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ORIGINAL ARTICLE

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A comparative study of congenital and postnatally acquired human cytomegalovirus infection in infants: lack of expression of viral immediate early protein in congenital cases

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Abstract Postmortem tissues from infants with congenital and postnatally acquired human cytomegalovirus (HCMV) infection were examined by routine histology, immunohistochemistry (IHC) and in situ hybridization (ISH) to determine the dynamics of viral replication in vivo. Histologically, infants in both groups showed characteristic inclusion-bearing cells most commonly in lung, kidney, liver and pancreas. IHC for late proteins using a rabbit polyclonal antibody and ISH for viral genomes detected most of the infected cells as nuclear and/or cytoplasmic signals. However, immunostaining with a monoclonal antibody against viral immediate early (IE) proteins was variable depending on the stage of viral replication within an individual infected cell. In tissues of infants with postnatal HCMV infection, many cells harboured IE antigens, while in tissues from congenital cases most of the affected cells lacked IE antigens and only a few showed cytoplasmic staining. The difference was not caused by the antigenic diversity among viral strains as confirmed by in vitro study. Our findings suggested that congenital infections exhibited uniformly late stage proteins with inactive viral replication at death, while acquired ones remained active. The different viral activity may reflect the immune status of congenital and acquired HCMV infections.

Key words Cytomegalic inclusion disease
Viral replication · Viral regulatory proteins
Immunohistochemistry · In situ hybridization

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Introduction

Human cytomegalovirus (HCMV), a member of the herpesvirus family, is the most common cause of congenital viral infection, affecting from 0.4% to 2.3% of live-born infants (Stagno et al. 1986; Demmler 1991). Approximately 10% of infants with congenital HCMV infection exhibit clinically apparent disease at birth, and nearly 12% of these infants die during the newborn period (Conboy et al. 1987; Boppana et al. 1992).

In utero infection with HCMV (congenital HCMV infection) is one of the paradigms of vertical transmission of viruses. The strict species specificity of HCMV has prevented the development of adequate animal models. Thus, the dynamics of HCMV infection and the mechanisms of viral replication in vivo remain largely unknown. Investigation of pathological material is a reliable way to address this issue and the morphological detection of characteristic inclusion-bearing cells by light microscopy is of diagnostic importance. However, they are thought to represent fully developed features of infected cells (Becroft 1981) and histopathological examination without the use of specialist techniques is insufficient for our purpose.

Following HCMV infection the temporal expression of the virus genome is strictly controlled. Expression proceeds by a sequential synthesis of mRNA and proteins designated immediate early (or α), early (or β) and late (or γ). In general, the products of immediate early (IE) genes (or IE proteins) are those the virus requires to take over control of host cell macromolecular synthesis, while the early proteins including DNA polymerase are required to control production of daughter virions and the late proteins form the viral structural components. Therefore, the earlier products are required for the expression of later products (Wathen and Stinski 1982). In this regard, the detection of different viral proteins in vivo may provide a more precise analysis of the dynamics of infection. For example, since IE proteins can act as trans-activating factors for early gene viral transcription leading to viral replication

(Hermiston et al. 1987; Tevethia et al. 1987), their detection may be useful for identifying newly infected cells in vivo. Accordingly, Grefte et al. (1993) inferred that IE protein expression in endothelial cells was indicative of active HCMV infection.

To clarify the dynamics of congenital and postnatally acquired HCMV infection, we studied postmortem tissues by combining immunohistochemistry (IHC) for IE protein and for viral structural proteins and in situ hybridization (ISH) for viral genomic nucleic acids with routine histopathological examination. This is the first report demonstrating IE protein of HCMV for the systemic analysis of autopsy cases with congenital infection.

Materials and methods

Specimens of different organs were obtained at autopsy from one fetus and 12 infants with disseminated HCMV infection. Clinical data were obtained from the autopsy protocols. None of the infants received antiviral chemotherapy. Diagnosis of HCMV infection was based on the presence of characteristic morphological changes, including inclusion bodies or 'owl eye' appearing-cells in haematoxylin and eosin (H&E)-stained tissue sections. The presence of HCMV-specific antigens and viral nucleic acids was also investigated in paraffin-embedded (4 μm thick) serial sections as follows.

