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Human cytomegalovirus induces JC virus DNA replication in human fibroblasts

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ABSTRACT **JC virus, a human papovavirus, is the causative agent of the demyelinating brain disease progressive multifocal leucoencephalopathy (PML). PML is a rare but fatal disease which develops as a complication of severe immunosuppression. Latent JC virus is harbored by many asymptomatic carriers and is transiently reactivated from the latent state upon immunosuppression. JC virus has a very restricted host range, with human glial cells being the only tissue in which it can replicate at reasonable efficiency. Evidence that latent human cytomegalovirus is harbored in the kidney similar to latent JC virus led to the speculation that during episodes of impaired immunocompetence, cytomegalovirus might serve as helper virus for JC virus replication in otherwise nonpermissive cells. We show here that cytomegalovirus infection indeed leads to considerable JC virus DNA replication in cultured human fibroblasts that are nonpermissive for the replication of JC virus alone. Cytomegalovirus-mediated JC virus replication is dependent on the JC virus origin of replication and T antigen. Ganciclovir-induced inhibition of cytomegalovirus replication is associated with a concomitant inhibition of JC virus replication. These results suggest that reactivation of cytomegalovirus during episodes of immunosuppression might lead to activation of latent JC virus, which would enhance the probability of subsequent PML development. Ganciclovir-induced repression of both cytomegalovirus and JC virus replication may form the rational basis for the development of an approach toward treatment or prevention of PML.**

Herpesviruses can induce papovavirus replication in nonpermissive cells. Herpes simplex virus (HSV) has been shown to induce simian virus 40 (SV40) DNA replication in rodent cells (1, 2). Likewise, human and mouse cytomegalovirus induce SV40 DNA replication in human and mouse fibroblasts, respectively (ref. 3; R.H., unpublished work). Thus, by acting as helper viruses, herpesviruses appear to be able to extend the host range for papovavirus replication. These findings led us to speculate that the replication of the human papovavirus JC virus (JCV) could similarly be enhanced by human herpesviruses. We chose to investigate a potential interaction of human cytomegalovirus (HCMV) and JCV for several reasons: Both viruses are important human pathogens which are highly prevalent in the adult population, with seropositivity ranging between 60% and 90% for either of the two viruses (4, 5). In immunocompetent subjects, primary infection with either JCV or HCMV is typically asymptomatic. However, after primary infection either virus can persist in the latent state in the kidney and possibly at other sites of the human body. Reactivation occurs under conditions of impaired immunocompetence, mostly in advanced stages of AIDS, as a consequence of immunosuppressive therapy, or in the course of certain malignancies (4–6), when shedding of either virus in the urine is a frequent finding. Systemic HCMV infection can involve many different organs. Clearly, HCMV-induced encephalitis is the most threatening complication. JCV, too, induces a severe, fatal neurological disorder, called progressive multifocal leukoencephalopathy (PML), which used to be a rare disease before the introduction of extensive therapeutic immunosuppression and the advent of AIDS (7). Neurological symptoms arise from infection of glial cells, predominantly oligodendroglia, a cell type for which JCV has a strong tropism and which is the site of viral replication in the brain (5).

JCV has an extremely restricted host range. Although a large number of permanent cell lines have been tested for their permissiveness for JCV, primary human fetal glial cells and two transformed derivatives thereof represent the only cell systems in which the virus can be propagated with some efficiency (8–10). But even in glial cells virus titers are relatively low, which reflects the high tissue specificity of JCV replication *in vivo*. To approach the question of a putative interaction between the two viruses, we asked whether HCMV might serve as a helper virus for JCV replication in nonpermissive cell systems. Here we show that this is indeed the case: high-level JCV DNA replication can be found in HCMV-infected fibroblasts, which supports the idea that also *in vivo*, especially during phases of immunosuppression, HCMV might exert helper activity for JCV replication in otherwise nonpermissive cells, which in turn might enhance the probability of PML development.

