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THYMIDINE KINASE ACTIVITY IN MOUSE EMBRYO FIBROBLAST CELLS INFECTED WITH MURINE CYTOMEGALOVIRUS

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SUMMARY: Thymidine kinase activity was found in whole cell extracts of growing and stationary mouse embryo fibroblast cells after infection with murine cytomegalovirus. Determination of the kinetic constants and heat stability characteristics indicated that the enzyme activity from infected cells was different to that found in uninfected cells in the growth phase. The expression of thymidine kinase activity during virus replication was reflected by the incorporation of (6-^{3}H) thymidine into acid precipitable fractions of infected cell cultures. Preliminary data from kinetic studies showed a reduction in the phosphorylation of thymidine by this enzyme activity in the presence of Acyclovir, a potent inhibitor of herpes virus replication.

INTRODUCTION

The compound 9-(2'-hydroxyethoxymethyl) guanine (Acyclovir), an acyclic nucleoside analogue of guanosine, is a potent inhibitor of HSV replication (1). This compound is selectively phosphorylated to acyclo-GMP by the virus-coded thymidine kinase (E.C.2.7.1.75) (1,2). Conversion to the triphosphate form is dependent on cellular enzyme activity and acyclo-GTP is a potent competitive inhibitor for the virus coded DNA polymerase (1,3,4,5).

It is apparent from 50% effective-dose (ED50) estimates in cell culture that a number of strains of human CMV are much less sensitive than HSV 1 and HSV 2 to the action of Acyclovir (6,7). The insensitivity of human CMV can be explained by

ABBREVIATIONS: HSV: herpes simplex virus, CMV: cytomegalovirus, MEF: mouse embryo fibroblast.

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insufficient acyclo-GTP in the cell, which is probably due to the absence of a suitable thymidine kinase in infected cells (8,9), because the DNA polymerase coded for by human CMV is very sensitive to inhibition by acyclo-GTP (5). We have reported that murine CMV is more sensitive than human CMV to Acyclovir in cell culture, although the apparent difference in sensitivity may be a reflection of the obligatory use of different host cell systems (7). The sensitivity of murine CMV to the action of Acyclovir observed in cell culture was borne out by observations that mice were protected from murine CMV induced disease by the administration of this compound (Tyms, A.S., manuscript in preparation). On the basis of the established mode of action against HSV infections, the sensitivity of murine CMV infections to Acyclovir was surprising in view of the reported lack of thymidine kinase activity in cells infected with this virus (10,11). The present paper reports the investigation of thymidine kinase activity in MEF cells infected with the Osborn strain of murine CMV.

MATERIALS AND METHODS

MEF cells were derived from mouse embryos by trypsinization and grown in Eagle's minimum essential medium containing 10% or 2% foetal calf serum for growth and maintenance, respectively. Cells used in the 'growing' phase were infected within 24 hours of seeding. 'Starved' cell monolayers had the growth medium replaced by maintenance medium on reaching confluency and were infected 4 or 5 days later. The Osborn strain of murine CMV was obtained from Professor C.A. Mims (Department of Microbiology, Guy's Hospital Medical School, London) and virus stock was prepared from the extracellular fluid from infected MEF cells. In all experiments, the multiplicity of infection was at least 5 plaque forming units per cell.

Pyrimidine deoxyribonucleoside kinase (thymidine kinase) activity was measured in whole cell extracts using (6-^{3}H) thymidine (specific activity 30.Ci/mmol) which was obtained from the Radiochemical Centre, Amersham, England. Cell