For IHC, two antibodies specific for HCMV antigens were used as primary antibodies. One was a rabbit anti-HCMV polyclonal antibody (pAb), obtained after immunization with HCMV (AD169) virions collected from virus-infected human embryonic lung fibroblast (HEL) cultivated in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. pAb recognized viral antigens in HCMV-infected HEL beginning 48 h postinfection, and infected cells treated with both cycloheximide (which inhibits translation of IE gene) and actinomycin D (which prevents transcription of early gene; Griffiths and Grundy 1987) were not reactive with the antibody, suggesting that pAb predominantly recognized late proteins of HCMV. In vitro, both nuclear and cytoplasmic antigens were detected by pAb. The other antibody was a monoclonal antibody (mAb), designated E5, which recognized an IE protein of HCMV. Briefly, spleen cells from BALB/c mice immunized with sonicated AD169-infected HEL were fused with SP2/O Ag14. The reactivity with nuclear antigens in cells as early as 2 h postinfection and in infected cells treated with cycloheximide and actinomycin D indicated that E5 was a mAb against IE protein. Immunoprecipitation analysis revealed that E5 predominantly recognized a 78 kDa protein in AD169-infected cell lysate. To our knowledge, E5 is the most reliable mAb to the IE protein of HCMV available for staining formalin-fixed, paraffin-embedded sections (data to be published).

Staining was perfomed by the avidin-biotin complex (ABC) immunoperoxidase method (Sata et al. 1986). Incubations with primary antibodies were conducted overnight at 4° C, and for colour development, a substrate consisting of 0.02% 3,3-diaminobenzidine (Dohjin, Kumamoto, Japan) in 0.05M TRIS-hydrochloride (pH 7.2) and 0.015% hydrogen peroxide was employed. Counterstaining was performed in 2% methyl green solution for 3 min.

Probes for the detection of HCMV sequences consisted of a mixture of eight fragments from AD169 strain, 62.3 kb in total, encoding major IE gene (MIE), DNA polymerase or early gene, phosphoprotein 28, major capsid protein, glycoprotein H, glycoprotein B, and regions of terminal repeating long and L–S junction (Greenaway et al. 1982; Chee et al. 1990). All these were cloned DNA fragments inserted into plasmid, pUC19. As verified by Southern blot analysis, the probes were specific for HCMV and showed no hybridization with placental or other herpesvirus DNA (data not shown). The cloned fragments and plasmid DNA, which was used as a negative control, were biotinylated by the random-priming method (Boehringer Mannheim, Germany).

ISH was performed as described previously (Maeda et al. 1993). Briefly, deparaffinized sections were treated with 100 μg/ml proteinase K (Boehringer Mannheim), then fixed in 4% paraformaldehyde and dehydrated. Thereafter each section was covered with a hybridization mixture including 10 ng biotinylated probe, then denatured at 98° C for 5 min, cooled on ice for 5 min and incubated at 42° C for 14 h. Following hybridization, sections were washed three times in 0.2 × SSC (30 mM sodium chloride, 3 mM sodium citrate) at 57° C, blocked with 2% nonfat dry milk, and the biotinylated probes were detected by streptavidin-alkaline phosphatase complex.

To examine the expression of MIE gene transcripts (mRNA), the signal intensity of ISH with the MIE gene probe omitting the heat-denaturation step (which prevents dissociation of DNA into a single-stranded form, and enables exclusive detection of single stranded mRNA) was compared with that of ISH including denaturation.

In vitro, the expression of IE protein was compared in cells infected with clinical isolates of HCMV derived from congenitally infected patients by immunofluorescence (IF); case 4, Yada and YAN–3 kindly supplied by Dr. Eizuru (Eizuru et al. 1988) and with laboratory strains (AD169, Town, and Davis) using the mAb, E5, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG(Fab')$_2$ (Tago, Burlingame, Calif., USA).