MATERIALS AND METHODS

Recombinant Plasmid DNAs. Cloned JCV DNA (pJC-GS/B and pJC-GS/K) was kindly provided by K. Dörries (11). JCV DNA was subcloned into the *Eco*RI site of pBluescript KS(+) (Stratagene), yielding pJCm (brain variant) and pJCk (kidney variant). The circular JCV genome is disrupted at the *Eco*RI site within the late gene region, leaving the origin and T-antigen sequence intact. Mutants carrying defects in the origin of replication (pJCΔori), large T antigen (pJCΔT-ag), or small t antigen (pJCΔt-ag) were derived from pJCm by partial *Hin*dIII digestion and filling in the ends with T4 DNA polymerase, thereby introducing frameshifts at position 5112, 4498, or 4914, respectively (nucleotide numbering according to refs. 9 and 12). Plasmids were purified by the alkali lysis protocol with two successive bandings in CsCl (13).

Preparation of Covalently Closed Circular JCV DNA. JCV DNA was separated from vector sequences by digestion of pJCm with *Eco*RI and preparative agarose gel electrophoresis. The eluted 5.2-kb JCV DNA band was recircularized by ligation with T4 DNA ligase at a DNA concentration of 1–10

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Abbreviations: JCV, JC virus; PML, progressive multifocal leukoencephalopathy; HCMV, human cytomegalovirus; HSV, herpes simplex virus; SV40, simian virus 40.

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ng/μl. DNA was concentrated by repeated extractions with 2-butanol and dialyzed. Covalently closed, circular DNA was separated from unligated DNA by CsCl/ethidium bromide gradient centrifugation.

Cells and Viruses. Human embryo fibroblasts at passages 15–20 were grown in Dulbecco's modified Eagle's medium supplemented with antibiotics and 10% heat-inactivated fetal bovine serum. BJAB cells were grown in RPMI 1640 medium with similar supplements. HCMV strain AD169 was propagated on human embryo fibroblasts. Virus stocks had titers of 1–2 × 10^6 tissue culture infectious doses (TCID)/ml.

Transfections. Cells (1–2 × 10^7) were suspended in 600 μl of phosphate-buffered saline and electroporated with 2–10 μg of DNA in cuvettes of 0.4-cm electrode distance at 960 μF and 250V, with a Bio-Rad Gene Pulser. Cells were plated in complete medium. Sixteen hours later cells were infected with HCMV at a multiplicity of infection of 0.1. Cells were harvested for extraction of genomic DNA at the indicated time points. In one set of experiments ganciclovir (gift of H. Freye, Syntex, Aachen, F.R.G.) was added to interfere with HCMV replication.

Southern Blot Analysis. Genomic DNA was isolated as described (13). JCV DNA replication was analyzed on Southern blots of *Eco*RI- and *Dpn* I-digested DNA by using vector-free JCV DNA as a probe, labeled with [α-^{32}P]dCTP by random priming. For analysis of HCMV DNA replication, blots were rehybridized with the 8-kb *Eco*RI-M fragment of HCMV strain AD169. The amount of replicated vector sequences was monitored by rehybridization of the blot with ^{32}P-labeled pUC19 DNA.

