human DNA Quantification Kit (product code: 4343895; Life Technologies, USA). Five nanograms of hDNA were processed for HCMV-DNA quantification, carried out using a real-time PCR assay, CMV ELITE MGB™ kit (product code: RTK015PLD; ELITech Group, Italy). The tissue viral load was reported as number of copies/5ng of hDNA. The lower limit of quantification (LLoQ) was equal to 13 copies/5 ng of hDNA. Positive results below the LLoQ were censored with a value equal to 10 copies/5ng hDNA (Gabielli et al. 2017).

Detection and distribution of HCMV- infected brain cells

One section of 3 microns/brain area was analyzed by immunohistochemical staining, performed using anti-CMV [8B1.2, 1G5.2, 2D4.2] mouse monoclonal primary antibody (product code: 213M-26; Cell Marque, USA), that reacts with immediate early, delayed early, and late HCMV-antigen preparation. The HCMV-infected cells distribution in the brain was assessed analyzing 5 fields/brain regions at 10 HPF. The results were expressed as mean of HCMV-positive cells/encephalic area (Gabielli et al. 2019 and 2020).

Differentiation stage of the HCMV-infected neural/neuronal cells

Serial sections of 3 microns/brain area were analyzed by double immunohistochemical staining for simultaneously detection of HCMV-antigens with markers of neural/neuronal cells. In particular, nestin was used as a marker to recognize cells in the early stage of differentiation, DCX was used to identify neural cells with lineage determined and NeuN for mature neurons detection. The first immunohistochemical reaction was the anti-HCMV staining (pre-diluted mouse monoclonal anti-CMV, clone 8B1.2 1G5.2&2D4.2, Cell Marque USA), visualized using the OptiView DAB detection kit (brown color), followed by the second antibody represented by monoclonal anti-nestin clone 10c2 (product code: SC23927; Santa Cruz Biotech, USA) diluted 1:400, monoclonal anti-DCX clone 2G5 (product code: MABN707; Millipore USA) diluted 1:600 or polyclonal anti-NeuN(product code: ab128886; Abcam Ltd, UK) diluted 1:300. The second immunoreaction was visualized using the Alkaline phosphatase UltraView detection Kit (product code: 760-501; red color). The double-labelled cells were assessed by analyzing 5 fields/brain regions at 10 HPF (Chen et al. 2010).

Statistical analysis

The Mann-Whitney test was used to compare the HCMV-DNA levels detected in severe and moderate brain damage *versus* mild brain damage cases. The differences in HCMV DNA levels and HCMV-infected cells of each brain regions were analyzed with Kruskal-Wallis test and One-Way ANOVA test, respectively. The mean values of HCMV-infected cells found in severe and moderate brain damage were compared with those detected in mild brain damage cases by independentt-test.

Results

HCMV-positive cells and HCMV-DNA were found in the brain of 8/10 (80%) fetuses. These results together with the data obtained by invasive (detection of HCMV-DNA in amniotic fluid) and non-invasive (ultrasound examination) prenatal diagnosis, performed at 21 WG, are reported in Table 2. For the subsequent examinations, the attention was focused on the cases with an encephalic involvement of HCMV infection. In the remaining 2 fetuses, no HCMV-positive cells and no viral DNA in the brain were observed. In control cases, no HCMV-positive cells and HCMV-DNA were detected in the brain.

Detection of pathological findings and evaluation of brain injury

Microglial activation, diffuse astrocytosis and vascular changes were detected in all 8 cases (Fig. 1), without differences in the brain region distribution. These evidences showed a diffuse encephalic inflammatory reaction. However, as previously reported, considering the severity and the frequency of some histological encephalic abnormalities, such as necrosis and microglial nodules (Fig. 1), a different degree of brain injury was identified. The brain damage was classified as severe, moderate and mild in 1 (12.5%), 3 (37.5%) and 4 (50%) cases, respectively (Table 2). In particular, the brain with severe damage showed cortical necrosis, mainly detected in layer III, diffuse macrophage infiltration of the leptomeninges, polymicrogyria and periventricular leukomalacia. For the same case the cerebellum showed an extensive parenchymal hemorrhage with scarce residual tissue identified. In all cases with severe/moderate encephalic damage, viral load higher than $>10^6$ copies/ml in amniotic fluid were found, and the 50% of these cases showed pathological findings involving the brain via ultrasound (Table 2).