Results

The cases examined were categorized into two groups depending on the mode of infection (Table 1). HCMV infection acquired in utero was defined by the isolation of HCMV from urine or throat swab within the first 3 weeks of life or the presence of clinical manifestations at birth which were characteristic of HCMV infection with no apparent underlying disease leading to immune dysfunction, as previously described (Dobbins et al. 1992), with minor modifications. By our definition virus isolation was not necessary for the identification of congenital infection. Several clinical manifestations observed in our cases were considered to be consistent with HCMV infection, and these cases were confirmed by subsequent postmortem examination. Thus, cases 1–8 were classified as congenital infections. The remaining cases (9–13) had underlying diseases which may have caused immune insufficiency, such as congenital leukaemia, congenital heart disease, or severe bacterial infection. These 5 cases had had frequent blood transfusions and this history of multiple transfusions is often the common factor resulting in fatal HCMV infection, especially in premature infants (Sandler and Grumet 1982). Blood given to infants is not screened for HCMV in Japan and cases 9–13 were thus classified as postnatally acquired infections.

The most commonly noted clinical manifestations in the infants with congenital HCMV infections were intrauterine growth retardation and petechiae. Hepatosplenomegaly was noted in three infants and jaundice was present in two. Microcephaly and seizures were present in two infants, one of whom also had intracranial calcification and chorioretinitis (case 8). In three cases (4, 5 and 8) definitive clinical diagnosis of HCMV

Table 1 Clinical data of human cytomegalovirus (HCMV)-infected cases (*CHD* congenital heart disease)

Case number	Age at death/Sex	Gestation (w)	Birth weight (g)	HCMV-related clinical features at birth	History of transfusions	Underlying disease	Mode of HCMV transmission[a]
1	Sillbirth/F	34	528	Not applied	–	–	Congenital
2	2 days/M	26	480	Petechia	–	–	Congenital
3	7 weeks/M	25	940	Hepatosplenomegaly	–	–	Congenital
4	7 weeks/M	37	1,970	Petechia, jaundice, hepato-splenomegaly	–	–	Congenital[b]
5	12 weeks/M	31	1,261	Thrombocytopenia, microcephaly	+	–	Congenital[b]
6	14 weeks/M	39	3,600	Petechia, jaundice	–	–	Congenital
7	19 weeks/M	40	3,108	Hepatosplenomegaly	–	–	Congenital
8	29 weeks/M	38	1,790	Microcephaly, petechia, retinitis intracranial calcification	–	–	Congenital
9	4 weeks/M	24	609	–	+	CHD	Acquired
10	8 weeks/F	29	1,040	–	+	CHD	Acquired
11	16 weeks/M	25	925	–	+	Sepsis	Acquired
12	22 weeks/F	37	3,000	–	+	Leukaemia	Acquired
13	42 weeks/M	32	1,394	–	+	CHD	Acquired

[a] Congenital infection was defined by HCMV isolation within the first 3 weeks of life, or the presence of HCMV-related clinical manifestations at birth (cases 1–8), and other cases were considered as postnatally acquired infection because of the presence of underlying diseases and history of frequent blood transfusions (case 9–13)

[b] HCMV was isolated from throat swab or urine within 3 weeks after birth

infection was established by virus isolation or serological studies (elevated HCMV-specific IgM).

Tissues were examined for inclusion-bearing cells by H&E staining. Lung, kidney, liver and pancreas were the most commonly affected organs in infants with congenital and acquired HCMV infections. Inclusion-bearing cells were less common in the adrenal gland, brain, intestine, heart and thymus and were absent in bone marrow.

In the lung, the target cells were alveolar epithelial cells in all cases and bronchiolar epithelial cells only in case 2. Inflammation was mild in most cases, and focal interstitial infiltration by lymphocytes and plasma cells was absent. In the kidney, epithelial cells of distal convoluted tubules were major sites of infection, and proximal tubules and Bowman's capsules were also involved in some cases. Intense, focal interstitial infiltration (consistent with interstitial nephritis) was detected in four cases. In the liver, epithelial cells of small bile ducts and hepatocytes were infected. Bile plugs and periportal fibrosis were seen in several cases with jaundice. Typical inclusion-bearing cells in the brain were detected only in case 13. Periventricular calcification was evident in case 8, periependymitis in cases 3 and 5, and microglial nodules were seen in four cases (1, 3, 10 and 13).