RESULTS

HCMV Induces Replication of Cloned JCV DNA. Human diploid fibroblasts have repeatedly been shown to be nonpermissive for JCV replication (9, 14), whereas HCMV replicates to high titers so that a helper effect for JCV replication should be readily detectable. Since high-titer JCV stocks are difficult to obtain, cloned JCV DNA (pJC-GS/B) carrying the origin of replication and the T-antigen sequence was transfected into human fibroblasts which were infected with HCMV 16 hr later. To test for HCMV-induced JCV replication, total genomic DNA was extracted 3, 6, and 9, days after infection. *Eco*RI/*Dpn* I-digested DNA was probed with ^{32}P-labeled JCV DNA. *Dpn* I selectively digests transfected plasmid DNA which has been methylated during prokaryotic replication, whereas DNA replicated in eukaryotic cells is unmethylated and thus *Dpn* I-resistant. Upon digestion with *Eco*RI, replicated JCV DNA is separated from vector sequences and is visible as unit-length molecule of 5.2 kb. HCMV induced JCV DNA replication, which increased in parallel with increasing levels of HCMV DNA replication, as detected by hybridization of the same blot first with a JCV-specific DNA probe and then with a HCMV-specific probe (Fig. 1). As expected, no trace of JCV DNA replication was found after transfection of JCV without subsequent HCMV infection. The amount of JCV DNA produced 9 days after HCMV infection was in the range of 10^3–10^4 copies per cell. This was calculated by comparison of the strong JCV signal at 9 days postinfection with that of 2.5 ng of *Eco*RI-digested pJC-GS/B used as sensitivity marker (Fig. 1). The amount of JCV DNA produced was in the range of that described for fully permissive fetal glial cells (10). Recently it has been described that the B-lymphoma cell line BJAB can be infected with JCV to produce JCV DNA replication (15). We were able to confirm this finding. Yet, upon cocultivation of BJAB cells with JCV-transfected and HCMV-infected fibroblasts we failed to detect production of infectious JCV even after prolonged incubation periods (data not shown). The experiment in Fig. 1 was repeated with pJC-GS/K,

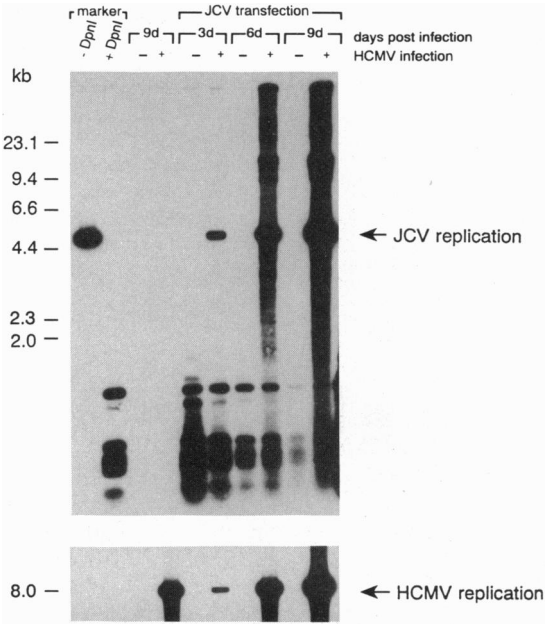

FIG. 1. HCMV induces JCV DNA replication. Human embryo fibroblasts were electroporated with cloned JCV DNA (pJC-GS/B) and 16 hr later infected with HCMV at a multiplicity of infection of 0.1. Cells were harvested at 3, 6, or 9 days postinfection as indicated. A Southern blot of *Eco*RI/*Dpn* I-digested genomic DNA (5 μg per lane) was hybridized to ^{32}P-labeled JCV DNA (*Upper*). The film was exposed for 4 hr with an intensifying screen. HCMV-induced JCV replication yields *Dpn* I-resistant unit-length molecules of 5.2 kb. Bands in the low molecular weight range represent *Dpn* I-digested DNA, which decreases with increasing time postinfection. The blot was rehybridized with a HCMV-specific probe (*Lower*). Sensitivity markers were 2.5 ng of pJC-GS digested with *Eco*RI or with *Eco*RI plus *Dpn* I, respectively.

which had been cloned from the kidney of the same patient (16), leading to comparable JCV replication levels in the presence of HCMV (data not shown).

