Journal of General Virology (2003), 84, 1437–1441 DOI 10.1099/vir.0.18979-0

Short Communication

Human cytomegalovirus requires cellular deoxycytidylate deaminase for replication in quiescent cells

Giorgio Gribaudo,[1] Ludovica Riera,[1] Patrizia Caposio,[1] Frank Maley[2] and Santo Landolfo[1]

[1]Department of Public Health and Microbiology, University of Torino, Via Santena 9, 10126 Torino, Italy

[2]Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York, USA

Correspondence
Giorgio Gribaudo
giorgio.gribaudo@unito.it

Received 15 November 2002
Accepted 3 February 2003

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.

Human cytomegalovirus (HCMV) is a ubiquitous herpesvirus which generally causes asymptomatic infections in healthy individuals but may lead to severe diseases in immunologically immature or immunocompromised hosts (Mocarski & Courcelle, 2001; Landolfo *et al.*, 2002). Its success as an opportunist largely depends on its ability to establish latent life-long infections, counteract host antiviral defence mechanisms and replicate in a wide variety of cells and tissues, including differentiated post-mitotic cells such as mesenchymal cells, endothelial cells, epithelial cells, smooth muscle cells and monocytes/macrophages (Hengel *et al.*, 1998; Fortunato *et al.*, 2000; Bissinger *et al.*, 2002).

Unlike other herpesviruses, HCMV does not encode specific dNTP biosynthetic enzymes, such as thymidine kinase (TK), dihydrofolate reductase (DHFR), thymidylate synthase (TS) or an active form of ribonucleotide reductase (Chee *et al.*, 1990). It thus depends mainly on host cell metabolism for a sufficient concentration of dNTPs for the replication of its DNA. However, the biochemical pathways needed for such replication are expressed at very low levels in non-dividing cells. To explain this apparent paradox, it has been hypothesized that in these cells HCMV infection stimulates the relevant host dNTP-synthesizing enzymes. We have indeed demonstrated that this occurs for some of the enzymes involved in thymidylate biosynthesis, since infection of quiescent fibroblasts with both murine and human CMV increases cellular DHFR and TS content, and inhibition of these activities abrogates virus replication (Lembo *et al.*, 1998, 1999; Gribaudo *et al.*, 2000, 2002). TS catalyses the *de novo* biosynthesis of thymidylic acid (dTMP) by reductive transfer of the methylene group from 5,10-methylenetetrahydrofolate to the 5-position of the substrate, deoxyuridylic acid (dUMP), to form dTMP and dihydrofolate (Maley & Maley, 1990; Johnson, 1994). Therefore, the intracellular availability of dUMP may be the rate-limiting step in dTMP biosynthesis and hence critical for efficient CMV replication in quiescent cells. In mammalian tissues, dUMP may be produced by the deamination of deoxycytidine 5′-monophosphate nucleotide (dCMP) catalysed by deoxycytidylate aminohydrolase (dCMP deaminase) (EC 3.5.4.12). Alternatively, dUMP generation may involve the reduction of UDP to dUDP by ribonucleotide reductase, followed by phosphorylation of dUDP to dUTP and finally hydrolysis of dUTP to dUMP and pyrophosphate by deoxyuridine triphosphate pyrophosphatase (dUTPase) (Fig. 1) (Maley & Maley, 1990; McIntosh & Haynes, 1997).

Since elucidation of the mechanisms of virus-mediated regulation of host biosynthetic pathways may lead to the identification of new targets suitable for the design of molecules with antiviral activity, we have further analysed the effects of HCMV infection on the expression of enzymes involved in dTMP synthesis by addressing the source of the TS substrate dUMP in HCMV-infected cells. In this study, we have demonstrated that HCMV infection stimulates

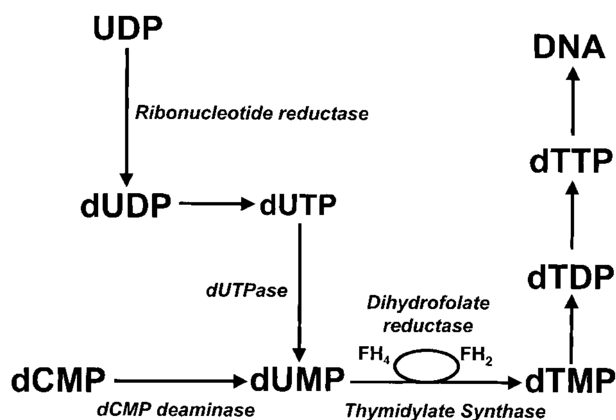

Fig. 1. Partial schematic diagram of *de novo* dTMP synthesis. The roles of thymidylate synthase, dihydrofolate reductase, ribonucleotide reductase, dCMP deaminase and dUTPase are illustrated. The activities of dCMP deaminase and dUTPase supply dUMP pools for thymidylate synthase. FH_2, dihydrofolate; FH_4, tetrahydrofolate.