IHC staining using E5 revealed IE protein in a subpopulation of inclusion-bearing cells, as well as in a few morphologically normal cells. Three distinct staining patterns reflecting the developmental stage of HCMV infection were demonstrated. In the first or 'nuclear type', positive signals were confined within nuclei, either as homogeneous or doughnut-shaped staining (Figs. 1b, 2b) of cells which appeared somewhat larger but were otherwise morphologically normal (Fig. 1a) and of cells with intranuclear inclusions lacking a clear zone or 'halo' (Fig. 2a). No signal was detected in these cells by IHC with pAb and or by ISH for HCMV genomes (Fig. 1d) but, if present, signal was predominantly within nuclei (Figs. 1c, 2c, 2d). These findings were consistent with early stages of infection (Becroft et al. 1981). In the second or 'cytoplasmic type' (Fig. 3b), cytoplasmic granular staining was seen selectively in cells containing intranuclear inclusions with a 'halo' and cytoplasmic inclusion bodies (Fig. 3a). By IHC using pAb and ISH, positive signals were detected predominantly in the nuclei (Fig. 3c, d). In the third or 'negative type', no IE protein staining was detected in obviously well-developed cytoplasmic and nuclear inclusion-bearing cells in which inclusion bodies appeared rather eosinophilic (Fig. 4a, b). But by ISH and IHC with pAb positive signals of these cells were detected in nuclei and cytoplasm (sometimes they were stronger in cytoplasm than in nuclei. Fig. 4c, d).

No hybridization was noted using the labelled plasmid vector, and positive immunostaining was absent by IHC omitting the respective primary antibody.

By combining IHC and ISH the dynamic status of HCMV infection was studied in postmortem tissues from 13 infants with either congenital or postnatal HCMV infections. IHC data are summarized in Table 2. In infants with postnatally acquired HCMV infection, IE proteins were detected in almost all tissues in which HCMV infections were confirmed by other methods. In contrast, despite widespread infection, IE antigens were not demonstrable in tissues from infants with congenital HCMV infection, except in cases 1 (stillbirth) and 2 (died 2 days after delivery) and in lung and kidney tissues from case 6.

In general, the results of ISH using a mixture of HCMV probes paralleled those of IHC with pAb, except in splenic tissue from cases 5, 11 and 12 in which ISH was more sensitive than IHC in detecting infected

