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Production of a variant virus with different growth characteristics

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Human Cytomegalovirus Persistent Infection in a Human Central Nervous System Cell Line: Production of a Variant Virus with Different Growth Characteristics

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SUMMARY

The susceptibility of human central nervous system cell lines to human cytomegalovirus (HCMV) and the fate of infected cultures were studied. Significant amounts of infectious progeny virus were produced in 118MGC glioma and IMR-32 neuroblastoma, but not in KGC oligodendroglioma cells when the cultures were infected with wild-type virus (HCMVwt) at an m.o.i. of 10 p.f.u. per cell. Further passage of infected 118MGC cells resulted in the establishment of a long-term persistent infection. This infection, designated 118MGC/Towne, continuously produced infectious virus (HCMVpi) with titres ranging from 10^2 to 10^5 p.f.u./ 10^6 cells up to 360 days post-infection (corresponding to 50 subcultures). Since no temperature-sensitive mutants, defective interfering particles or interferon-like activity were found in the 118MGC/Towne cultures, maintenance of the persistent infection seemed to be due to a balance between the release of infectious virus and the growth of uninfected cells. The HCMVpi produced in long-term persistently infected cultures was shown to be different from the HCMVwt originally used to infect by the following characteristics: (i) HCMVpi replicated slowly and yielded lower amounts of progeny virus than HCMVwt; (ii) HCMVpi induced a 73000 mol. wt. immediate early protein that was not synthesized in HCMVwt-infected cells; (iii) HCMVpi had a different DNA structure from that of HCMVwt. These results suggest that HCMVpi is a slower growing variant of HCMVwt and probably plays an important role in the maintenance of the persistent infection.

INTRODUCTION

Human cytomegalovirus (HCMV) is the commonest agent causing foetal infection. Congenital HCMV infection causes a wide range of diseases including cytomegalic inclusion disease (CID), congenital defects and interstitial pneumonia (Rapp, 1980). Particularly in the infant whose brain is severely damaged by HCMV infection, symptoms such as microcephalus, epileptic encephalitis, cerebral palsy and optic atrophy are exhibited (McCracken *et al.*, 1969; Berenberg & Nankervis, 1970). Moreover, infants infected congenitally with HCMV asymptotically have also been shown to exhibit mental retardation, sensorineural hearing loss, low intelligence and deafness (Hanshaw *et al.*, 1976).

Studies on the response of murine nerve tissue cultures *in vitro* to murine cytomegalovirus indicated that all cell types of the nerve tissues showed cytopathic effects with typical cytomegalovirus inclusion and cell fusion (Schneider *et al.*, 1972). However, little information is available concerning the interaction between HCMV and human central nervous system (CNS) tissue cultures. Therefore, in the experiments reported here, we have studied the susceptibility to HCMV of various cell lines derived from human CNS and examined the fate of HCMV-infected cultures. The results obtained show that certain human CNS lines are permissive for

HCMV replication and that one in particular, 118MGC glioma cells, can support productive replication as well as long-term persistent infection. The virus produced in the persistent infection is shown to be a slower growing variant of the input HCMV.

METHODS

Cells and cultures. Human CNS cell lines used in these studies were neuroblastoma cells (IMR-32; Tumilowicz *et al.*, 1970), glioblastoma cells (118MGC; Ponten & Macintyre, 1968) and oligodendroglioma cells (KGC; Miyake, 1979). Both IMR-32 and 118MGC cells were cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. KGC cells were cultured and maintained in DM-160 medium (Kyokuto, Japan) containing 10% foetal calf serum. Human embryonic lung (HEL) cells, prepared as previously described (Tanaka *et al.*, 1984a), were cultured with DMEM supplemented with 10% calf serum. All cultures except KGC cells were maintained after virus infection in DMEM containing 5% calf serum.

Virus. The Towne strain of HCMV was used in these studies. Stocks of HCMV were prepared in HEL cells as described by Tanaka *et al.* (1984a).

Virus growth studies. Subconfluent monolayers of CNS cells grown in 1 oz prescription bottles were infected with HCMV at an m.o.i. of 10 p.f.u. per cell, and adsorbed for 90 min at 37 °C. At various intervals post-infection, the total amount of infectious virus was measured by the method described in our previous papers (Tanaka *et al.*, 1984a, b). Infectious centre assay was carried out as described previously (Tanaka *et al.*, 1985).

Immunofluorescent (IF) staining. HCMV-specific antigens were examined by indirect IF techniques using human convalescent serum (Tanaka *et al.*, 1981, 1985) which contained antibodies to viral immediate early antigens (IEA) and late antigens (LA), with titres of 1:16 and 1:640, respectively.

Polyacrylamide gel electrophoresis (PAGE). Synthesis of immediate early (IE) proteins was analysed by SDS-PAGE according to the method described by Blanton & Tevethia (1981). HEL cells grown in 25 cm² culture flasks were infected with HCMV at an m.o.i. of 5, or mock-infected. After 1 h adsorption, IE proteins were immediately labelled for 2 h or pulse-labelled from 1 to 7 h after infection at 2 h intervals with [³⁵S]methionine (30 µCi; sp. act. 1110 Ci/mmol, Amersham) in methionine-free maintenance medium. IE proteins were extracted and immunoprecipitated by anti-IEA-positive human convalescent serum. Immunoprecipitated proteins were separated by electrophoresis on 10% slab gels (Tanaka *et al.*, 1984a, 1985). Cycloheximide (CH)-enhanced IE proteins were also examined according to the method described by Jeang & Gibson (1980). ¹⁴C-labelled proteins [myosin H chain, mol. wt. 200000 (200K); phosphorylase b, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; lysozyme, 14K] were co-electrophoresed in each gel as mol. wt. standards.