Table 2 Results of prenatal diagnosis performed at 21 WG in correlation with histological and virological brain findings.

Case No.	Prenatal diagnosis at 21 WG			Autopsy		
	HCMV-DNA in AF (copies/mL)	Ultrasound findings		Brain tissue		
		Brain	Other organs	HCMV-IHC	HCMV-DNA	Damage
9	>1,250,000	Periventricular hyperechogenicity	Hyperechogenic bowel	Positive	Positive	Severe
1	>1,250,000	Periventricular hyperechogenicity Corpus callosum hypoplasia	Hyperechogenic bowel	Positive	Positive	Moderate
2	>1,250,000	Normal	Normal	Positive	Positive	Moderate
7	>1,250,000	Normal	Normal	Positive	Positive	Moderate
3	182,000	Normal	Normal	Positive	Positive	Mild
4	>1,250,000	Normal	Normal	Positive	Positive	Mild
5	948,473	Normal	Normal	Positive	Positive	Mild
8	489,000	Normal	Normal	Positive	Positive	Mild
6	323,300	Normal	Normal	Negative	Negative	/
10	270,000	Normal	Normal	Negative	Negative	/

Quantification and distribution of tissue viral load into the brain

The median HCMV-DNA levels detected in the brain of cases with severe and moderate damage were higher than those with mild encephalic injury: 92 copies/5ng hDNA (range: 20-380 copies/5ng hDNA) and 87 copies/5ng hDNA (range: 100-7,505 copies/5ng hDNA) *versus* 10 copies (range: 10-248 copies/5ng hDNA), respectively ($Z=4.827$, $p<0.0001$). The median level of tissue viral load in each brain area was different ($H=13.795$ $p=0.08$) and the highest viral load, equal to 212 copies/5ng hDNA (range 10-7,505 copies/5ng hDNA), was detected in the hippocampus (Fig. 2).

Stratifying the median HCMV-DNA levels in correlation with the degree of brain damage, the highest values were also identified in the hippocampus. Values equal to 380 copies/5ng hDNA, 910 copies/5ng hDNA (range 105-7,505 copies/5ng hDNA) and 93 copies/5ng hDNA (range 10-248 copies/5ng hDNA) were detected in the cases with severe, moderate and mild brain damage, respectively (data not shown). Tissue viral load in severe brain damage was referred to the only case available. In addition, in this case, the HCMV-DNA values found in the cerebellum may be biased because it was severely hemorrhagic.

Quantification and distribution of HCMV-positive cells into the brain

HCMV-positive cells, including neural/neuronal, glial and endothelial cells were present as both scattered and grouped together in clusters. The HCMV-infected cells were also detected along the migration pathway probably affecting radial glia (Fig. 3).

The mean values of HCMV-positive cells, counted in each field over the different brain regions, in the cases with severe and moderate encephalic injury were higher than those found in the fetuses with mild brain damage: 2.49 cells (range: 0-9 cells) and 1.57 cells (range: 0-23 cells) *versus* 0.22 cells (range: 0-11 cells), respectively ($t=8.443$ $p<0.00001$). The mean value of HCMV-infected cells in each brain area was different ($F=2.311$, $p=0.02$) and the highest value was found in the hippocampus with 2.9 cells (range: 0-23 cells) followed by subventricular zone (including the periventricular areas in each lobe and the ganglionic eminence), with 1.8 cells (range: 0-19 cells), Fig. 4. In addition, the mean values of HCMV-positive cells detected in the germinal matrix were higher than those observed in the cortical area and in white matter (3.5 cells in germinal matrix [range 0-19 cells], 0.8 cells in white matter [range: 0-7 cells] and 0.5 cells in cortex [range: 0-4 cells]). In particular, this was observed in each brain lobe with the highest value in the germinal matrix of temporal lobe (data not shown).