HCMV-Induced JCV Replication Is Origin- and T-Antigen-Dependent. In permissive cells JCV DNA replication is dependent on an intact origin of replication in cis and T antigen in trans. To evaluate whether these functions are similarly required for HCMV-induced JCV DNA replication, mutant JCV genomes derived from the wild-type JCV sequence subcloned into pBluescript (pJCm) were transfected. pJCΔori carries a 4-bp insertion at position 5112. Small deletions around this site have been shown to destroy JCV origin function in permissive human glial cells without affecting T-antigen expression (17). The frameshift within the T-antigen open reading frame of pJCΔT-ag leads to a premature translational stop at codon 33. As expected, neither pJCΔori nor pJCΔT-ag was replicated upon HCMV infection (Fig. 2). pJCΔt-ag was replicated at levels similar to wild-type pJCm. This mutant differs from wild type only at the ultimate C terminus of small t antigen, which appears not to be essential for JCV DNA replication. Rehybridization of the blot with the HCMV-specific probe (Fig. 2 *Lower*) demonstrated equal levels of HCMV replication in all transfections. In another set of experiments it was confirmed that HCMV did not induce replication of the vector (pUC19) alone (Fig. 3 *Middle*). In the cloned plasmid (pJC-GS/B) the vector portion was replicated at equimolar amounts compared to JCV. However, this was a passive effect, since Fig. 2 shows that HCMV-induced replication of cloned JCV depended on a functional JCV origin of replication and on T antigen. On the other hand, vector-free, covalently closed, circularized JCV DNA replicated to similar levels as cloned JCV DNA upon infection with HCMV (Fig. 3 *Top*).

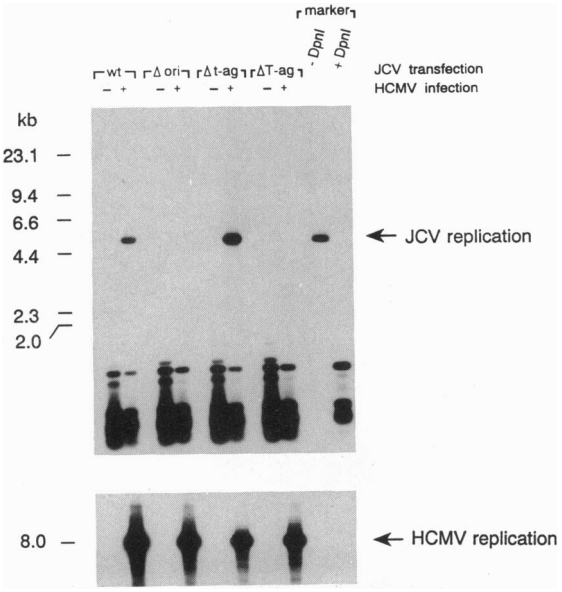

FIG. 2. HCMV-induced JCV replication is origin- and T-antigen-dependent. The experiment was performed as in Fig. 1, except that the following JCV constructs were transfected: pJCm (wild type, wt), pJCΔori (Δori), pJCΔt-ag (Δt-ag), and pJCΔT-ag (ΔT-ag). Genomic DNA was extracted for Southern blot analysis 5 days after infection.

Ganciclovir Inhibits HCMV-Induced JCV Replication. Experiments with neutralizing antibodies against HCMV virions showed that active HCMV infection was required for the induction of JCV replication (data not shown). This excluded the possibility that an HCMV-induced factor present in the AD169 stocks was responsible for the observed effects, rather than HCMV itself. A separate specificity control was performed with the nucleoside analog 9-(1,3-dihydroxy-2-propoxymethyl)guanine (ganciclovir), a drug with high selectivity for HCMV (18–20). In uninfected cells ganciclovir is inactive, since its mechanism of action involves the formation of the nucleoside triphosphate which is catalyzed by the HCMV-encoded kinase UL97 (21, 22). The active compound is a strong inhibitor of the HCMV-encoded DNA polymerase, with a significantly lower inhibitory activity toward cellular DNA polymerase α (23). Ganciclovir-mediated reduction of HCMV DNA replication was paralleled by a concomitant reduction in JCV DNA replication (Fig. 4), showing that JCV replication was dependent on efficient HCMV replication.