Pulse-chase analysis of IE protein synthesis. Pulse-chase studies of IE protein synthesis were performed according to the method described by Gibson (1981). HEL cells were treated with CH (50 µg/ml) 1 h before, during the 1 h viral adsorption period, and from 1 to 18 h after infection. The cultures were then radiolabelled for 2 h with [³⁵S]methionine (20 µCi/ml) in methionine-free maintenance medium containing actinomycin D (act D; 5 µg/ml). The radiolabelled cultures were rinsed three times with growth medium, incubated for an additional 18 h in act D-containing medium, and then processed for analysis of SDS-PAGE.

Purification of virions and extraction of viral DNA. Purification of virions was carried out by the method described by Talbot & Almeida (1977). The purified virions were lysed in 500 mM-EDTA, pH 8.0, 100 µg/ml proteinase K, 0.5% Sarkosyl NL97 at 50 °C for 3 h. DNA was extracted three times with phenol and then exhaustively dialysed against 50 mM-Tris-HCl pH 7.4, 1 mM-EDTA. The dialysed DNA solution was stored at 4 °C until use.

Digestion of HCMV DNA with restriction endonucleases and electrophoresis. The restriction endonucleases used in these experiments were *Eco*RI, *Bam*HI, *Hind*III and *Xba*I (Takara Reagents, Japan). Digestion of viral DNA was carried out in the appropriate buffers with sufficient concentrations (usually 4 units/µg DNA) of the enzymes to accomplish complete digestion in 2 h at 37 °C. The digested DNA fragments were separated by electrophoresis in horizontal 0.6% agarose gels. The mol. wt. of the fragments were determined from their electrophoretic migration relative to *Hind*III-digested fragments of phage lambda DNA (Murray & Murray, 1975). After electrophoresis the DNA fragments in the gel were visualized by staining with ethidium bromide (1 µg/ml) and by illumination with a u.v. transilluminator (Ultra-Violet Products, San Gabriel, Ca., U.S.A.), and photographed using a Polaroid MP4 camera.

Blotting and hybridization procedures. DNA fragments were transferred to nitrocellulose filters according to Southern (1975). The DNA fragments on the filter were hybridized with HCMV wild-type DNA labelled *in vitro* with [³²P]dCTP (sp. act. 3000 Ci/mmol, Amersham) by nick translation according to the method described by Rigby *et al.* (1977).

RESULTS

Growth kinetics of HCMV in CNS cell lines

The susceptibility of three CNS lines to HCMV was first examined by infecting subconfluent monolayers of IMR-32, 118MGC and KGC cells at an m.o.i. of 10. At various intervals post-

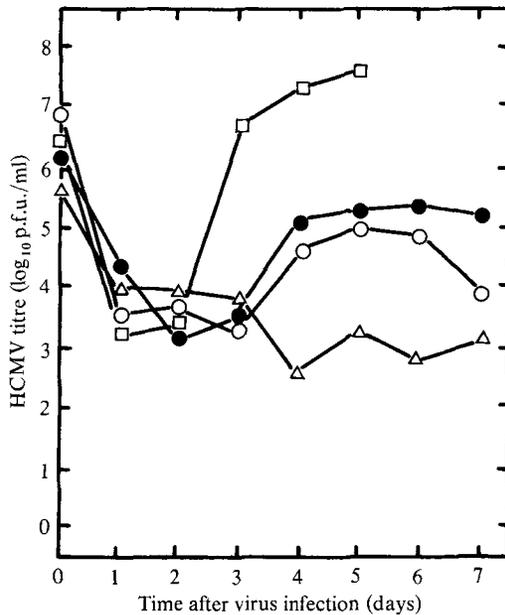


Fig. 1. Replication of HCMV in various human CNS cell lines. Subconfluent monolayers of IMR-32 (○), 118MGC (●), KGC (△) or HEL (□) cells were infected with HCMV at an m.o.i. of 10. After 90 min, cells were washed with Hanks' balanced salts solution and maintenance medium was added. At the indicated times, the cells were disrupted by freezing and thawing once, and by sonication. The total amount of infectious virus was then measured by plaque assay on HEL cells.

infection, the total amount of infectious virus was determined. For comparison, HEL cells fully permissive for HCMV replication were also infected at the same m.o.i. (Fig. 1). In both IMR-32 and 118MGC cultures synthesis of infectious virus began to increase at 4 days and reached a titre of approximately 10^5 p.f.u./ml at 5 days. However, the amount of infectious virus did not significantly increase in KGC cells. IF staining at 5 days post-infection revealed that intranuclear inclusion bodies (IA) were found in more than 95% of HEL, 118MGC and IMR-32 cells. In contrast, IA-positive cells were detected in only 3.3% of KGC cells. These results indicate that the CNS cell lines 118MGC and IMR-32 are able to support HCMV replication, although the maximum titres produced in these lines were more than 100-fold lower than that in HEL cells.

Establishment of persistent infection with HCMV in CNS cell lines

The fate of HCMV-infected CNS lines was examined next. IMR-32, 118MGC and KGC cells were infected with HCMV and cultured in growth medium with passage at a split ratio of 1:3 once a week. The amount of infectious virus and the number of antigen-positive cells were determined. In KGC cells, less than 10% of the cells showed c.p.e. at 7 days post-infection. However, neither infectious virus nor viral antigens could be detected in cultures passaged more than twice. More than 90% of the IMR-32 cells were destroyed within 7 days after virus infection. The surviving cells produced infectious virus with titres ranging from 10^1 to 10^4 p.f.u./ 10^6 cells for at least 60 days (over eight passages). However, both virus and viral antigens became undetectable within 2 months after infection.