Differentiation stage of the HCMV-infected neural/neuronal cells

The differentiation stage of HCMV-infected neural/neuronal cells was evaluated in 6 out of the 8 fetuses with HCMV-positive cells in the brain (cases in Table 2: 1,2,5,7,8,9).

The double immunohistochemical staining for simultaneous detection of HCMV-antigens and neural/neuronal cell markers showed that the 63.3% of HCMV-infected cells expressed nestin (441 nestin-

HCMV-positive cells/696 HCMV-positive cells) and 94% expressed DCX (601 DCX-HCMV-positive cells/639 HCMV-positive cells) (Fig. 5). In all cases, nestin used as marker of neural cells in early stage of differentiation, was mainly expressed in the subventricular zone and hippocampus, while in the white matter and cortex was weakly detected. In thalamus, hypothalamus and basal ganglia, this marker was not expressed. However, the positive cells for both HCMV-antigens and nestin were found in all brain regions, including the area where nestin was not usually expressed by non-infected neural cells (Fig. 5b). Considering the results obtained using DCX as marker of neuronal progenitor cells with determined lineage, almost all HCMV-positive cells (94%) expressed this marker. In the brain of the fetuses studied, DCX was diffusely expressed in each region, furthermore, positive-cells for both HCMV-antigens and DCX were detected in all encephalic areas. Finally, no HCMV-positive cells expressing NeuN were found (Fig. 5d). This marker, used to identify mature neurons, were mainly detected in the cortex, it was less expressed in white matter and was not found in the subventricular zone and hippocampus of studied cases.

When comparing the same brain region for fetuses with different degree of brain damage, in the case with severe encephalic injury, the expression of NeuN resulted almost absent compared to fetuses with moderate or mild brain damage (Fig. 6).

In the brain of control cases, no pathological findings were found. The nestin was mainly expressed in the hippocampus and the subventricular zone, weakly expressed in the white matter and cortical area and was absent in the thalamus, hypothalamus and basal ganglia (Fig. 7). DCX was diffusely found in all brain regions. NeuN was mainly detected in the cortical area, less expressed in white matter, thalamus and hypothalamus and almost absent in the subventricular zone and in the hippocampus.

Discussion

Although cCMV infection is the leading cause of significant damage in brain development, little is known about the neuropathogenic mechanisms by which this viral infection leads to human fetal cerebral injury (Cheeran et al. 2009). The results obtained by this study show encephalic viral infection in 80% (8/10) of examined fetuses, proved by the detection of HCMV-positive cells and HCMV-DNA in the brain. This suggests that not all HCMV-infected fetuses show cerebral involvement of viral replication, as reported in previous studies (Gabrielli et al. 2009, 2012, 2013).

In this study, we focused on the 8 cases with HCMV-positive cells and HCMV-DNA detected in the brain. The presence of diffused microglial activation, astrogliosis, and vascular changes observed in all brain regions, suggest a disseminate inflammatory response (Cotran and Pober 1989; Fernández-Arjona et al. 2017; Robillard et al. 2016). In addition, microglial nodules in the same areas analyzed were also found. These lesions are a neuropathological characteristic of viral CNS infections, especially encephalitis caused by HSV-1 and HCMV. These were the result of microglial cells involved in phagocytosis of infected brain cells killed by CD8⁺ cytotoxic T-lymphocytes (Chen et al. 2019; Langford et al. 2002; Ludlow et al. 2016; Nebuloni et al. 2000; Rock et al. 2004; Tröscher et al. 2019). In a previous study, characterizing the