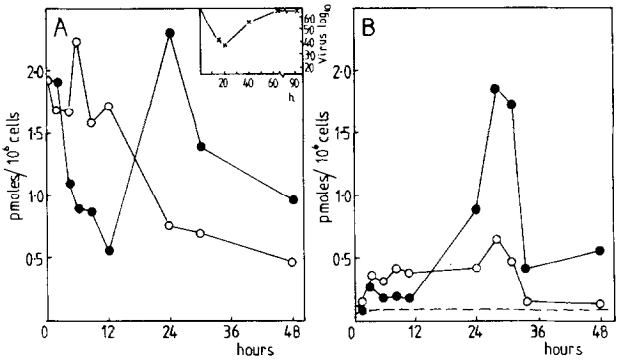


Figure 1. Time-course appearance of thymidine kinase activity in MEF cells infected with murine CMV (closed circles) and sham-infected (open circles). The rate of enzyme activity was measured in extracts of 'growing' cells (Panel A) and 'starved' cells (Panel B). The broken line shows the level of enzyme activity in uninfected 'starved' cells during the course of the experiment.
Inset: Single-step growth curve for murine CMV.

Ordinate: pmoles (6-^{3}H) thymidine phosphorylated/10^{6} cells.
Abscissa: time in hours post-infection.

extracts were prepared from sham-infected and virus infected cells, reacted with substrate and the radiolabelled product separated on Whatman DEAE-cellulose paper discs (DE81). The method was essentially as described by Závada et al. (8). For the study of enzyme kinetics, initial velocity was measured at varying substrate concentrations in the presence or absence of Acyclovir (Wellcome Research Laboratories, Beckenham, Kent, England) at a fixed concentration. For heat inactivation studies, enzyme preparations were incubated in a 63°C waterbath for various times before residual enzyme activity was determined. Protein estimations were made with the Folin phenol reagent (12). To measure the incorporation of (6-^{3}H) thymidine into high molecular weight material, extracts from sham-infected or virus-infected cells were precipitated with 10% trichloroacetic acid and processed according to the method of Archard and Williamson (13).

RESULTS

Initial experiments were set up to determine whether or not thymidine kinase activity was detectable in MEF cells after infection with murine CMV. In figure 1 is shown the results of time-course studies of enzyme activity measured in extracts of infected and sham-infected MEF cells. After sham-infection of 'growing' cells, the raised enzyme activity associated with

Table 1. Incorporation of (6-^{3}H) thymidine into acid-precipitable material from MEF cells infected with murine CMV or sham-infected. Cells were pulse labelled for one hour at various times post-infection.

Hours	Infected	Sham-infected
1	1.8*	0.5*
8	10.0	1.2
15	16.2	4.3
17	24.3	3.5
24	22.4	2.7
38	15.6	0.8

* DPM x 10^{-4}

such cells was maintained for about 12 hours before the level of activity began to decline. However, the characteristic thymidine kinase activity associated with 'growing' cells was rapidly reduced soon after infection with murine CMV but a peak of enzyme activity was detected in these cells beginning about 12 hours post-infection (Panel A). If 'starved' MEF cells were either infected with murine CMV or sham-infected, thymidine kinase activity was increased in both cases with maximum activity at about 28 hours post infection. However, the level of enzyme activity measured in the infected cells was greater than that associated with sham-infected cells (Panel B).

Evidence for intracellular thymidine kinase activity was obtained from studies of the incorporation of radiolabelled thymidine into acid-precipitable material from 'starved' cells (table 1). The results suggest that the rate of incorporation of the radiolabelled nucleoside into DNA was significantly greater in the murine CMV infected cells than in the corresponding sham-infected cells. This shows that there is a functional thymidine kinase present during the replication of murine CMV.

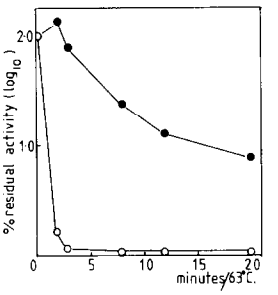


Figure 2. Stability of thymidine kinase after incubation at 63°C. Enzyme preparations were prepared from 'growing' MEF cells (open circles) or 'starved' MEF cells infected with murine CMV (closed circles) and adjusted for protein content. The plots of residual enzyme activity show the enzyme from infected cells had greater stability at 63°C.