On the other hand, in HCMV-infected 118MGC cells virus-specific cytopathology progressed rapidly and more than 95% of the cells were destroyed within 6 days after infection. Continuous culture of virus-infected 118MGC cells, however, resulted in the establishment of a persistent infection with HCMV. As shown in Fig. 2 the persistently infected line, referred to as 118MGC/Towne, continuously produced infectious virus (approximately 10^2 to 10^5 p.f.u./ 10^6

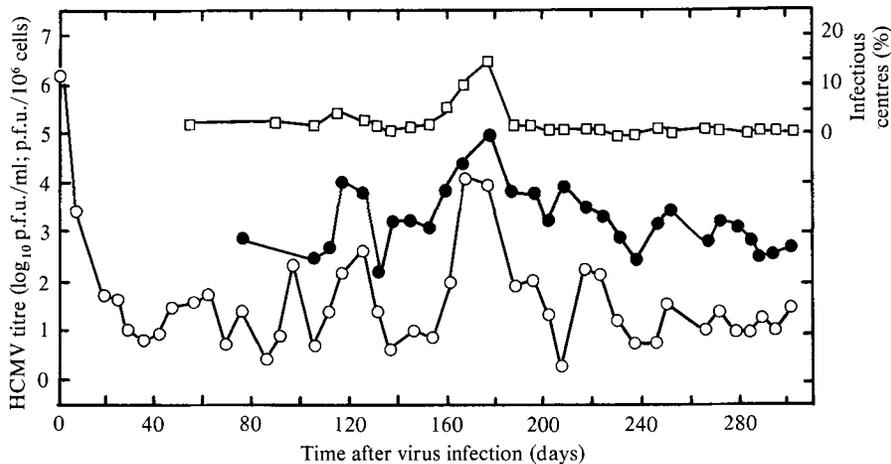


Fig. 2. Production of infectious HCMV in persistently infected 118MGC cultures. 118MGC cells were infected with HCMV at an m.o.i. of 10 and subcultured once a week at a split ratio of 1:3. At the indicated times after infection, virus titres in the culture supernatant (○) and cell-associated (●), or infectious centre titres (□) were determined.

cells) and released virus into the culture medium with titres ranging from 2×10^0 to 10^4 p.f.u./ml up to 360 days after virus infection (over 50 subcultures). Infectious centre assays performed at each passage level demonstrated that 0.1 to 15% of the cells were able to produce infectious virus (Fig. 2). The proportion of LA-positive cells at each passage level closely correlated with the infectious centre titre.

Characteristics of 118MGC/Towne cultures

The 118MGC/Towne cultures grew at the same rate as uninfected 118MGC cells. The morphological appearance of the cells resembled that of uninfected parent cells. However, HCMV-specific cytopathology was consistently observed in less than 10% of the cultures. These cells were found to contain typical intranuclear inclusion bodies by IF staining. To determine whether 118MGC/Towne cells produced temperature-sensitive (*ts*) mutants, the virus produced during the persistent infection (hereafter referred to as HCMV_{pi}) was tested for its plaquing efficiency on HEL cells at 34 or 39 °C. No significant difference was found in plaque number at the two temperatures, nor was there a difference between HCMV_{pi} and the wild-type HCMV (hereafter referred to as HCMV_{wt}) originally used for infection. The lack of temperature sensitivity of HCMV_{pi} was further confirmed by the finding that maintenance of 118MGC/Towne cultures at a non-permissive temperature (39 °C) for 7 days did not significantly influence the production of infectious virus or synthesis of viral antigens.

Interferon-like activities were not detected in the culture fluids from 118MGC/Towne cultures. Moreover, defective interfering particles (DIP) were not produced in 118MGC/Towne cells, because HCMV_{pi} did not interfere with the growth of HCMV_{wt} in HEL cells. In addition, DNA analysis by isopycnic centrifugation in CsCl indicated that viral DNA synthesized in 118MGC/Towne cultures was found at 1.718 g/ml, a density identical to that of standard HCMV_{wt} DNA synthesized in HEL cells. The peak that corresponds to DIP DNA (Ramirez *et al.*, 1979) was not observed for HCMV_{pi} DNA.

Culturing of 118MGC/Towne cells in the presence of HCMV-positive human convalescent serum for 21 days resulted in curing of the persistent infection. Synthesis of infectious virus and viral antigens (IEA and LA) could no longer be detected in the cured 118MGC/Towne cultures after the removal of the antiserum, suggesting that extracellular virus is more important in the maintenance of the persistent state than cell-to-cell virus spread. To study whether the cells in 118MGC/Towne cultures in which no viral antigen was detectable retained the HCMV genome in a latent state, 118MGC/Towne cultures were cloned by limiting dilution. These cell clones