encephalic inflammatory infiltrate in human fetuses with cCMV infection, the presence of microglial nodules containing HCMV-positive cells surrounded by activated CD8+ T-cells was demonstrated (Gabrielli et al. 2012). Except for necrosis, all pathological findings were not localized in specific brain areas, but uniformly distributed, probably due to diffuse inflammatory reaction and immune responses into the encephalon. The necrosis, found in the brain with severe damage and mainly localized in cortical layer III, could be due not only to direct viral replication, but also to hypoxia caused by HCMV-induced placental injury (Gabrielli et al. 2012). In the studied cases, the different degree of encephalic injury was classified as severe (12.5%), moderate (37.5%) and mild (50%). In cases with severe/moderate brain damage, prenatal diagnosis performed at 21 WG, showed a high viral load in amniotic fluid (values more than 10^6 copies/mL) and in 50% of cases, brain pathological findings detected by ultrasound examination. These findings are in agreement with published data reporting that the presence of high viral loads in amniotic fluid, sampled at the appropriate time, combined with ultrasound evidence of abnormalities in the CNS, are highly suggestive of fetal cCMV infection with poor outcome (Guerra et al. 2008; Lazzarotto et al. 2014; Pass and Arav-Boger 2018). In addition, in cases with severe/moderate encephalic damage, higher median HCMV-DNA values and mean values of HCMV-positive cells were found compared to those detected in cases with mild brain injury. This confirms a correlation between the degree of damage and the level of viral replication, as already reported (Gabrielli et al. 2012). Among brain areas, the highest viral load was identified in the hippocampus (median value 212 copies/5ng hDNA, range: 10-7,505 copies/5ng hDNA) as well as the highest HCMV-infected cells (mean value 2.9 cells, range: 0-23 cells), followed by subventricular zone (1.8 cells, range: 0-19). In addition, considering each cerebral lobe, the highest mean value of HCMV-positive cells was detected in the germinal matrix (part of subventricular zone) with respect to cortex and white matter. In acute encephalitis due to other neurotropic viruses, such as HSV-1, involvement of select brain regions are reported, including the limbic structure where the hippocampus is a susceptible target of infection (Damasio and Van Hoesen 1985; Harris and Harris 2018; Kopp et al. 2009; Lathe and Haas 2017; Potel et al. 2003). For HCMV infection, the main viral localization in the hippocampus (dentate gyrus) and cerebral subventricular zone was proven in few studies involving human subjects, such as adult immunocompromised patients and premature infants with lethal cCMV infection (Perlman and Argyle 1992; Yoon et al. 2017). Yin X et al., observed that during human fetal life, the highest proportion of NSPCs reside in the hippocampus, followed by the amount of these cells detected in subventricular zone (Yin et al. 2013). Considering these findings, though identified in a low number of fetuses studied, the highest viral load detected in hippocampus and the higher HCMV-infected cell values found in both hippocampus and in subventricular zone could probably suggest a preferential HCMV replication in the cerebral areas where NSPCs reside. Murine models of MCMV infection, showed that the immature neural cells were the main target of viral replication (Mutnal et al. 2011; Tsutsui et al. 2002). Moreover, studies performed on human NSPCs cultures, demonstrated the greatest susceptibility to HCMV of these cells, in which the effects of viral replication depend on their differentiation state (Hernández-Salinas et al. 2015; Li et al. 2009; Odeberg et al. 2006). In order to explore these findings, the differentiation stage of HCMV-infected neural/neuronal cells was investigated. In our small population, the main neural/neuronal marker expressed by HCMV-positive cells was DCX (94%), the antigen identifying the neural progenitor cells with a determined lineage (as neuroblasts). Nestin, that is the