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 G_0/G_1 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

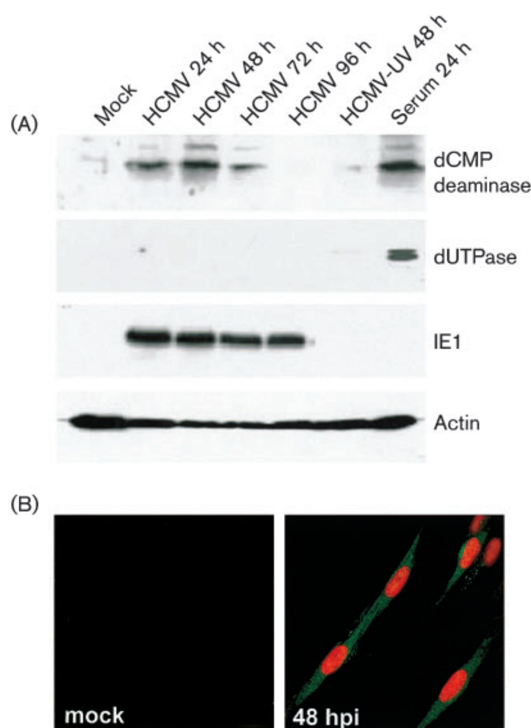

Fig. 2. Differential regulation of dCMP deaminase and dUTPase expression by HCMV infection in quiescent fibroblasts. (A) HCMV increases dCMP deaminase protein levels. HELF cells were growth-arrested in 0·5 % foetal bovine serum and then infected with HCMV or UV-irradiated HCMV, mock-infected, or serum-stimulated to re-enter the cell cycle. Total cell extracts were prepared at the indicated times p.i. and fractionated by SDS-PAGE (50 μg protein per lane). Proteins were analysed by immunoblotting with rabbit anti-human dCMP deaminase serum (Maley *et al.*, 1993; Weiner *et al.*, 1993), with rabbit serum raised against the human recombinant dUTPase N protein (Ladner *et al.*, 1996) or with anti-IE1 mAb. Actin immunodetection with a mAb was performed as an internal control. Cell extracts were isolated from mock-infected cells, cells that had been infected with HCMV for 24, 48, 72 and 96 h, cells infected with UV-treated HCMV for 48 h, or cells stimulated with 10 % serum for 24 h. (B) dCMP deaminase protein localization in HCMV-infected quiescent HELF cells by indirect immunofluorescence. HELF cells grown on coverslips were growth-arrested in 0·5 % foetal calf serum for 48 h and then mock-infected or infected with HCMV (m.o.i. of 0·2). At 48 h p.i., cells were fixed with acetone/methanol, permeabilized and costained with anti-dCMP deaminase antibodies and mouse anti-HCMV IE1 mAb. The merged image shows the combination of dCMP deaminase expression (green fluorescence) and HCMV IE1 expression (red fluorescence) in infected cells.

was used as a marker of virus infectivity. A pulse of 1·2 J cm^{-2} completely abolished 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 G_2 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 (EC_{50} and EC_{90}) 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 EC_{90} zebularine to infected cells. Fig. 3(B) shows that 2′-deoxyuridine reversed the drug's antiviral activity.