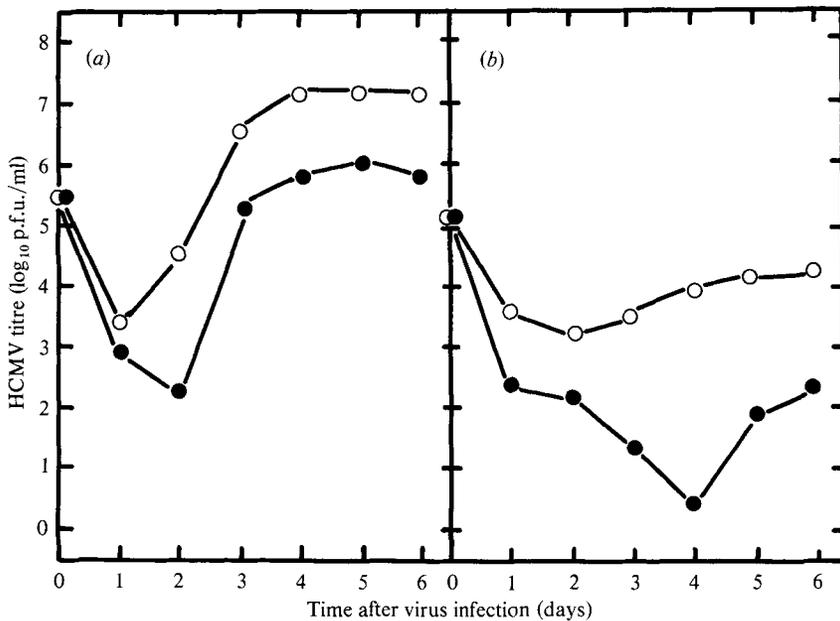


Fig. 3. Replication of HCMVwt (O) and HCMVpi (●) in (a) HEL or (b) 118MGC cells. Subconfluent monolayers of cultures were infected with HCMVwt or HCMVpi at an m.o.i. of 1. HCMVpi was obtained from 118MGC/Towne culture at 430 days post-infection and grown once in HEL cells at an m.o.i. of 0.1. After a 90 min adsorption period, cells were washed with Hanks' balanced salts solution and maintenance medium was added. At the indicated times the total amount of infectious virus was measured.

were tested for production of infectious virus and HCMV-specific antigens. Neither infectious virus nor IEA and LA was found in more than ten cell clones examined. Superinfection of these clones with HCMVwt produced the same amount of infectious virus as in the uncloned parent 118MGC cultures.

Slower replication of persistently infecting virus

During the virus titration experiments, we noticed that individual plaques formed by HCMVpi on HEL cells appeared later and were smaller as compared with HCMVwt, suggesting that 118MGC/Towne cultures may produce an HCMV variant with respect to growth characteristics. To test this possibility, we compared the growth rate of HCMVpi and HCMVwt in HEL and 118MGC cultures infected as subconfluent monolayers at an m.o.i. of 1. At 24 h intervals after infection, the total amount of infectious virus was determined. The results, shown in Fig. 3, indicate that synthesis of infectious progeny HCMVwt and HCMVpi in HEL cells was first observed at 2 and 3 days after infection, respectively; that is, there was a 1 day delay in the appearance of progeny HCMVpi compared to that of HCMVwt. Moreover, the maximum titre of HCMVpi produced 4 to 6 days after infection of HEL cells was approximately tenfold lower than that of HCMVwt (Fig. 3a). The lower yield and slower replication of HCMVpi compared to HCMVwt were also observed in 118MGC cultures. In these cultures, there was a 2 day delay in synthesis of progeny HCMVpi and an approximately 100-fold reduction in the maximum HCMVpi yield when compared to HCMVwt (Fig. 3b).

Synthesis of IE proteins by persistently infecting virus

The molecular basis of the decreased replication of HCMVpi was examined. Synthesis of IE proteins by HCMVwt and HCMVpi was compared in HEL and 118MGC cells by SDS-PAGE. As can be seen in Fig. 4(a), two major IE proteins of apparent mol. wt. 72K and 78K [corresponding to the 73K and 78K IE proteins reported by Blanton & Tevethia (1981)] were