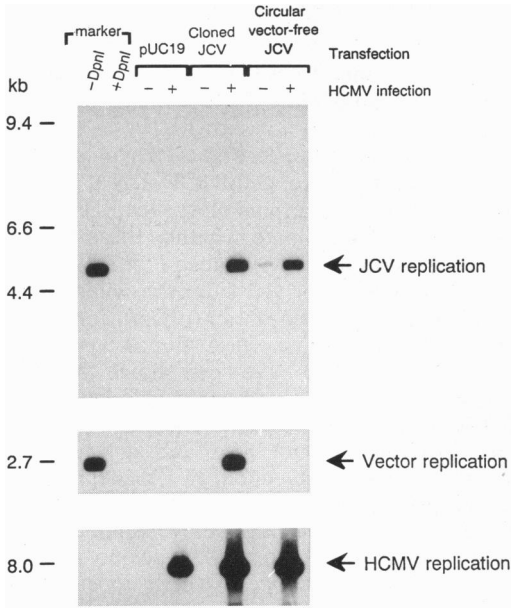

FIG. 3. HCMV induces replication of circularized, vector-free JCV DNA. The experiment was performed as described in Fig. 1. The following DNAs were transfected: pUC19; pJC-GS/B (cloned JCV); unit-length JCV DNA freed from vector sequences, recircularized *in vitro*, and gradient-purified (circular, vector-free JCV). Genomic DNA was extracted 5 days postinfection. After hybridization with the JCV-specific probe (*Top*), the blot was rehybridized with pUC19 DNA to control for vector replication (*Middle*) and then with the HCMV-specific probe to document comparable levels of HCMV replication (*Bottom*). The faint band seen upon transfection of circular JCV DNA without HCMV infection represents minimal nonspecific *Dpn* I resistance due to DNA damage upon extensive purification of *in vitro* circularized JCV DNA.

DISCUSSION

In this report we identify HCMV as a potent helpervirus for JCV replication in human fibroblasts that are nonpermissive for the replication of JCV alone. It is not clear at present whether fibroblasts are sites of either JCV or HCMV persistence in the human body. However, both JCV and HCMV have been shown to establish latency in the kidney, and there is evidence that both viruses can infect tubular epithelial cells. Immunosuppression consistently causes viral reactivations which are associated with shedding of viral particles in the urine (4, 6). HCMV reactivation may either lead to replication of latent JCV genomes residing in the same cell or, alternatively, lead to productive HCMV infection so that HCMV circulating in the bloodstream may infect cell types in which JCV resides latent. This interaction is not necessarily confined to the kidney. PML results from productive JCV replication in glial cells which eventually are destroyed. HCMV can infect glial cells as well, which opens the possibility that HCMV directly enhances JCV replication in the cells relevant for the pathogenesis of PML. In support of this assumption we have found that HCMV enhances JCV replication in human glioblastoma cells (I.A. and R.H., unpub-

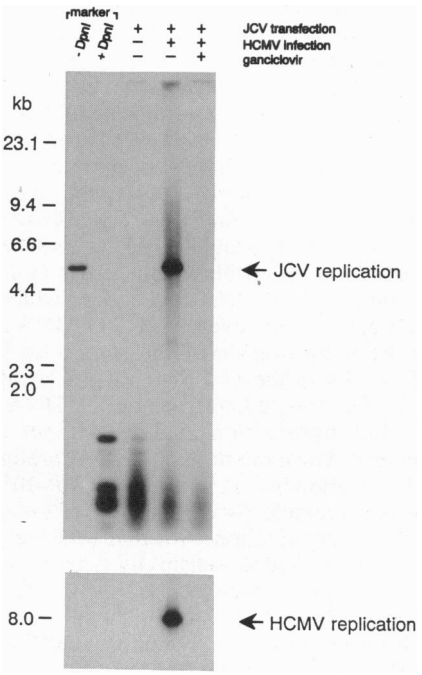

FIG. 4. Ganciclovir inhibits HCMV-induced JCV replication. The experiment was performed as in Fig. 1. pJC-GS/B was transfected and 16 hr later cells were infected with HCMV strain AD169 as indicated. Parallel cultures were incubated with or without ganciclovir (100 μM) for 5 days with daily changes of medium.

lished observation). Furthermore, by the use of the polymerase chain reaction technique it has become possible to demonstrate that JCV resides in the brains of many asymptomatic JCV carriers (24, 25). Thus, the presence of latent JCV in brain cells, in conjunction with repeated episodes of immunosuppression resulting in transient HCMV viremia, offers ample opportunity for a productive interaction of the two viruses which may eventually enhance the probability of PML development.

