Murine cytomegalovirus induces expression and enzyme activity of cellular dihydrofolate reductase in quiescent cells

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Murine cytomegalovirus (MCMV) productively infects quiescent fibroblasts in which the levels of nucleoside triphosphate precursors and cell functions involved in DNA metabolism are minimal. It appears that MCMV has evolved molecular pathways in order to ensure the presence of nucleoside triphosphate precursors for the viral DNA polymerase. Here, we report that MCMV infection of quiescent NIH 3T3 cells markedly stimulates transcription, expression and activity of the cellular dihydrofolate reductase (DHFR), a key enzyme in the synthesis of DNA precursors. DHFR stimulation by MCMV is sensitive to UV irradiation and seems to depend on expression of the viral immediate-early protein pp89. Finally, it has been demonstrated that suppression of virus-induced DHFR activity by the specific inhibitor methotrexate prevents MCMV DNA replication. These observations indicate that induction of host cell DHFR activity by MCMV is required for viral DNA synthesis in quiescent fibroblasts.

The cytomegalovirus (CMV) genome encodes enzymes involved in DNA and nucleotide metabolism, including a DNA polymerase, uracil-DNA glycosylase, ribonucleotide reductase and dUTPase (Rawlinson et al., 1996). However, CMV must largely rely upon host cell metabolism to supply precursors and enzymes utilized in the replication of viral DNA. In contrast with this notion, CMV efficiently replicates in quiescent cells (Dittmer & Mocarski 1997; D. Lembo & S. Landolfo, unpublished results), in which the levels of nucleoside triphosphate precursors and cell functions involved in DNA synthesis are minimal. One possible explanation is that the virus may fulfill its needs by inducing the relevant host enzymes of the required biosynthetic pathways.

Dihydrofolate reductase (DHFR) has a central role in the synthesis of DNA precursors. This enzyme is responsible for the NADPH-dependent reduction of folic acid and dihydrofolic acid to tetrahydrofolic acid. Derivatives of tetrahydrofolate are required for the biosynthesis of purines and thymidylate acid. The enzyme is also the target of the chemotherapeutic drug methotrexate (MTX) (Schweitzer et al., 1990). Expression of DHFR is cell cycle-regulated. When quiescent serum-starved cells are induced to re-enter the cell cycle by serum addition DHFR enzyme levels are low throughout the G1 phase, but increase sharply at the G1-S boundary (Johnson et al., 1978).

Among herpesviruses, only herpesvirus saimiri, herpesvirus ates and human herpesvirus-8 have been found to encode their own DHFR (Trimble et al., 1988; Nicholas et al., 1997). Since neither human CMV (HCMV) nor murine CMV (MCMV) encode the DHFR enzyme, it is conceivable that they have evolved a pathway to increase the activity of cellular DHFR in order to ensure that there are nucleoside triphosphate precursors for the viral DNA polymerase in quiescent cells. Although previous studies have established that HCMV infection induces transcriptional activation of the DHFR promoter in a transient co-transfection assay (Margolis et al., 1995; Wade et al., 1992), increases in the DHFR protein and its enzymatic activity remain to be demonstrated. In this study, we demonstrate that MCMV infection does indeed stimulate DHFR activity and that this activity is required for viral DNA synthesis in quiescent cells.

The murine fibroblast cell line NIH 3T3 was transfected by calcium phosphate precipitation with the indicator plasmid pWT-LUC (which contains the luciferase cDNA driven by the murine DHFR promoter), serum-starved and then infected with MCMV at an m.o.i. of 3 p.f.u. per cell for 18 h. Fig. 1(a) shows the average luciferase induction determined in several experiments with different virus stocks. Induction levels ranged from approximately 25- to 35-fold indicating that the DHFR promoter is highly induced by MCMV infection in quiescent cells. Reporter gene activity was normalized to the amount of plasmid DNA introduced into recipient cells by DNA dot blot analysis as described by Abken & Reifenrath (1992). We also noted that co-transfection of pWT-LUC with an MCMV genomic fragment encoding the immediate-early protein 1 (IE-1) pp89 strongly stimulated DHFR promoter activity (data not...
Fig. 1. Transactivation of the DHFR promoter and accumulation of DHFR mRNA upon MCMV infection. (a) NIH 3T3 cells were co-transfected with pWT-LUC (2 µg), pCH110 (2 µg), and pBlueScript SK (8 µg), serum-starved for 60 h and infected with 3 p.f.u. per cell of UV-irradiated or untreated MCMV. Luciferase activity was assayed at 18 h p.i. The average fold induction of four independent experiments is shown. An aliquot of transfected cells was also analysed for pp89 expression by immunoblotting (insert). Lanes: 1, mock; 2, MCMV; 3, MCMV UV-irradiated 0 ± 2 J/cm²; and 4, MCMV UV-irradiated 0 ± 6 J/cm². (b) Total RNA was extracted from mock-infected cells (lane 1), from MCMV-infected cells at different times after infection (lane 2, 6 h p.i.; lane 3, 24 h p.i.; lane 4, 48 h p.i.), and from 10% serum-stimulated cells for 6 h (lane 5) and 24 h (lane 6). The Northern blot was sequentially hybridized with a DHFR and a β-actin radiolabelled cDNA. (c) Protein levels of the pp89 during MCMV infection (at 0, 6, 24 and 48 h p.i.) as determined by Western blotting. Generation of the pp89 antiserum has been previously described (Gariglio et al., 1997).