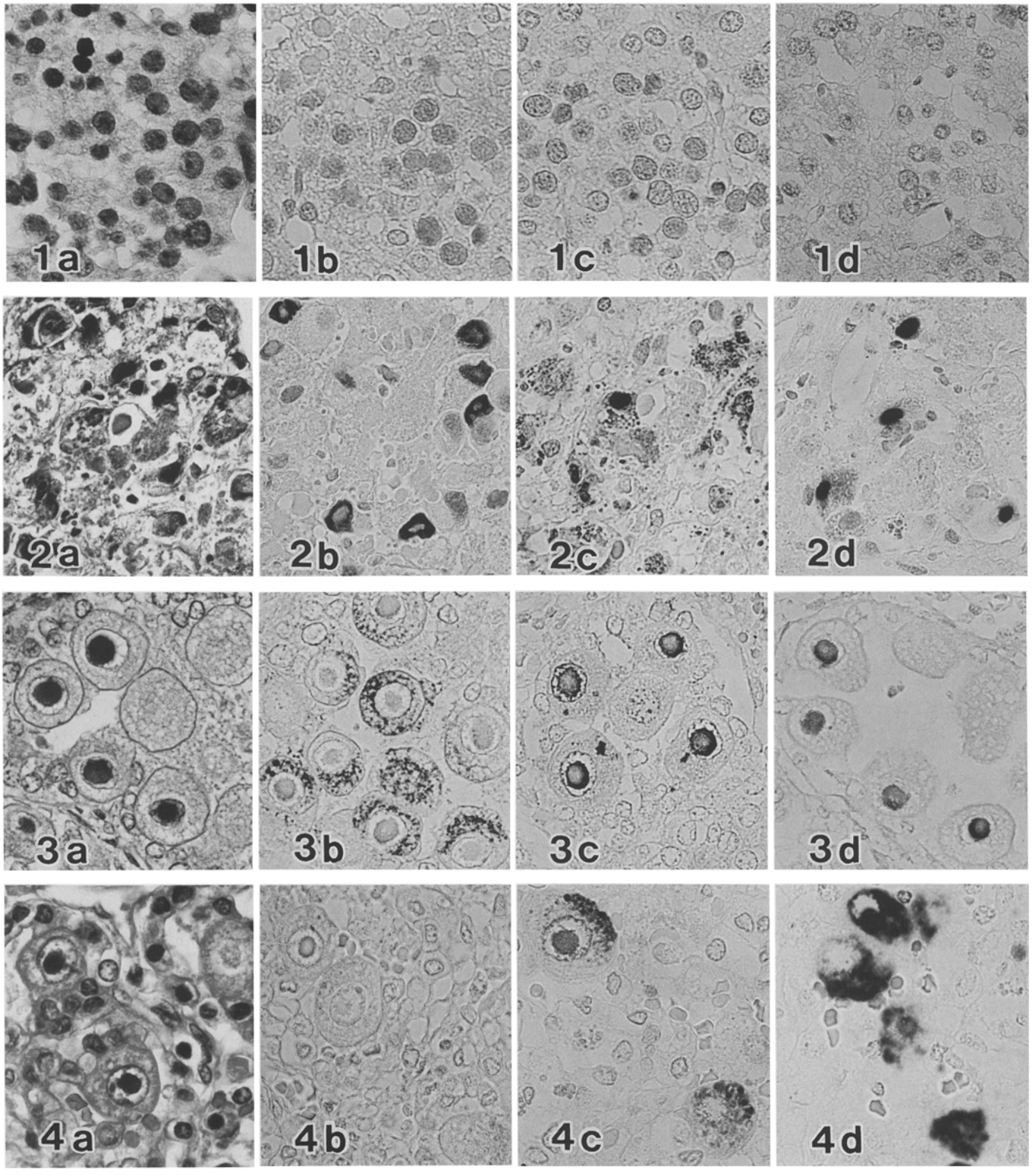

Fig. 1a–d Adrenal cortex of case 9 with postnatally acquired human cytomegalovirus (HCMV) infection. The cells were considered to be in the earliest stage of infection. **a** Morphologically almost normal cells with somewhat larger and blurry nuclei, haematoxilin and eosin, **b** Immunohistochemistry (IHC) with monoclonal antibody against immediate early protein (E5); homogeneous positive reaction in nuclei, referred to as 'nuclear type'. **c** IHC with anti-HCMV polyclonal antibody; weak staining of nuclei. **d** In situ hybridization with mixed probe (ISH) detecting HCMV-specific nucleic acid sequences; no hybridization signal, ×400

Fig. 2a–d Adrenal medulla of case 12 with postnatally acquired HCMV infection. **a** Intranuclear inclusion bodies lacking clear halo, haematoxylin and eosin **b** IHC with E5; homogeneous and doughnut-shaped staining in nuclei (nuclear type). **c** IHC with polyclonal antibody; positive signals in nuclei and cytoplasm. **d** ISH; hybridization signals confined within nuclei, ×400