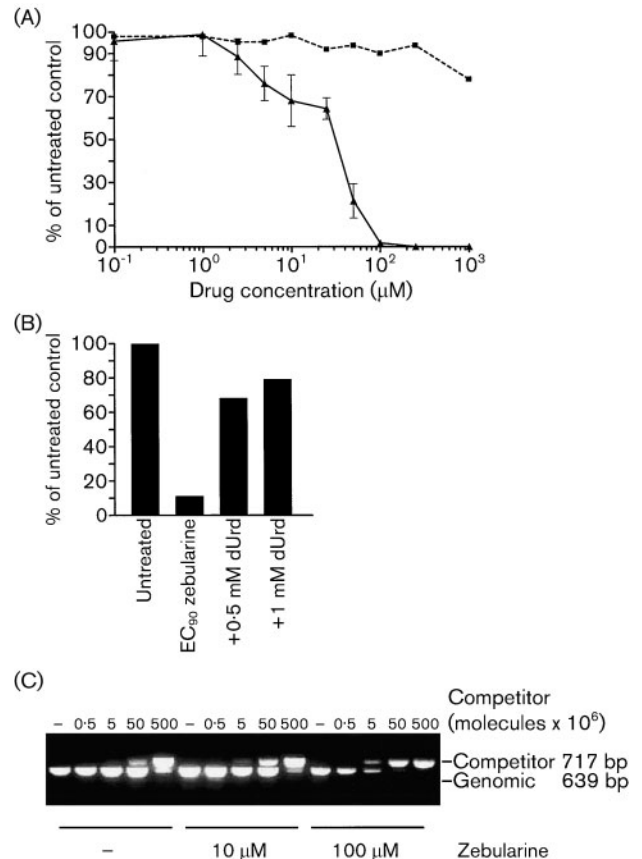

Fig. 3. Inhibition of HCMV replication and viral DNA synthesis by zebularine. (A) Inhibition of HCMV replication. Cultures of HELF cells were growth-arrested in 0·5 % foetal bovine serum, then mock-infected or infected with HCMV at an m.o.i. of 1. After virus adsorption, cells were incubated with increasing concentrations of zebularine until an extensive viral CPE was observed in the untreated control. The infectivity of the supernatants was then evaluated in a standard plaque assay. Values are the means of three independent experiments. To determine cell viability, HELF cells were growth-arrested in 0·5 % foetal bovine serum and then incubated with increasing concentrations of zebularine. After 4 days incubation, the number of viable cells was determined by the MTT method, as described by Pauwels *et al.* (1988). ■, Cell viability; ▲, HCMV yield. (B) Effect of 2′-deoxyuridine on zebularine anticytomegaloviral activity. Growth-arrested HELF cells were infected with HCMV at an m.o.i. of 1 and then exposed to 1 EC_{90} (75 μM) of zebularine in the absence of or with increasing concentrations of 2'-deoxyuridine (dURd) until an extensive viral CPE was observed in the untreated control. Supernatants were collected and titrated as described above. (C) Inhibition of HCMV DNA synthesis. HELF cells were growth-arrestedin 0·5 % foetal bovine serum and then mock-infected or infected with HCMV at an m.o.i. of 1. After virus adsorption, cells were incubated with increasing concentrations of zebularine. Total genomic DNA was then purified at 96 h p.i. and 50 ng was mixed with increasing concentrations of competitor DNA ranging from 5×10^5 to 5×10^8 molecules and subjected to PCR. PCR products were fractionated by 2 % agarose gel electrophoresis, visualized by ethidium bromide staining and the bands corresponding to competitor and genomic DNA were quantified by densitometric scanning. The positions of genomic and competitor DNA are indicated on the right.

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 10^8 molecules. However, for DNA extracted from cells treated with zebularine at 100 μM, the equivalence was reached at about 5×10^6 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 $1EC_{90}$ 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 parvovirus 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 deoxycytidine 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. This compound is a potent inhibitor of human cytidine/2′-deoxycytidine deaminase and also of dCMP deaminase following its intracellular metabolism to the 2′-deoxy-5′-monophosphate derivative (Maley *et al.*, 1993; Barchi *et al.*, 1995). 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.

ACKNOWLEDGEMENTS

We thank Bob Ladner for providing the anti-dUTPase N serum and Victor Marquez for a generous sample of zebularine. This work was supported by grants from MURST-CNR Biotechnology programme L. 95/95, MURST (40 % and 60 %) to G. G. and S. L., from the AIDS Research Project and from the Ricerca Sanitaria Finalizzata (Regione Piemonte) to S. L., and by grants to F. M. from the National Institutes of Health/NCI, CA 44355.

REFERENCES

Aduma, P. J., Gupta, S. V. & De Clercq, E. (1990). Antiherpes virus activity and effect on deoxyribonucleotide triphosphate pools of (E)-5-(2-bromovinyl)-2′-deoxycytidine in combination with deaminase inhibitors. *Antivir Res* **13**, 111–126.

Aduma, P. J., Gupta, S. V., Stuart, A. L. & Tourigny, G. (1991). Deoxyribonucleotide triphosphate pools of herpes simplex virus infected cells: the influence of selective antiherpes agents and the role of deaminase pathway. *Biochem Cell Biol* **69**, 409–414.

Barchi, J. J., Jr, Cooney, D. A., Hao, Z., Weinberg, M., Taft, C., Marquez, V. E. & Ford, H., Jr (1995). Improved synthesis of zebularine [1-(β-D-ribofuranosyl)-dihydropyrimidin-2-one) nucleotides as inhibitors of human deoxycytidylate deaminase. *J Enzym Inhib* **9**, 147–162.

Bissinger, A. L., Sinzger, C., Kaiserling, E. & Jahn, G. (2002). Human cytomegalovirus as a direct pathogen: correlation of multiorgan involvement and cell distribution with clinical and pathological findings in a case of congenital inclusion disease. *J Med Virol* **67**, 200–206.

Chee, M. S., Bankier, A. T., Beck, S. & 12 other authors (1990). Analysis of the protein-coding content of human cytomegalovirus strain AD169. *Curr Top Microbiol Immunol* **154**, 125–169.