marker detecting the neural cells in an early stage of differentiation, was found in the 63.3% of HCMV-positive cells, while no HCMV-infected cell resulted positive for NeuN, the antigen expressed by mature neurons. These findings showed that the HCMV-infected cells were mainly NSPCs, confirming the preferential tropism of HCMV for immature and proliferating cells of neuronal lineage. On the other hand, the total absence of HCMV-infected cells expressing NeuN, could be explained by previous human cell culture studies, which showed that mature neurons are refractory to HCMV replication (Cheeran et al. 2009). The HCMV-positive cells that did not express neural/neuronal markers, were probably cells different from those of neuronal lineage. This is in agreement with literature, which reports the ability of HCMV to infect different type of cells in the brain (Gabrielli et al. 2012). Different hypothesis could explain the high proportion of HCMV-positive cells expressing DCX. The first is that it could reflect the diffuse expression of this marker in all brain regions at 21 WG, as observed in both control cases and published data. Secondly, it could also speculate that the massive DCX expression among the HCMV-positive cells may be due to a preferential viral replication in neuronal precursor cells rather than in neural stem cells. Finally, the high HCMV-positive cells expressing DCX could be due to an altered ability of infected neuronal progenitor cells to differentiate into mature neurons. The total absence of HCMV-positive cells expressing NeuN, in the cases studied, may be aligned with the latter hypothesis. Studies performed on human cell cultures demonstrated that the HCMV infection in NSPCs led to either the inhibition or delay of differentiation in these cells (Cheeran et al. 2009; Hernández-Salinas et al. 2015; Rahimi-Balaei et al. 2018). The detection of HCMV-infected cells expressing nestin in all brain regions, also in areas where this marker was not present at 21 WG, may suggest that HCMV infection could interfere with neuronal differentiation. In control cases, NeuN is mostly expressed in the cortex and weakly in the white matter. On the other hand, NeuN cortical expression in the only fetus with severe brain damage was almost absent, but preserved in fetuses with moderate/mild injury. NeuN immunohistochemistry can individuate signs of neuronal suffering; in fact immunoreactivity has been reported to decrease under several pathologic conditions such as fetal hypoxia and maternal smoking (Lavezzi et al. 2013).

The presence of HCMV-infected cells in the pathways of neuronal migration, may suggest that these cells could retain their ability to migrate, although may be aberrant, as reported by Cheeran et al (2009). Moreover, because HCMV-infected cells were found in all subventricular zone, including periventricular region and ganglionic eminence, where immature cells depart for radially and tangentially neuronal migration, respectively, both migration modes could be affected. In conclusion, although in a small number of cCMV infected fetuses, this study suggests a preferential viral tropism for NSPCs, that may lose or delay their capacity to differentiate, while may retain their ability to migrate. All these findings may result in reduced proliferation of immature neural cells, cellular signaling alterations, cell death, and aberrant neuronal migration. The latter may lead to abnormal brain development, such as intracranial calcification, polymicrogyria, lissencephaly, and other consequences frequently detected in cCMV infection. The pathological findings detected in this study showed brain features at 21 weeks of gestation and it is difficult to establish the subsequent evolution of the injury. However, the only fetus with severe pathological findings could probably have had a poor outcome, since necrosis, periventricular leukomalacia and polymicrogyria are parenchymal brain lesions associated with permanent neurological sequelae (Krstanovic et al. 2021, Leruez-Ville et al. 2020).

In light of these results suggesting the direct role of HCMV in the pathogenesis of cCMV-related brain damage, additional features of cerebral injury will have to be investigated in all encephalic regions, such as the effects of the immune responses and the development degree of additional infected cell types involved in the neurogenesis, such as glial cells.

Abbreviations

HCMV
human cytomegalovirus
cCMV
congenital HCMV
IHC
immunohistochemistry
DCX
doublecortin
NeuN
neuronal nuclei
hDNA
humanDNA
NSPCs
neural stem/progenitor cells
CNS
central nervous system
MCMV
murine cytomegalovirus
HSV-1
herpes simplex virus-1
WG
week of gestation
LLoQ
lower limit of quantification
AF
amniotic fluid.