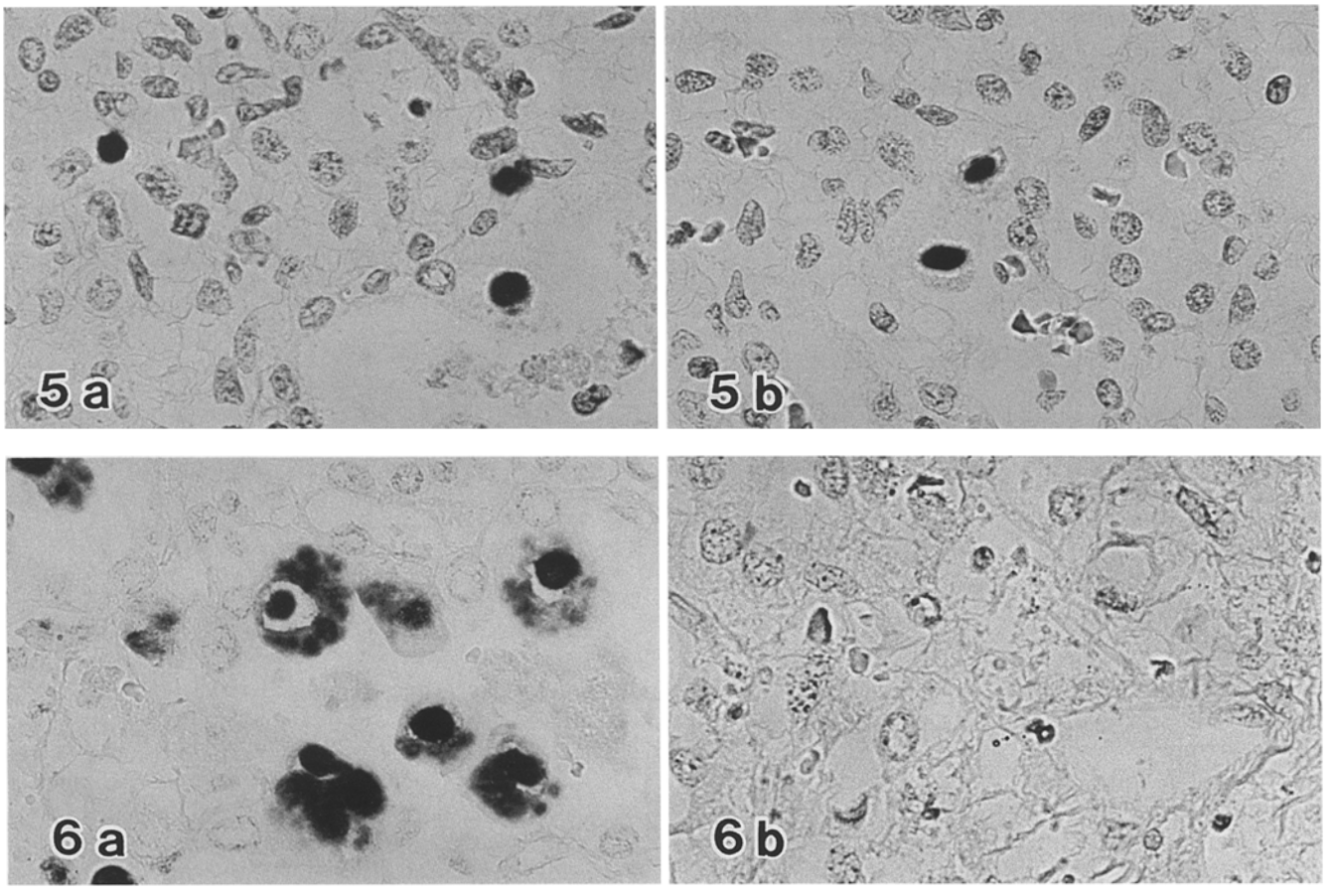

Fig. 5 Lung of case 10 containing cells with immediate early (IE) protein. In situ hybridization using major immediate early gene as a probe (MIE–ISH) with (**a**) and without (**b**) heat denaturation of section. **a** Hybridization signals originated by both DNA and mRNA or whole nucleic acids. **b** Weaker signals generated by mRNA as compared with **a**. The presence of IE protein proved to be accompanied by transcription of MIE gene, × 400

Fig. 6a, b Lung of case 4 lacking immediate early (IE) protein-positive cells. **a** MIE–ISH detection of whole nucleic acids; numerous cells with intense signals predominantly in cytoplasm. **b** MIE–ISH for mRNA; very faint signals, much weaker than **a**. The lack of IE protein expression was consistent with no MIE-mRNA, × 400

◀ **Fig. 3a–d** Kidney of case 3 with congenital HCMV infection. **a** Tubular epithelial cells containing intranuclear inclusion with halo and cytoplasmic inclusion bodies, haematoxylin and eosin **b** IHC with E5; granular staining in cytoplasm referred to as 'cytoplasmic type'. **c, d** IHC with polyclonal antibody and ISH; positive signals predominantly in nuclei, × 400

Fig. 4a–d Lung of case 4 with congenital HCMV infection. **a** Fully developed inclusion-bearing cells, containing distinct halo in alveolar epithelia, considered to be in the late stage of infection, haematoxylin and eosin **b** IHC with E5; no positive signal referred to as 'negative type'. **c, d** IHC with polyclonal antibody and ISH; weak nuclear staining and strong cytoplasmic granular signals, × 400

cells. Particularly in cases 5 and 11, numerous HCMV-positive cells were detected by ISH despite the absence of inclusion-bearing cells and of any detectable HCMV antigen by IHC.