To determine whether MCMV-induced stimulation of DHFR promoter activity by MCMV was a consequence of viral gene expression, cells were also infected with UV-inactivated virus and expression of the viral protein pp89 was used as a marker of virus infectivity. Preliminary experiments were conducted to establish conditions for efficient UV inactivation. As shown in a representative immunoblot (Fig. 1a, insert, lane 4), pp89 was not detected in extracts from cells infected at an m.o.i. of 3 p.f.u. per cell after UV irradiation of 0±6 J/cm², whereas UV irradiation of 0±2 J/cm² resulted in a partial virus inactivation (Fig. 1a, insert, lane 3). These results demonstrate that induction of DHFR promoter activity correlates with MCMV gene expression, and indicate that serum contamination of viral preparations or binding and entry of virus alone are not sufficient to activate the murine DHFR promoter.

To determine whether MCMV-induced stimulation of DHFR promoter activity correlates with an increase in the steady-state levels of DHFR mRNA, Northern blot analysis of total RNA from mock- or MCMV-infected cells at various times after infection was performed. Time-course examination of MCMV induction in quiescent cells (Fig. 1b) showed that, compared with mock-infected cells, levels of DHFR mRNA began to increase 6 h after infection (Fig. 1b, lane 2) and rise to a maximum after 48 h (Fig. 1b, lane 4). To check the inducibility of the DHFR mRNA in our system, we exposed quiescent mock-infected cells to 10% serum for 6 and 24 h. This resulted in a significant induction of the DHFR transcripts at 24 h (Fig. 1b, lane 6) but not at 6 h (Fig. 1b, lane 5), indicating that MCMV infection increases the expression of DHFR mRNA earlier than exposure to serum does. Western blot analysis performed in parallel (Fig. 1c) demonstrated that pp89 is already detectable at 6 h post-infection (p.i.) and continues throughout infection (24 and 48 h p.i.). Moreover, treatment of infected cells with ganciclovir (GCV), an inhibitor of MCMV DNA synthesis, did not affect accumulation of the DHFR transcripts following MCMV infection (data not shown). This result demonstrates that DHFR mRNA induction by MCMV occurs independently of viral DNA replication and rules out the involvement of newly synthesized viral late gene products.

The observation that MCMV stimulates expression of the DHFR gene prompted us to verify whether this corresponded to an increase in DHFR activity. Fig. 2 shows a representative...
time-course study of DHFR activity in quiescent cells infected with MCMV at an m.o.i. of 5 p.f.u. per cell and harvested at 6, 24 and 48 h p.i. DHFR activity was assayed radioenzymically as described by Rothenberg (1966) in cytosol extracts and the results are expressed as percentage of reduced tritiated folate. Assays on infected cell extracts demonstrated an increase in DHFR activity from 24 h p.i. (about 2.5-fold) to a maximum at 48 h p.i. (about 5-fold) compared with mock-infected cells. No such increase was found with extracts from cells infected with UV-inactivated MCMV. Altogether, the present results demonstrate that MCMV infection of quiescent fibroblasts specifically induces an increase in cellular DHFR gene expression and enzyme activity. This conclusion is based on the following observations: (i) dependence of DHFR increase on virus m.o.i. (data not shown); (ii) dependence of this effect on virus viability, because UV-inactivated virus cannot trigger any stimulation of the DHFR promoter and enzyme activity; and finally (iii) DHFR promoter transactivation by a specific MCMV genomic fragment (i.e. the IE-1 coding region).