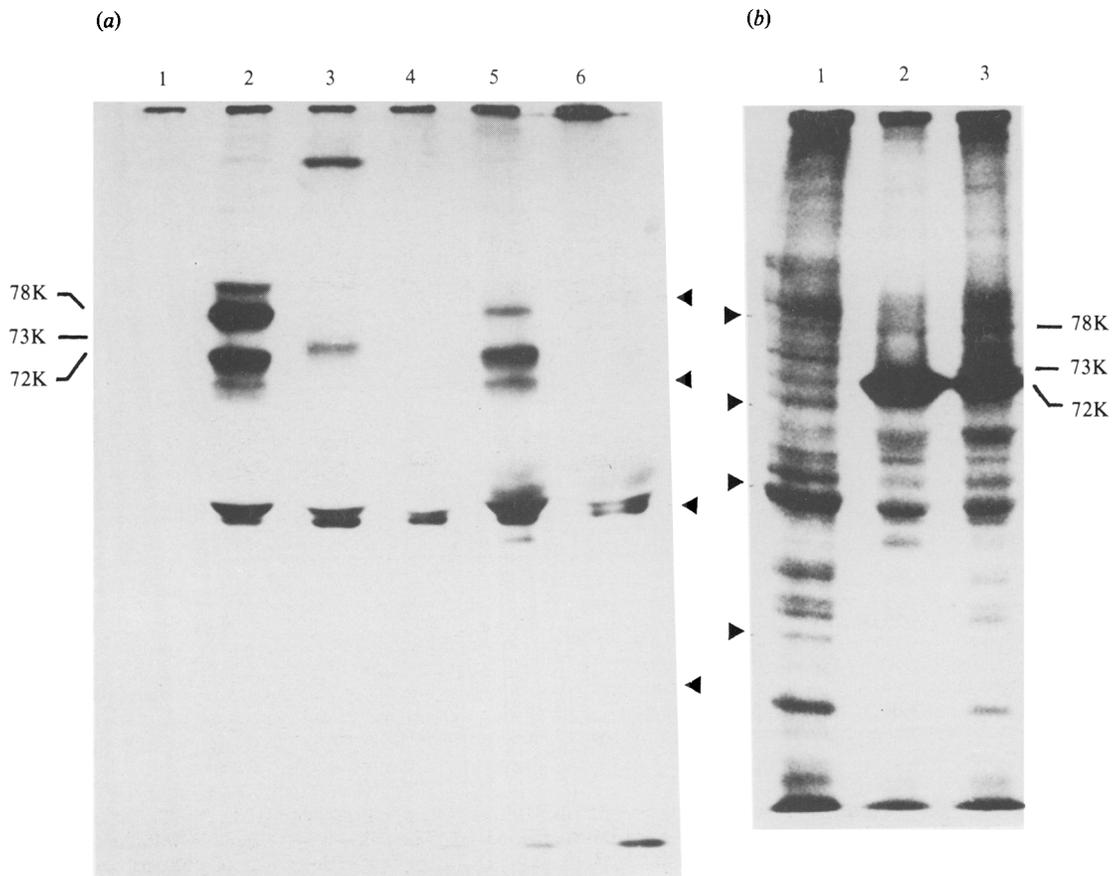


Fig. 4. Analysis of IE proteins synthesized in HEL or 118MGC cells infected with HCMVwt or HCMVpi. (a) Subconfluent monolayers of HEL (1, 2, 3) or 118MGC (4, 5, 6) were infected with HCMVwt (2, 5) or HCMVpi prepared as described in the legend to Fig. 3 (3, 6) at an m.o.i. of 5, or mock-infected (1, 4). After a 1 h adsorption period unadsorbed virus was removed and the cultures were pulse-labelled with [35 S]methionine for 2 h in methionine-free maintenance medium. IE proteins were extracted, immunoprecipitated by human serum, and separated by SDS-PAGE as described under Methods. (b) Subconfluent monolayers of HEL cells were infected with HCMVwt (2) or HCMVpi (3) or mock-infected (1) in the presence of CH. At 18 h post-infection the CH-containing medium was removed, and cells were labelled for 2 h with [35 S]methionine in methionine-free maintenance medium containing act D. The radiolabelled cultures were immediately solubilized, and proteins were separated by SDS-PAGE. The mol. wt. of IE-specific proteins are presented, and the arrowheads indicate the mol. wt. of standards (92.5K, 69K, 46K and 30K).

found in HCMVwt-infected HEL (lane 2) and 118MGC cells (lane 5), whereas the level of synthesis of the 72K and 78K IE proteins was less in both HEL (lane 3) and 118MGC cells infected with HCMVpi (lane 6) when compared to HCMVwt. However, it is noteworthy that in HEL cells HCMVpi induced an IE protein with a mol. wt. (73K) different from that induced by HCMVwt (lane 3). The same difference in electrophoretic mobility of the IE proteins induced by HCMVwt and HCMVpi could also be detected when CH-enhanced IE proteins were analysed by SDS-PAGE (Fig. 4 b).

Kinetic experiments on IE protein synthesis revealed that in HCMVwt-infected cells the major IE proteins (72K and 78K) were synthesized at 1 to 3 h after infection (Fig. 5, lane 2), and the relative amount of these proteins increased at 3 to 5 h (lane 3). Major early (E) proteins of apparent mol. wt. 118K, 88K and 68K which corresponded to the major E virus proteins

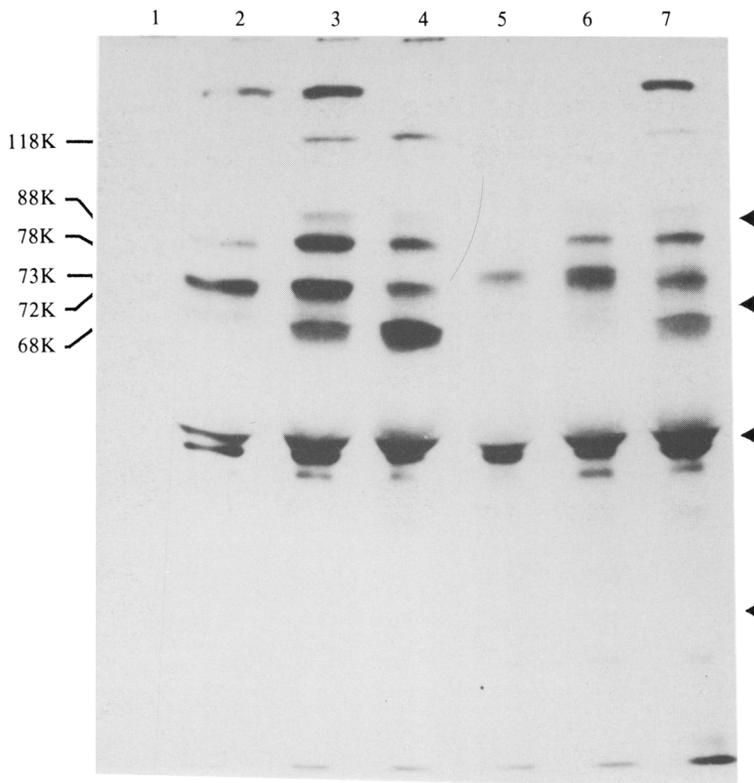


Fig. 5. Time course of IE and E protein synthesis. HEL cells were infected with HCMVwt (2, 3, 4) or HCMVpi (5, 6, 7) at an m.o.i. of 5, or mock-infected (1). The cultures were pulse-labelled with [³⁵S]methionine at 1 to 3 h (2, 5), 3 to 5 h (3, 6), and 5 to 7 h (4, 7) after infection. IE proteins were extracted, immunoprecipitated, and separated by SDS-PAGE. The mol. wt. of IE- and E-specific proteins are presented, and the arrowheads indicate the mol. wt. of standards (92.5K, 69K, 46K and 30K).

described by Blanton & Tevethia (1981) were also detectable at this time (lane 3). On the other hand, in HCMVpi-infected cells, only the 73K IE protein was detected at 1 to 3 h (lane 5) and the relative amount of 73K protein synthesized decreased with time after infection. Conversely, the 72K and 78K proteins were synthesized at 3 to 5 h after infection (lane 6). Moreover, in HCMVpi-infected cells it required 2 h longer to synthesize the major E proteins at the same molar ratio as in HCMVwt-infected cells (lane 7).

