Human cytomegalovirus requires cellular deoxycytidylate deaminase for replication in quiescent cells

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We have previously observed that the expression of two thymidylate biosynthesis enzymes, dihydrofolate reductase and thymidylate synthase (TS), is upregulated in quiescent human fibroblasts infected with human cytomegalovirus (HCMV). Here, we have demonstrated that HCMV increases expression of the cellular deoxycytidylate deaminase (dCMP deaminase), which provides the substrate for TS by converting dCMP to dUMP. We observed an increase in dCMP deaminase protein levels, whereas deoxyuridine triphosphatase (dUTPase), another cellular enzyme that may provide dUMP by hydrolysing dUTP, was undetectable. The essential requirement of cellular dCMP deaminase for productive HCMV replication was further emphasized by showing that a precursor of a potent dCMP deaminase inhibitor, zebularine, suppressed virus replication and DNA synthesis. These results suggest that HCMV exploits the host’s dCMP deaminase activity to replicate in quiescent cells.
dCMP deaminase expression and that its activity is required for efficient virus replication and DNA synthesis in quiescent cells.

To investigate whether HCMV infection of quiescent cells correlates with the regulation of cellular dCMP deaminase and/or dUTPase expression, quiescent HELF cells (low-passage human embryonic lung fibroblasts) were infected with HCMV AD169 (m.o.i. of 5) and at different time points post-infection (p.i.), cell extracts were prepared and examined for the presence of these enzymes by immunoblotting with specific antisera. Quiescent cells (arrested in G0/G1 phase) were obtained by incubating confluent HELF monolayers for 48 h in medium containing 0.5 % serum. Flow cytometry confirmed that more than 90 % were growth-arrested. As shown in Fig. 2(A), dCMP deaminase protein was undetectable in mock-infected cells. In HCMV-infected cells, dCMP deaminase was detected at 24 h p.i., peaked at 48 h p.i. and then declined to the basal level by 96 h p.i. and as expected, its level was upregulated by serum stimulation of quiescent cells. This HCMV-stimulated polypeptide immunostained by the anti-dCMP deaminase serum was also identified as dCMP deaminase by its comigration with the 20 kDa recombinant dCMP deaminase protein (Weiner et al., 1993). In contrast, when the same extracts were probed with an anti-dUTPase antiserum, expression of this dUMP-synthesizing enzyme appeared not to be affected by HCMV infection (Fig. 2A), as it was only detectable in extracts prepared from mock-infected cells stimulated with 10 % serum for 24 h.

To determine whether stimulation of dCMP deaminase expression by HCMV was a consequence of viral gene expression, quiescent cells were also infected with UV-inactivated HCMV and expression of the viral IE1 protein was used as a marker of virus infectivity. A pulse of 1.2 J cm\(^{-2}\) completely ablated expression of IE1 (Fig. 2A) and no dCMP deaminase protein was detected at 48 h p.i., demonstrating that active viral gene expression is required to induce cellular dCMP deaminase expression.
Further evidence of this requirement was obtained by immunofluorescence assays on HCMV-infected cells, which were costained with anti-dCMP deaminase and anti-IE1 antibodies. Confocal laser microscopy showed that only cells expressing the IE1 antigen reacted with the anti-dCMP deaminase serum (Fig. 2B). The dCMP deaminase staining pattern was specific for HCMV-infected cells, since mock-infected cells were not stained.

These results demonstrate that HCMV infection of quiescent HELF cells enhances dCMP deaminase, but does not stimulate dUTPase expression. Furthermore, stimulation of dCMP deaminase expression depends on active HCMV gene expression, rather than simply binding and entry of the virus particle.

dCMP deaminase is a key enzyme in pyrimidine deoxyribonucleotide metabolism. It provides the nucleotide substrate for TS and its activity is allosterically regulated by the ratio of dCTP to dTTP, with dCTP as activator and dTTP as inhibitor in the cell (Maley & Maley, 1990). As with the TS enzyme, dCMP deaminase activity is associated with cell proliferation and regulated by cell cycle progression, since it is highest in S phase and then declines in the G2 phase (Maley & Maley, 1990). Moreover, the demonstration of its increased activity in a wide variety of experimental animal tumours and human neoplasia makes it a potential target for anticancer chemotherapy.