Several studies have reported that CMV infection leads to the activation of a number of cellular genes involved in DNA synthesis and differentiation, probably to optimize cellular conditions for efficient virus replication. It has been observed that cellular gene activation occurs either as a result of virus binding to the cell surface (Bolodog et al., 1991) or as a result of virus IE protein expression (Burns et al., 1993; Colberg-Poley et al., 1992; Dudding et al., 1989; Geist et al., 1993; Hayhurst et al., 1995; Koszinowski et al., 1986; Margolis et al., 1995; Schickedanz et al., 1988). In experiments where MCMV was partially or completely inactivated by UV exposure, we found that transactivation of the DHFR promoter and induction of DHFR activity strictly correlated with virus infectivity, suggesting that interaction of viral particles with the cell surface is not sufficient to stimulate DHFR gene expression. The kinetics of DHFR mRNA induction by MCMV, as well as the observation that inhibition of viral DNA replication by GCV has no effect upon DHFR mRNA accumulation, would be consistent with a role for IE viral proteins. Moreover, there is a considerable temporal overlap between increases in DHFR mRNA levels and the expression of pp89 throughout infection. When considered together with the data reported for the transactivating effect of pp89 on the DHFR promoter, these findings point to a causal relationship between pp89 and activation of DHFR gene expression.

The ability of CMV to stimulate the macromolecular synthesis of the host cell (Furukawa et al., 1973, 1975; Tanaka et al., 1975) raised the question of whether the effect on DHFR expression is specific in nature, or whether we are simply measuring a more generalized effect on total mRNA and protein cellular content where DHFR is a marker. To address this question, we verified whether suppression of DHFR activity by the specific inhibitor MTX affected MCMV DNA synthesis. Quiescent cells were infected at an m.o.i. of 3 p.f.u. per cell and, after virus adherence for 1 h, medium containing various MTX concentrations (0–2.2 µM) was added. Forty-eight hours after infection, intracellular viral DNA levels were quantified by dot blot analysis and hybridization with a radiolabelled viral probe. As shown in Fig. 3, a marked inhibition of MCMV DNA levels was observed in cultures treated with MTX concentrations of 0.22 and 2.2 µM. The 50% effective dose of MTX, determined from three independent experiments, appeared to be 0.14 µM. MTX-induced cytotoxicity was assessed by a trypan blue exclusion assay. No significant effects on cell survival were observed at the MTX concentrations required to inhibit MCMV DNA synthesis. The inhibitory effect of MTX on viral DNA replication and the finding that folic acid abrogates the antiviral effect of MTX (data not shown) demonstrate that DHFR is required for efficient virus replication in quiescent cells and is therefore a true cellular target of virus infection. Previous studies reported antiviral activity of MTX on growing cells infected by HCMV and MCMV (Shanley & Debs, 1989; Wachsman et al., 1996), but the mechanism of action remained undefined. The use of quiescent cells that express DHFR to
very low levels allowed us to demonstrate that the virus itself can induce DHFR activity to the extent required for its efficient DNA replication. Since most adult animal cells are differentiated and actively regulated to remain in a quiescent state, the ability of CMV to modulate the expression of the de novo pathways of nucleotide biosynthesis in quiescent cells may have a substantial role in the pathogenesis of CMV diseases. MTX antiviral activity in quiescent cells confirms that the ability of MCMV to increase DHFR expression is critical to virus growth.

DHFR is a known target for drug action (Schweitzer et al., 1990). Inhibitors of DHFR have proved useful in the treatment of cancer (Bertino, 1993; Fleming & Schilsky, 1992), bacterial infections (Salter, 1982), malaria (Hitchings, 1978), and Pneumocystis carinii pneumonia (Allegra et al., 1987; Fallon et al., 1990). Studies to control other opportunistic organisms such as Toxoplasma gondii and Candida albicans with DHFR inhibitors are also under way (Kuyper et al., 1996).

HCMV is an important opportunistic pathogen causing diseases in immunocompromised patients associated with the AIDS epidemic and organ transplantation (Huang & Kovalik, 1993). Treatment options for HCMV diseases remain limited and currently available therapies have significant toxicity (Bean, 1992). Moreover, clinically isolated HCMV strains that are resistant to the conventional drugs are becoming increasingly common (Baldanti et al., 1996; Chou et al., 1995). Wachsmann et al. (1996) have demonstrated that MTX is preferentially accumulated in HCMV-infected cells. They suggest that although MTX is an inhibitor directed against a host cell function, virus perturbation of the host cell can increase its action sufficiently to produce a virus-specific effect. These considerations and the recent availability of new high affinity inhibitors of DHFR endowed with an improved tissue distribution (Kuyper et al., 1996) indicate that the potential of DHFR inhibitors as antiviral agents for HCMV infections merits further investigation. This paper provides the theoretical basis for the setting up of in vivo animal models to study the efficacy of DHFR inhibitors in MCMV-infected mice.

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References
DHFR induction by MCMV


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