The presence of a virus-coded thymidine kinase is a key factor in the selectivity and potency of Acyclovir against HSV infections (1,2). Initial characterisation of the enzyme activity associated with murine CMV infections was attempted using enzyme kinetic studies with material prepared from growing MEF cells or 'starved' cells infected with murine CMV.

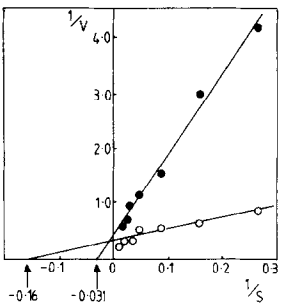
Enzymes with similar catalytic properties may be distinguished by protein stability tests at supra-optimal temperature. Samples of enzyme preparations were exposed to 63°C for different times. The residual thymidine kinase activity was assayed and plotted as a function of time (figure 2). The enzyme activity prepared from the virus infected cells was consistently more stable at 63°C. The results in figure 3 show Lineweaver-Burk plots of the initial velocity at varying thymidine concentrations for both enzyme preparations. The results illustrate the different affinities for thymidine by the enzyme prepared from infected cells (Km 28.2 σ 7.7 x 10^{-6} M, n=6) and the enzyme from MEF cells in the growth phase (Km 6.9 σ 1.5 x 10^{-6} M, n=6). On the basis of the substrate affinity and the enzyme stability data, it is concluded that

the enzyme activity associated with cells infected with murine CMV is not normally present in MEF cells during cell growth.

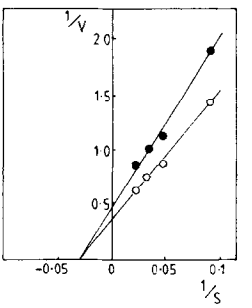
Experiments to decide whether or not there is an association between the thymidine kinase present in cells infected with murine CMV and the effectiveness of Acyclovir against the replication of this virus, requires a homogeneous enzyme preparation. Purification of the enzyme has not been achieved so far to test for phosphorylation of Acyclovir directly but information has been obtained which demonstrates affinity of the thymidine kinase for this compound. Experiments were set up to measure the initial velocity of enzyme reactions using varying thymidine concentrations with Acyclovir added at a fixed concentration. Alteration of the kinetic constants in such experiments can act as an indicator of the affinity of an enzyme for a potential inhibitor. The velocity of the enzyme reaction, at substrate concentrations close to the estimated Km value, was consistently reduced by the presence of Acyclovir at a concentration of 200μM or more. In figure 4 is shown experimental data expressed as Lineweaver-Burk plots from which the kinetic constants have been determined. The changes in the double-reciprocal plots suggested that the presence of Acyclovir (600μM) had little effect on the affinity of the enzyme for thymidine but consistently lowered the maximum velocity (Vm) for this reaction (Vm reduction 26-42%, n=3). This implies that Acyclovir reacts at a different active site to thymidine and this is characteristic of simple non-competitive inhibition.

DISCUSSION

Murine CMV replicates in continuous mouse cells deficient in thymidine kinase but does not induce a thymidine kinase



(3)



(4)

Figure 3. Lineweaver-Burk plots of data for thymidine kinase preparations from 'growing' MEF cells (open circles) and 'starved' MEF cells infected with murine CMV (closed circles).

Figure 4. Lineweaver-Burk plots of data for thymidine kinase preparations from 'starved' MEF cells infected with murine CMV. The initial velocity was measured at varying thymidine concentrations alone (open circles) or in the presence of 600μM Acyclovir (closed circles).

V is pmoles (6-^{3}H) thymidine phosphorylated/mg.protein and S is the concentration of thymidine (x 10^{-6}M).

activity during replication (10, 11). However, exogenous radiolabelled thymidine was incorporated into viral DNA during the replication of murine CMV in MEF cells (14, 15) although the rate of uptake of thymidine was decreased in these cells after infection (16). We have shown that thymidine is incorporated readily into acid-insoluble material when 'starved' MEF monolayers were infected with the Osborn strain of murine CMV. All this information implies that thymidine kinase activity is associated with MEF cells infected with murine CMV. This premise was verified by our experimental data that showed thymidine kinase activity present in these cells during the exponential phase of virus replication. Also, it was evident that the thymidine kinase activity measurable in 'growing' MEF cells was lost soon after infection and this is consistent with observations of thymidine incorporation (16) and DNA analysis (15).