The data in Fig. 2 raised the question of whether induction of dCMP deaminase expression by HCMV is needed for viral DNA replication in quiescent cells, or whether it stems from a more generalized stimulation of host transcription and translation in HCMV-infected cells. We therefore examined the effects on HCMV replication of zebularine [1-(β-D-ribofuranosyl)-dihydropyrimidin-2-one], a compound that can be metabolized to zebularine 2′-deoxyribose 5′-monophosphate, a potent dCMP deaminase inhibitor (Maley et al., 1993). Quiescent HELF cells were infected with HCMV at an m.o.i. of 1 and after virus adsorption, medium containing various concentrations (0.1–500 μM) of zebularine was added. Cultures were incubated until the controls displayed 100% CPE and supernatants were then assayed for infectivity by a standard plaque assay. Zebularine produced a significant dose-related reduction in HCMV yield at concentrations much lower than those producing cytotoxic effects (Fig. 3A). The calculated 50% and 90% antiviral effective concentrations (EC50 and EC90) were 30 μM and 75 μM, respectively. Evaluation by the MTT test (Pauwels et al., 1988) after 4 days demonstrated that zebularine did not significantly affect the viability of quiescent mock-infected cells (the 50% cytotoxic concentration was more than 1 mM, Fig. 3A). Its effects on HCMV replication, therefore, were not due to a generalized cellular toxicity. To confirm that dCMP deaminase was the drug’s sole target, 2′-deoxyuridine was added in combination with 1 EC50 zebularine to infected cells. Fig. 3(B) shows that 2′-deoxyuridine reversed the drug’s antiviral activity.
The effects of zebularine on HCMV DNA synthesis were evaluated by quantifying viral DNA levels at 96 h p.i. by competitive PCR. The target for PCR amplification was a segment of IE1 exon 4 generating an amplification product of 639 bp and the competitor DNA was a fragment of the same sequence as the target except for a 78 bp insertion to allow identification after electrophoresis. As shown in Fig. 3(C), the equivalence between target and competitor corresponding to a 1 : 1 molar ratio, and therefore indicating the number of target molecules initially present in the reaction, was obtained for control DNA at a concentration of about 10⁸ molecules. However, for DNA extracted from cells treated with zebularine at 100 μM, the equivalence was reached at about 5 × 10⁶ molecules, showing that zebularine inhibited viral DNA synthesis more than 50-fold. The observation that a dCMP deaminase inhibitor suppresses HCMV DNA synthesis strongly supports the conclusion that virus-induced dCMP deaminase enzyme activity is critical for efficient virus replication in quiescent cells.

Finally, to determine whether inhibition included other events in the HCMV growth cycle, the expression of immediate-early (IE1), early (UL44) and late (UL99) proteins was examined by immunofluorescence analysis. Neither IE1 nor UL44 expression was inhibited by 1EC₃₀ zebularine, but late protein expression was (data not shown).

Earlier studies have established that infection with other DNA viruses such as simian virus 40, herpes simplex virus type 1 (HSV-1) and X14 or H-1 parovirus stimulates host dCMP deaminase activity (Hatanaka & Dulbecco, 1966; Rolton & Keir, 1974; Ricceri et al., 1978). The significance of the dCMP deaminase pathway in HSV-1-infected cells has since been analysed by means of halogenated analogues of deoxyuridine and deaminase inhibitors. The results of these studies indicate that during virus replication the bulk of TTP for viral DNA synthesis is derived mainly from this pathway (Aduma et al., 1990, 1991). In the present study, we have shown that the cellular content of the dCMP deaminase protein is upregulated following HCMV infection of quiescent fibroblasts. The dependence of HCMV replication on dCMP deaminase activity is further supported by the results obtained with the pyrimidin-2-one nucleoside zebularine. However, cytidine deaminase expression is undetectable in human fibroblasts, suggesting that it plays a minor role in the synthesis of 2'-dCMP in these cells (Kuhn et al., 1993). This suggests that zebularine's anti-HCMV effects can be ascribed mainly to its dCMP deaminase inhibitory activity. The potent activity of zebularine against HCMV in quiescent HELF cells may depend on the fact that dCMP deaminase levels are much lower in quiescent uninfected cells than in proliferating cells (Maley & Maley, 1990). This observation could be exploited in HCMV-infected cells where elevated levels of dCMP deaminase could provide a specific target for anti-CMV drugs, such as zebularine, which may be only deleterious to the rapidly replicating viral DNA. A 90 % reduction in virus yield was in fact achieved with drug concentrations (75 μM) well below those required for its cytotoxic activity (> 1 mM) (Fig. 3).

We have also observed a differential regulation of the two main dUMP-providing enzymes in quiescent fibroblasts infected with HCMV (Fig. 2A) indicating that dCMP deaminase may be the major contributor of the TS substrate for de novo TTP synthesis. However, the lack of a significant stimulation of cellular dUTPase raises the question of whether this enzymatic activity is required for HCMV replication at all, since, in addition to its role in supplying dUMP, it plays a critical role in the maintenance of uracil-free DNA by reducing the availability of dUTP as a substrate for DNA replication. The occurrence within the HCMV genome of the UL72 gene, which is regarded as the evolutionary counterpart of the dUTPase gene in other herpesviruses, may suggest a role for this viral gene in compensating for the absence of stimulation of the cellular enzyme, but since the encoded protein lacks canonical amino acid sequence motifs it is probably not an active dUTPase (McGeoch & Davison, 1999).

These findings, along with previous studies showing increased DHFR and TS activities during HCMV infection (Lembo et al., 1999; Gribaudo et al., 2002), demonstrate that HCMV coordinately activates the expression of several cellular enzymes involved in the synthesis of dTMP and hence does not require an S-phase environment for its replication. Since most adult animal tissues are differentiated and actively regulated to remain in a quiescent state, the stimulation of de novo pathways of nucleotide biosynthesis in post-mitotic cells may have a significant role in the pathogenesis of CMV diseases.

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