Characteristics of cloned persistently infecting viruses

HCMVpi clones were isolated from plaques and purified by three successive plaque isolations from HEL cells. The representative four HCMVpi clones were examined for their growth rate and IE protein synthesis. The maximum titres of progeny virus of cloned HCMVpi were approximately five- to 10-fold lower than that of HCMVwt. All clones obtained here synthesized mainly the 73K IE protein at 1 to 3 h after infection (data not shown) quite similarly to that shown in Fig. 4 (lane 3). Thus, HCMVpi was different from HCMVwt with respect to growth rate and IE protein synthesis.

Comparison of IE protein synthesis and viral growth rate between short- and long-term persistent infections

To determine whether any difference in IE protein synthesis resulted from long-term persistent infection, synthesis of IE proteins by HCMVpi obtained from 118MGC/Towne

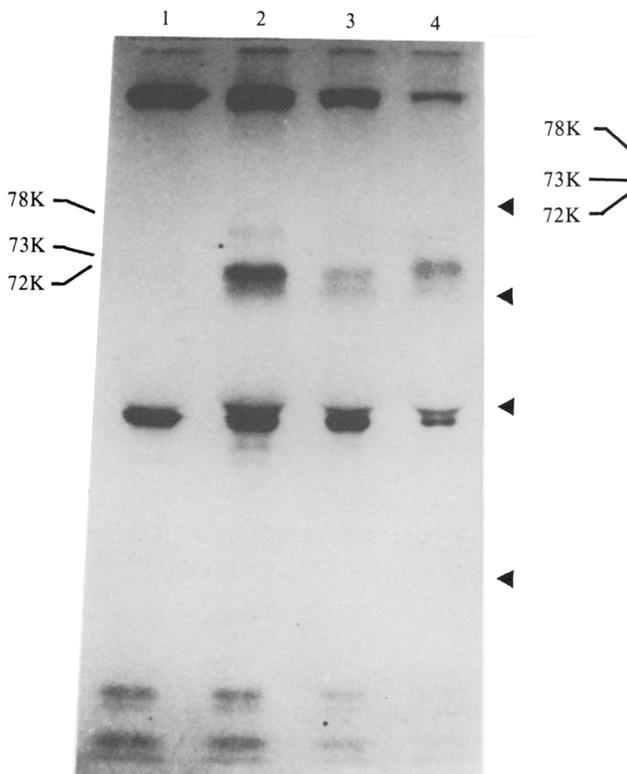


Fig. 6

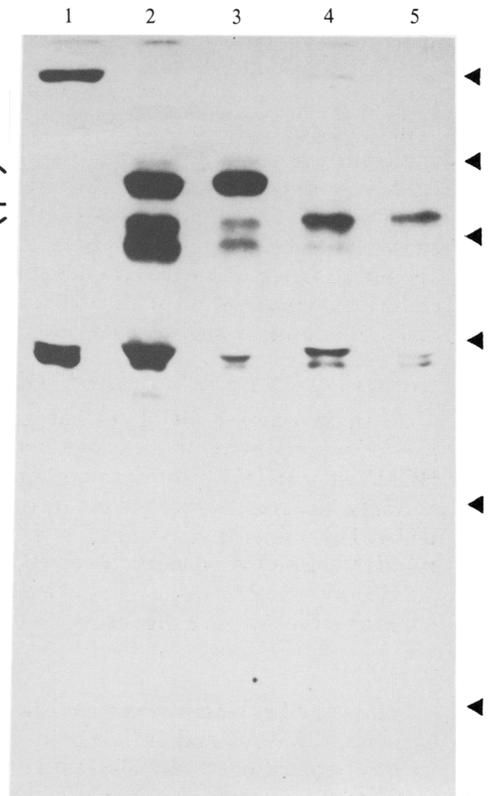


Fig. 7

Fig. 6. Comparison of IE protein synthesis between short-term and long-term persistent infections. HCMVpi was obtained from 118MGC/Towne cultures at 49 and 490 days after infection. Subconfluent monolayers of HEL cells were infected with HCMVwt or HCMVpi at an m.o.i. of 5, or mock-infected. After 1 h adsorption, infected cultures were radiolabelled with [35 S]methionine for 2 h. IE proteins were extracted, immunoprecipitated, and separated by SDS-PAGE. (1) Mock-infected cultures. (2) HCMVwt-infected cultures. (3) Cultures infected with HCMVpi obtained at 49 days. (4) Cultures infected with HCMVpi obtained at 490 days. The mol. wt. of IE-specific proteins are presented, and the arrowheads indicate the mol. wt. of standards (92.5K, 69K, 46K and 30K).