Two distinguishable thymidine kinase activities have been characterised in mouse cells (17). A cytosol activity is highest during rapid growth of cells and a mitochondrial matrix activity is associated with the stationary phase. In the present study, the enzyme preparations were not subjected to high-speed centrifugation to remove organelles. Hence, the differences observed between the enzyme activity could be accounted for by changes in cellular enzyme activity. Characterisation of the thymidine kinase must await isolation and purification of the enzyme protein from infected cells. Likewise, the question of phosphorylation of Acyclovir by a virus associated enzyme cannot be answered without further studies. However, we have provided some evidence in our kinetic data that the inhibitor does react with thymidine kinase but not at the same active site as thymidine. Support for this premise was obtained from thymidine reversal experiments. Competitive-type inhibitions are readily reversed by saturation with the normal substrate but other types of inhibition are not so readily reversed under these conditions (18). In plague reduction experiments with murine CMV we found that a ratio of thymidine to Acyclovir of 50:1 produced only 50% reversal of plaque inhibition (data not shown). This observation contrasts with reports for HSV which showed thymidine and Acyclovir present in a ratio of 5:1 reversed the inhibitory effect of the compound (1).

Further studies will relate the nature and role of thymidine kinase to the activity of Acyclovir against murine CMV infections.

Acknowledgement

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References

1. Elion, G.B., Furman, P.A., Fyfe, J.A., de Miranda, P., Beauchamp, L. and Schaeffer, H.J. (1977) Proc. Natl. Acad. Sci. U.S.A., 74: 5716-5720.
2. Fyfe, J.A., Keller, P.M., Furman, P.A., Miller, R.L. and Elion, G.B. (1978) J. Biol. Chem., 253: 8721-8727.
3. Miller, W.H. and Miller, R.L. (1980) J. Biol. Chem., 255: 7204-7207.
4. Furman, P.A., St. Clair, M.H., Fyfe, J.A., Rideout, J.L., Keller, P.M. and Elion, G.B. (1979) J. Virol., 32: 72-77.
5. St. Clair, M.H., Furman, P.A., Lubbers, C.M. and Elion, G.B. (1980) Antimicrob. Ag. Chemother., 18: 741-745.
6. Crumpacker, C.S., Schnipper, L.E., Zala, J.A., Levin, M.J. (1979) Antimicrob. Ag. Chemother. 15: 642-645.
7. Tyms, A.S., Scamans, E.M. and Naim, H.M. (1981) J. Antimicrob. Chemother., In press.
8. Závada, V., Erban, V., Řezáčová, D. and Vonka, V. (1976) Arch. Virol, 52: 333-339.
9. Estes, J.E. and Huang, E-S. (1977) J. Virol. 24: 13-21.
10. Muller, M.T. and Hudson, J.B. (1977) Virology 80: 430-433.
11. Eizuru, Y., Minamishima, Y., Hirano, A. and Kurimura, T. (1978) Microbiol. Immunol. (Tokyo) 22: 755-764.
12. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193: 265-275.
13. Archard, L.C. and Williamson, J.D. (1971) J. Gen. Virol., 12: 249-258.
14. Mosmann, T.R. and Hudson, J.B. (1973) Virology, 54: 135-149.
15. Moon, H.M., Sapienza, V.J., Carp, R.J. and Kim, K.S. (1976) Virology, 75: 376-383.
16. Hudson, J.B. (1980) Virology, 101: 545-548.
17. Kit, S., Leung, W-C, Jorgensen, G.N., Trkula, D. and Dubbs, D.L. (1975) Progr. Med. Virol., 21: 13-34.
18. Conn, E.E. and Stumpf, P.K. (1976) Outlines of Biochemistry, pp. 176-180, John Wiley, New York.