Fortunato, E. A., McElroy, A. K., Sanchez, V. & Spector, D. H. (2000). Exploitation of cellular signaling and regulatory pathways by human cytomegalovirus. *Trends Microbiol* **8**, 111–119.

Gribaudo, G., Riera, L., Lembo, D., De Andrea, M., Gariglio, M., Rudge, T. L., Johnson, L. F. & Landolfo, S. (2000). Murine cytomegalovirus stimulates cellular thymidylate synthase gene expression in quiescent cells and requires the enzyme for replication. *J Virol* **74**, 4979–4987.

Gribaudo, G., Riera, L., Rudge, T. L., Caposio, P., Johnson, L. F. & Landolfo, S. (2002). Human cytomegalovirus infection induces cellular thymidylate synthase gene expression in quiescent fibroblasts. *J Gen Virol* **83**, 2983–2993.

Hatanaka, M. & Dulbecco, R. (1966). Induction of DNA synthesis by SV40. *Proc Natl Acad Sci U S A* **56**, 736–740.

Hengel, H., Brune, W. & Koszinowski, U. H. (1998). Immune evasion by cytomegalovirus: survival strategies of a highly adapted opportunist. *Trends Microbiol* **6**, 190–197.

Johnson, L. F. (1994). Posttranscriptional regulation of thymidylate synthase gene expression. *J Cell Biochem* **54**, 387–392.

Kuhn, K., Bertling, W. M. & Emmrich, F. (1993). Cloning of a functional cDNA for human cytidine deaminse (CDD) and its use as a marker of monocyte/macrophage differentiation. *Biochem Biophys Res Commun* **190**, 1–7.

Ladner, R. D., McNulty, D. E., Carr, S. A., Roberts, G. D. & Caradonna, S. J. (1996). Characterization of distinct nuclear and mitochondrial forms of human deoxyuridine triphosphate nucleotidohydrolase. *J Biol Chem* **271**, 7745–7751.

Landolfo, S., Gariglio, M., Gribaudo, G. & Lembo, D. (2002). The human cytomegalovirus. *Pharmacol Ther* (in press).

Lembo, D., Angeretti, A., Gariglio, M. & Landolfo, S. (1998). Murine cytomegalovirus induces expression and enzyme activity of dihydrofolate reductase in quiescent cells. *J Gen Virol* **78**, 2803–2808.

Lembo, D., Gribaudo, G., Cavallo, R., Riera, L., Angeretti, A., Hertel, L. & Landolfo, S. (1999). Human cytomegalovirus stimulates cellular dihydrofolate reducatse activity in quiescent cells. *Intervirology* **42**, 30–36.

Maley, F. & Maley, G. F. (1990). A tale of two enzymes, deoxycytidylate deaminase and thymidylate synthase. *Prog Nucleic Acid Res Mol Biol* **39**, 49–80.

Maley, F., Lobo, A. F. & Maley, G. F. (1993). Properties of an affinity-column purified human deoxycytidylate deaminase. *Biochim Biophys Acta* **1162**, 161–170.

McGeoch, D. J. & Davison, A. J. (1999). The molecular evolutionary history of the herpesviruses. In *Origin and Evolution of Viruses*, pp. 441–465. Edited by E. Domingo, R. Webster & J. Holland. London: Academic Press.

McIntosh, E. M. & Haynes, R. H. (1997). dUTP pyrophosphatase as a potential target for chemotherapeutic drug development. *Acta Biochim Pol* **44**, 159–172.

Mocarski, E. S. & Courcelle, C. T. (2001). Cytomegalovirus and their replication. In *Fields Virology*, 4th edn, pp. 2629–2673. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Pauwels, R., Balzarini, J., Baba, M., Snoeck, R., Schols, D., Hederwijin, P., Desmyter, J. & De Clerq, E. (1988). Rapid and automated tetrazolium-based colorimetric assay for the detection of anti HIV compounds. *J Virol Methods* **20**, 309–321.

Ricceri, G., Ragusa, N., Avola, R., Barcellona, M. L. & Castro, A. (1978). Pyrimidine nucleotide synthesis in rat embryo cells infected with X14 or H-1 parvovirus. *Acta Virol* **22**, 104–112.

Rolton, H. A. & Keir, H. M. (1974). Deoxycytidylate deaminase. Evidence for a new enzyme in cells infected by the virus of herpes simplex. *Biochem J* **143**, 403–409.

Weiner, K. X. B., Weiner, R. S., Maley, F. & Maley, G. F. (1993). Primary structure of human deoxycytidylate deaminase and overexpression of its functional protein in *Escherichia coli*. *J Biol Chem* **268**, 12983–12989.