Fig. 7. Pulse-chase analysis of 73K IE protein synthesis. Subconfluent monolayers of HEL cells were infected with HCMVwt or HCMVpi (prepared as described in the legend to Fig. 3), or mock-infected in the presence of CH. At 18 h the CH-containing medium was removed, and cells were labelled for 2 h with [35 S]methionine in methionine-free maintenance medium containing act D. At the end of the labelling period, one culture was immediately processed for subsequent IE protein analysis by immunoprecipitation and SDS-PAGE. The other culture was rinsed three times with growth medium, and incubated for an additional 18 h in act D-containing medium. The cultures were then similarly processed for IE protein analysis. (1) Mock-infected cultures labelled for 2 h. (2) HCMVwt-infected cultures labelled for 2 h. (3) HCMVwt-infected cultures chased for 18 h. (4) HCMVpi-infected cultures labelled for 2 h. (5) HCMVpi-infected cultures chased for 18 h. The mol. wt. of IE-specific proteins are presented, and the arrowheads indicate the mol. wt. of standards (200K, 92.5K, 69K, 46K, 30K and 14.3K).

cultures at 49 and 490 days after infection was examined in HEL cells by SDS-PAGE. The IE protein synthesized by HCMVpi obtained at 49 days was electrophoretically indistinguishable from the 72K IE protein induced by HCMVwt (Fig. 6, lanes 2 and 3). In contrast, HCMVpi obtained from 118MGC/Towne cultures later after the establishment of the persistent infection (490 days) synthesized mainly the 73K IE protein (lane 4). In addition, growth experiments in HEL cells demonstrated that the maximum yield of HCMVpi obtained at 49 days was the same

as that of HCMVwt, whereas there was an approximate 20-fold reduction in the maximum yield of HCMVpi obtained at 490 days when compared to that in HCMVwt-infected cells.

Pulse-chase analysis of 73K protein synthesis

In the experiments described above we found that HCMVpi synthesizes a 73K IE protein, and that there was a 2 h delay in the appearance of 72K and 78K IE proteins as compared to HCMVwt, suggesting that the 73K protein is a precursor of the major 72K IE protein which is synthesized later. To test this possibility CH-enhanced IE proteins were pulse-labelled for 2 h, chased for 18 h and analysed by SDS-PAGE. As shown in Fig. 7, in HCMVpi-infected cells no apparent changes in the electrophoretic mobility of the 73K protein occurred during the 18 h chase period (lanes 4 and 5). These findings suggest that the 73K protein is not a precursor of the major 72K protein, and that the former is comparatively stable.

Analysis of DNA derived from HCMVpi and HCMVwt virions by restriction enzyme digestion

To study whether HCMVpi has a different genome structure from that of HCMVwt, restriction endonuclease cleavage analysis was performed. DNAs extracted from purified HCMVwt or HCMVpi virions were digested with restriction enzymes (*EcoRI*, *BamHI*, *HindIII* or *XbaI*), blot-transferred, and the fragments were hybridized with ³²P-radiolabelled HCMVwt DNA. The results are shown in Fig. 8. There was no detectable alteration in *EcoRI*, *BamHI* or *HindIII* digestion products between HCMVwt and HCMVpi DNA, but obvious differences in the electrophoretic movement of at least two fragments could be detected when HCMVpi and HCMVwt DNAs were digested with *XbaI*.

DISCUSSION

In these studies we have examined the interaction of HCMV with three human CNS cell lines. Of these, 118MGC and IMR-32, but not KGC, cultures were shown to be able to support HCMV replication when infected with virus at a high m.o.i. (10 p.f.u./cell), although the amounts of infectious virus produced in these two cultures were more than 100-fold lower than that in HEL cultures which are fully permissive for HCMV replication. The interaction of HCMV with human brain and choroid plexus cell cultures has been examined by Wroblewska *et al.* (1981), who found that these cell cultures have an ability to support HCMV replication and that the replicated virus remained cell-associated. Moreover, our experiments to study the fate of HCMV-infected CNS lines demonstrated that the 118MGC cultures enter into a long-term (for more than 1 year) persistent infection with HCMV. Because under natural conditions the CNS has often been shown to be involved in classic CID and subtle long-term effects on the CNS are brought about by HCMV infection (Ho, 1982), the persistent infection established in 118MGC cultures (118MGC/Towne) may provide a useful experimental model for study of the interaction of HCMV with the CNS *in vivo*.

Several characteristics of the 118MGC/Towne cultures were investigated. First, production of infectious virus, expression of viral antigens, and infectious centre titres varied widely during cell passage. Similar variation in virus synthesis has been observed in cell cultures persistently infected with another herpesvirus, equine herpesvirus (Dauenhauer *et al.*, 1982), in which DIP were continuously produced and it was indicated that the defective virus is probably necessary to maintain the state of persistence. In addition, Mocarski & Stinski (1979) have shown that a HCMV persistent infection established in human fibroblasts produces DIP. However, in our system we could not detect appreciable amounts of DIP by three experimental approaches: (i) the persistent virus had no interference activity against standard wild-type HCMV, (ii) the HCMVpi DNA synthesized in 118MGC/Towne cultures had a density (1.718 g/ml) identical to that of standard HCMVwt DNA and did not contain defective HCMV DNA species (density 1.67 to 1.68 g/ml) and (iii) digestion of HCMVpi DNA with *EcoRI* detected no unique DNA fragment associated with HCMV DIP DNA (Stinski *et al.*, 1979; Fig. 8). Thus, DIP do not seem to be involved in the maintenance of the persistent infection.