We do not know which HCMV functions mediate helper activity. Ganciclovir-mediated repression of JCV replication basically offers two alternative possibilities. Late HCMV genes, whose efficient expression relies on DNA replication, may represent the critical function(s) necessary for the induction of JCV replication. So far only a few HCMV genes with stringent late gene expression have been identified, most of which code for structural proteins (26). Although we cannot exclude a possible role of these genes in the helper effect for JCV, we would rather envisage regulatory proteins or proteins with enzymatic function as potential helper functions. Previous work on HSV-induced SV40 amplification in rodent cells which are nonpermissive for SV40 replication showed that a subset of six out of seven HSV replication genes was necessary and sufficient for the induction of SV40 amplification (13). By analogy one would speculate that the HCMV replication complex, or a component thereof, could be the critical factor for the replication of JCV in nonpermissive cells. So far, only the HCMV DNA polymerase, its accessory protein, and the major DNA-binding protein have been characterized in some detail (20, 27–32). Unambiguous definition and functional testing of the components of the HCMV replication complex have become possible with the recent identification of the HCMV origin of replication, oriLyt (33–35). Since oriLyt has a more complex structure than the HSV origins, in addition to a much slower replication cycle in HCMV compared with HSV, it is not surprising that some of the HCMV functions involved in DNA replication appear to be different from the respective HSV functions (ref. 30; D. G. Anders, personal communication). This assumption is supported by the finding that HSV infection only leads to marginal JCV DNA replication in human fibroblasts (R.H., unpublished observation).

The current view of the pathogenesis of PML development assumes an asymptomatic primary infection by a JCV archetypal strain, with establishment of latency in the immunocompetent host. Repeated reactivations lead to the generation of viral variants with altered tissue tropism. Indeed, JCV isolates from brains of PML patients differ from kidney isolates by rearrangements within the regulatory region, which may contribute to the process of adaptation to growth in an additional tissue or cell type (11, 16, 36–38). It is conceivable that HCMV might not only enhance replication of archetype JCV but in addition might promote this adaptation process by an active contribution to the rearrangement of the JCV regulatory region. This is suggested by the fact that HCMV has been shown to induce host-cell repair replication and chromosomal aberrations in human fibroblasts (39, 40). Likewise for HSV the induction of genetic instability, including point mutations and DNA amplification within the host cell genome, or recombination between duplicated target DNA sequences has been characterized. These activities are mediated by the HSV replication complex (13, 41, 42). A similar activity of HCMV, in conjunction with the ability to replicate JCV DNA, could greatly enhance the development of JCV variants from archetypal strains.

Although the pathogenesis of PML development is not entirely understood, it is obvious that the discrepancy between the high incidence of primary infection and the relative rarity of PML in conjunction with the long latency period between the two events calls for additional pathogenetic factors. Clearly immunosuppression represents the triggering event. But does it do so *per se*? The unexpectedly high incidence of PML in AIDS patients might in part be explained by a direct interaction of human immunodeficiency virus and JCV (43). HCMV reactivations due to immunosuppression may represent another factor which can directly activate latent JCV. Epidemiological studies and the direct demonstration of the coexistence of the two viruses in the same cells *in vivo* will help to clarify this point. Fortunately, therapeutic or prophylactic intervention appears feasible, as we have shown that ganciclovir inhibits JCV replication along with HCMV replication. Early and efficient treatment of HCMV reactivation in the presymptomatic patient would be required to reduce the risk of JCV reactivations and, it is hoped, PML development as well.

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