Detection of transcripts or mRNA of the MIE gene was undertaken on both IE protein-positive (adrenal gland from case 12; lung from case 10) and protein-negative sections (lung from cases 4 and 7; spleen from case 11). IE protein-positive cells were also positive by ISH for MIE mRNA but with weaker intensities than for both DNA and mRNA, indicating the presence of MIE transcripts in nuclei (Fig. 5a, b). In contrast, the IE antigen-negative specimen (lung section from case 4) showed no hybridization for mRNA, while whole nucleic acids were strongly positive both in nuclei and cytoplasm suggesting that MIE transcripts were absent (Fig. 6a, b). Another specimen lacking IE antigen (spleen from case 11) showed equally strong intensities regardless of heat denaturation, indicating that the signals were predominantly reflected by MIE mRNA (data not shown).

The possibility that antigenic differences prevented the recognition of IE protein was assessed. Three laboratory strains and three clinical isolates from infants with congenital HCMV infection including case 4, in whom IE antigen was not detected at all in tissue sections, were examined by IF in vitro. No remarkable difference was found in the reactivity with E5 among the viral strains (data not shown).

Table 2 Detection of human cytomegalovirus (HCMV) proteins and genomes (*n* nuclear, *c* cytoplasmic)

Organ	Congenital infection				Acquired infection			
	Number (examined cases)	Immediate early (IE) protein	(n/c)[a]	Late protein and viral genomes	Number	IE protein	(n/c)[a]	Late protein and viral genomes
Lung	8	2	(0/2)	8	5	5	(4/1)	5
Kidney	8	3	(1/2)	7	5	4	(3/1)	5
Liver	7	1	(1/0)	5	5	3	(3/0)	4
Brain	5	1	(1/0)	2	3	2	(2/0)	2
Adrenal	7	0		4	5	4	(4/0)	4
Pancreas	8	0		4	4	4	(4/0)	4
Spleen	7	0		3 (1)[b]	3	2	(2/0)	2 (1)[b]
Heart	5	0		2	2	1	(1/0)	1
Salivary gland	2	0		2	0	–		–

The numbers are those of specimens with positive staining by each method.
[a] Immunohistochemical (IHC) detection of viral IE proteins using monoclonal antibody; E5 showed nuclear (*n*) or cytoplasmic (*c*) granular patterns as described in parentheses. IHC with rabbit anti-HCMV polyclonal antibody dominantly recognizing late proteins was consistent with the result of in situ hybridization (ISH) except for spleens.
[b] Spleens from two patients (cases 5 and 11) showed positive signals only by ISH while viral antigens were absent

Discussion

Routine histopathology in conjunction with IHC for detecting IE and late viral antigens and ISH for viral genomes enabled us to determine the stage of HCMV infection in individual cells within different organs. Our data indicate that the dynamics of viral replication differs in congenital and postnatal HCMV infection.

Histopathologically, amphophilic nuclear inclusion bodies (sometimes with a halo) and eosinophilic granular cytoplasmic inclusion bodies are fully developed features of HCMV-infected cells (Becroft 1981). IHC with pAb, which recognizes viral structural or late proteins, demonstrated cells in the late stage of infection. ISH with a mixture of probes detects viral DNA, the amount of which increased during viral replication since the sensitivity of our ISH system was approximately 50 or more copies of viral genomic DNA per cell (data not shown), indicating that ISH-positive cells were in the late stage of infection. In contrast, IE protein-harbouring cells can be interpreted to be in the early stage of infection, as this protein is expressed immediately after infection and during viral replication. IE antigen-expressing cells infected with HCMV were found in three different patterns (nuclear, cytoplasmic and negative), which was reflected by the stage of infection. Our observations are consistent with a previous study using immunoelectron microscopy which demonstrated the subcellular distribution of IE1 products during infection in vitro. IE antigens were localized in the nuclei during the early phase and in the cytoplasm during the late phase (Tsutsui and Yamazaki 1991). Moreover, the transcripts of IE gene were also detected in the nuclei of IE protein-positive cells with nuclear and cytoplasmic type of staining. These methods, therefore, proved useful for investigating the dynamics of HCMV replication in vivo.