Secondly, the 118MGC/Towne cultures did not produce *ts* mutants or interferon-like substances both of which have been reported to be involved in the establishment and

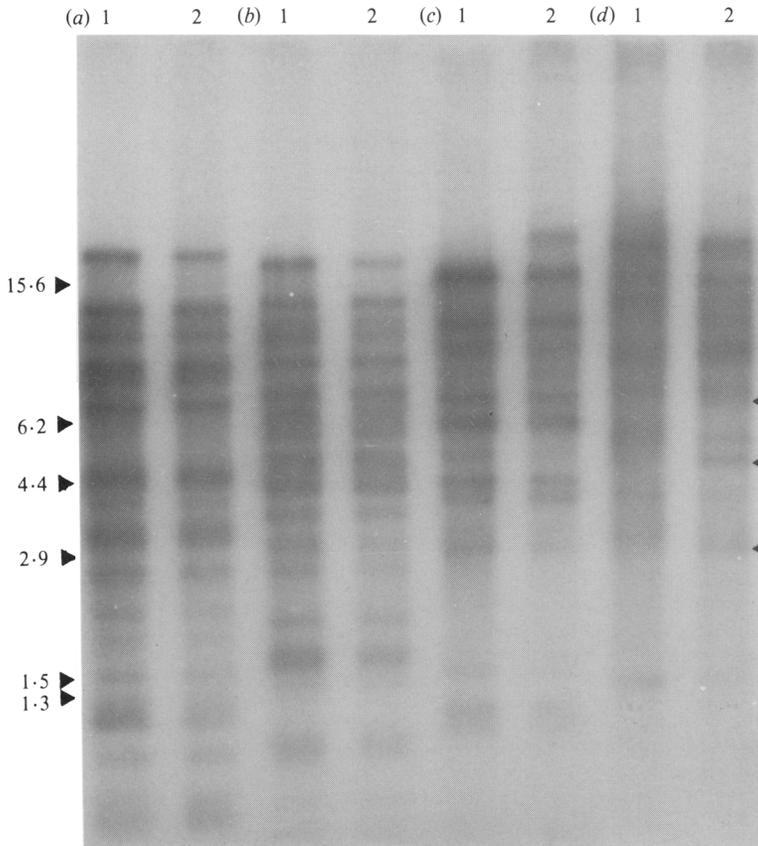


Fig. 8. Restriction enzyme analysis of DNA derived from HCMVwt (lanes 1) and HCMVpi (lanes 2) virions. DNAs were prepared and digested with (a) *EcoRI*, (b) *BamHI*, (c) *HindIII* or (d) *XbaI* as described under Methods. Digested DNA fragments were electrophoresed on a 0.6% agarose gel, and then transferred to nitrocellulose filters. The DNA fragments on the filters were blot-hybridized with ^{32}P -radiolabelled HCMVwt DNA. The blots were autoradiographed by exposure to Kodak X-Omat AR film at -70°C for 24 h. Molecular sizes in megadaltons for λ DNA fragments digested with *HindIII* are indicated at the left hand side. Arrowheads at the right hand side indicate DNA fragments present in HCMVpi and absent in HCMVwt.

maintenance of persistent infections with various viruses (Preble & Youngner, 1975; Dal Canto & Rabinowitz, 1981; Sekellick & Marcus, 1978, 1979). We have isolated uninfected cell clones from 118MGC/Towne cultures and all of these clones were shown to be negative for HCMV-specific IE gene expression and sensitive to superinfection with HCMVwt. Therefore, it is suggested that the maintenance of the 118MGC/Towne infection is due to a balance between the release of infectious virus and the growth of uninfected cells, as in other HCMV persistent infections established by Furukawa (1979, 1984).

Thirdly, we were able to demonstrate that HCMVpi released from 118MGC/Towne cultures is a variant of the virus originally used for infection. This idea was supported by the following evidence. (i) Replication of HCMVpi and its cloned derivatives in HEL and 118MGC cultures was slower, and lower amounts of infectious progeny virus were produced compared with HCMVwt infection (Fig. 3). (ii) HCMVpi and subsequent clones induced a 73K IE protein that was never detected in HCMVwt-infected cells (Fig. 4). (iii) Restriction enzyme analysis showed that HCMVpi had a different DNA structure from HCMVwt DNA (Fig. 8).

The characteristics associated with the variant virus described above were features of long-term persistent infections but not of short-term persistent infections. However, in the present

studies we could not determine whether these variants had been present as subpopulations of the original inoculum, whether persistently infected 118MGC cells might have been selected by enrichment or whether mutations had occurred during the long-term persistence period.

The molecular mechanisms causing HCMVpi to replicate slowly and persist for a long period in 118MGC cultures may be related to the induction of the 73K IE protein by HCMVpi, which has an altered genome. In HCMVpi-infected cultures synthesis of the 73K protein preceded that of the 72K and 78K major IE proteins (Fig. 5). There are two plausible explanations for this finding. One is that the 73K protein is a precursor of the 72K protein. Alternatively, the 73K protein may be non-functional and, consequently, may have an inhibitory effect on the synthesis of the major IE proteins. Our data on pulse-chase labelling of the 73K protein rule out the first possibility. Previous reports (Wathen *et al.*, 1981; Wathen & Stinski, 1982) have shown that HCMV IE proteins act as a trigger for further viral gene expression, and the major 72K IE protein autoregulates the transcription of the viral IE genes (Stenberg & Stinski, 1985). We speculate that the non-functional 73K IE protein results from a minor change in the 72K IE protein and that it may competitively inhibit the synthesis of the normal 72K protein. Analysis of the nature of the 73K IE protein induced by HCMVpi and the relationship between its synthesis and the altered DNA structure is currently being carried out.

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