Declarations

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The authors declare that they have no conflicts of interest/competing interests.

Availability of data and material

Data and materials are available with conditions for access, if the request is reasonable and justified.

Code availability

Not applicable.

Author's contributions

Piccirilli Giulia, Gabrielli Liliana, Bonasoni Maria Paola and Lazzarotto Tiziana designed the study. Bonasoni Maria Paola, Salfi Nunzio Cosimo Mario and Santini Donatella performed histological analysis. Piccirilli Giulia, Gabrielli Liliana, Chiereghin Angela, Turello Gabriele, Borgatti Eva Caterina, Simonazzi Giuliana, Felici Silvia and Leone Marta did data collection. Piccirilli Giulia, Gabrielli Liliana, Bonasoni Maria Paola and Turello Gabriele wrote the paper and assisted in generation of Figures. Lazzarotto Tiziana provided critical reading of the manuscript. All authors participated in the data interpretation and approved the final version of the manuscript.

Ethical approval (research involving human participants)

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

The study was approved by the Ethical Committee of IRCCS S. Orsola Polyclinic, University Hospital, Bologna, Italy (approval numbers: 14/2017/U/tess and 8/2010/O/Sper).

Informed consent

Informed consent was obtained from all individual participants included in the study.

Consent for publication

Not applicable.

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Figures

Figure 1

Histological findings in brain injury. Diffuse microglial activation (increased number of rod-shaped cells, arrows) [a], astrogliosis (cell with clear nuclei, named Alzheimer type II, arrow) [b] and vascular changes (plump endothelial cell protruding into the vascular lumen cell, arrow) [c] detected in all brain regions (hematoxylin-eosin staining 40 HPF, Scale bar 50 µm). Microglial nodules (arrow) [d] were occasional (<3/brain region) and multiple in brain with mild and moderate/severe damage, respectively (hematoxylin-eosin staining, 100 HPF, Scale bar 50 µm). Necrosis in cortical layer III (between the arrows) and polymicrogyria [e] were found in the brain with severe injury (hematoxylin-eosin staining, 4 HPF, Scale bar 500 µm).

Figure 2

HCMV-DNA levels in the brain. Distribution of tissue viral load in different brain regions.

Figure 3

HCMV-infected cells in temporal lobe. HCMV-infected cells (brown-stained cells) from subventricular zone (filled arrow) to cortex (dashed arrow). These positive cells, located along the migration pathway, show slender extension typical of radial glia (hematoxylin-eosin staining, 4 HPF, Scale bar 500 μ m).

Figure 4

HCMV-positive cells in the brain. Distribution of mean HCMV-infected cell values in different brain areas (total subventricular zone refers to both periventricular areas and ganglionic eminence)

Figure 5

HCMV-antigen and neural/neuronal cell marker expression. HCMV-positive cells expressing HCMV-antigens and nestin [a,b], DCX [c] or NeuN [d] are identified by both brown and red staining (filled arrow); uninfected cells expressing only nestin [a], DCX [c] or NeuN [d] are identified by red staining (dashed arrow); double immunohistochemistry, 40 HPF, Scale bar 50 μ m.

Figure 6

NeuN expression and brain damage. Expression of NeuN (red cells) in cortical area of temporal lobe in case with severe (a) moderate (b) and mild (c) encephalic damage (double immunohistochemistry, 4 HPF, Scale bar 500 μ m)

Figure 7

Expression of neural/neuronal cell markers in control cases. Expression of nestin in subventricular zone [a], white matter [b] and cortical area [c]; expression of DCX in cortical area [d] hippocampus [e] subventricular zone [f] and white matter [g]; expression of NeuN in cortical area [h] and subventricular zone [i] (immunohistochemistry, 4 HPF, Scale bar 500 μm).

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