Of 13 infants with HCMV infection, 8 cases were congenital and 5 were postnatally acquired. The clinical features of congenital HCMV infection in our cases were similar to those reported recently in the United States (Dobbins et al. 1992). The frequency of HCMV infection in each organ and the preferred histological sites of viral replication were also similar to those of the Auckland necropsy series (Becroft 1981), the only exceptions being more frequent involvement of the adrenal gland and spleen in our cases.

In infants with postnatally acquired HCMV infection many cells harboured IE proteins. In contrast, very few cells expressed IE antigens in infants with congenital infection. This difference was not caused by the altered reactivity of the antigens in sections after formalin fixation and paraffin embedding or by virus strain specificity, since E5 could detect IE proteins in all postnatally acquired cases and in three cases with congenital infection in vivo, and those in the cells infected in vitro with strains isolated from congenitally infected patients and laboratory strains in the same manner. These findings indicated that most of the virus-infected cells in congenital HCMV cases were uniformly in the late stage of viral replication or that the state of infection may be less active at death, while cells in postnatally acquired cases showed various stages of infection and active viral replication. Our conclusion is in accord with the study by Borisch et al. (1988), who demonstrated that the 150 kDa phosphoprotein of HCMV, a late gene product or viral structural protein, was detected more frequently in distinct inclusion-bearing cells in congenitally infected infants than in adults with HCMV infection. Since HCMV infection tends to be more virulent when acquired during the first half of gestation (Stagno et al. 1986), our congenital cases might have been infected in early gestation, and the lack of IE expression may be partially attributed to the duration of infection. Nevertheless, it was surprising that newly infected cells were absent despite widespread dissemination of HCMV leading to death. The expression of abundant IE antigens in two congenital HCMV cases (case 1 was stillborn and case 2 died 2 days after birth) may imply that

infection continued to be active in utero and after delivery activity had been rapidly suppressed. The other exception was case 6, in which IE protein-positive cells presenting a cytoplasmic pattern were found in lung and kidney. Especially in these organs, a long-lasting active infection may persist in some infants with congenital HCMV infection.

The significance of restricted transcription of the IE gene in splenic tissue of two infants without either translation or replication of viral genomes is not clear. Myerson et al. (1984) also reported that HCMV genomes were detected in normal-appearing splenic cells. IE1 transcripts of murine CMV have also been detected in the spleen of latently infected mice (Mercer et al. 1988; Pomeroy et al. 1991; Henry and Hamilton 1993). Splenic cells may serve as a reservoir of HCMV in the same way as they do for murine CMV, but further examination will be needed.

Differences in the dynamics of congenital and postnatal HCMV infection may be correlated with the immunological status. Immunological incompetence is a common feature in both groups. The immune response is immature in congenital HCMV cases and is compromised by chemotherapy or congenital heart disease leading to malnutrition in postnatally acquired cases. In general, the immune responsiveness of fetuses or babies is developing, while that of the infants with severe disease may be suppressed. Following delivery, a rapid expansion of the lymphocyte population occurs, resulting in the inversion of the ratio of lymphocytes to granulocytes (Vaughan and Litt 1990), which may reflect maturation of the cell-mediated immunity which is more important for the elimination of HCMV (Quinnan et al. 1982; Borysiewicz et al. 1988). Less active HCMV replication in congenital cases reminds us that the destructive viral replication itself is not the direct cause of death. Rather, HCMV infection-induced organ dysfunction probably plays a more important role leading to a fatal outcome in congenitally infected infants. In the treatment of congenital HCMV infection, supportive therapy for organ failure may be more essential than antiviral agents.

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