JOURNAL OF VIROLOGY, June 1983, p. 901-908
0022-538X/83/060901-08$02.00/0
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Vol. 46, No. 3

Alterations in Substrate Specificity and Physicochemical Properties of Deoxythymidine Kinase of a Drug-Resistant Herpes Simplex Virus Type 1 Mutant

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Received 28 December 1982/Accepted 18 March 1983

The deoxythymidine kinase (dTK) activity of a 5-methoxymethyldeoxyuridine-resistant mutant (MMdU^r-20) of herpes simplex virus type 1 was compared with that of the parental wild-type (WT) virus. The dTK activity induced by the mutant was consistently less than that induced by the WT virus, was inhibited by antibody specific for herpes simplex virus dTK, and was more thermostable than the WT dTK. Further, it was inhibited to a lesser degree than the WT dTK by the nucleoside analogs MMdU and arabinosylthymine (araT), which suggests that one of the effects of the mutation was a selective alteration in substrate recognition by the dTK. The loss of ability to inhibit the mutant dTK by *E*-(2)-5-bromovinyldeoxyuridine was not as great as that seen with araT and MMdU. This agrees well with our previous observation that the MMdU^r-20 mutant of herpes simplex virus is only partially resistant to this analog, as compared with araT and MMdU (V. Veerisetty and G. A. Gentry, Virology **114**:576–579, 1981). [2-^{14}C]araT was used to explore further the resistance to araT. Extracts of cells infected with the mutant, although producing a small amount of [^{14}C]araTMP, were unable to produce [^{14}C]araTTP, in contrast to extracts of cells infected with the WT virus. Both extracts, however, produced [^{14}C]dTTP from [^{14}C]deoxyribosylthymine. Finally, the ability of the extracts to phosphorylate [^{14}C]dTMP was examined. It was found that this activity was greatly reduced relative to dTK activity in the case of the mutant. These findings suggest that a mutation in the dTK polypeptide has affected recognition not only of nucleoside substrates but of the nucleotide substrate dTMP as well, which agrees with the suggestion of Chen et al. that both activities are located on the same polypeptide (M. S. Chen and W. H. Prusoff, J. Biol. Chem. **253**:1325–1327, 1978; M. S. Chen, J. Walker, and W. H. Prusoff, J. Biol. Chem. **254**:10747–10753, 1979; M. S. Chen, W. P. Summers, J. Walker, W. C. Summers, and W. H. Prusoff, J. Virol. **30**:942–945, 1979).

Since the discovery that herpes simplex virus (HSV) induces a new virus-coded deoxythymidine kinase (dTK) (12), considerable attention has been given to this enzyme because it can be used to enhance the selectivity of anti-herpesviral agents (10, 16). Unfortunately, drug-resistant mutants of HSV arise readily both in vitro and in vivo (2, 7, 8, 15, 18, 23, 26) and may already be present in previously untreated patients (22). Two types of mutants have been detected, one with a mutation in the dTK gene and the other with a mutation in the DNA polymerase gene (7, 15, 23, 26).

We have recently isolated a number of mutants of HSV type 1 (HSV-1) by using the antiviral nucleoside 5-methoxymethyldeoxyuridine (MMdU) (26). All mutant viruses were cross-resistant to the other nucleoside analogs acyclovir (ACV), *E*-(2)-5-bromovinyldeoxyuridine (BVdU), and arabinosylthymine (araT), but were as sensitive as the parent virus to phosphonoacetate. Because HSV-induced dTK is required for the antiviral activity of many of the known nucleoside analogs (1, 11, 13, 17), the failure of induction of viral dTK would make the virus resistant to them. Those mutants, however, that induce dTK but are resistant to nucleosides deserve further attention because they may be as virulent as the wild type (WT) virus, whereas those that lack dTK are known to be relatively avirulent (9, 14, 25). The majority of our MMdU-resistant (MMdU^r) mutants failed to induce detectable levels of dTK activity. One, however, MMdU^r-20, induced appreciable amounts of dTK (up to 70% of WT dTK) but was also resistant to nucleoside analogs. Because

this virus is sensitive to phosphonoacetate, we inferred that the mutation probably was in the dTK gene and altered the substrate specificity for nucleoside analogs. In this report we show that this enzyme is distinctly different from the parental virus-induced enzyme. The data suggest a correlation between the extent of phosphorylation of the nucleoside and the level of resistance of the virus.

MATERIALS AND METHODS

Cells. Baby hamster kidney (BHK-21) cells were used for virus growth and titration. Mouse cells (3T3) and L-Kit cells, both of which are negative for host cytosol dTK (dTK^-), were used for dTK experiments. All were grown in Eagle medium supplemented with 5% fetal calf serum. For routine passage of dTK^- cells, 30 μg of bromodeoxyuridine per ml was included in the medium.

Buffers. Lysis buffer consisted of 0.01 M Tris-hydrochloride (pH 7.5), 0.01 M KCl, 1 mM $MgCl_2$, and 0.002 M dithiothreitol. Reaction buffer consisted of 0.15 M Tris-hydrochloride (pH 7.5), 2.5 mM ATP, 2.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 10 mM sodium fluoride, 1 mg of bovine serum albumin per ml, 3 mM phosphocreatine, and 1.0 U of creatine phosphokinase per ml. Reaction mixture was reaction buffer containing [2-^{14}C]deoxyribosylthymine (dT) (1.0 μC/ml, 0.02 mM) or [2-^{14}C]araT (17 μC/ml, 1.7 mM, unless otherwise indicated) or [2-^{14}C]dTMP (1.2 μC/ml, 0.024 mM). For thin-layer chromatography (TLC), a 4 M sodium formate (pH 3.4) buffer stock was prepared; appropriate dilutions were made when necessary.

Nucleosides and antiviral compounds. AraT was purchased from Raylo Chemicals (Edmonton, Alberta, Canada). MMdU, BVdU, phosphonoacetate, and ACV were gifts from Lorne Babiuk (Department of Veterinary Microbiology, University of Saskatchewan, Saskatoon, Canada), Eric De Clercq, (Rega Institute for Medical Research, Katholeiek Universiteit, Louvain, Belgium), Ronald Duff (Abbott Laboratories, Chicago, Ill.), and G. B. Elion (Burroughs Wellcome Co., Research Triangle Park, N.C.), respectively. [2-^{14}C]araT (specific activity, 10 mC/mM) was synthesized by Raylo Chemicals. [2-^{14}C]dT (specific activity, 50 mC/mM) and [2-^{14}C]dTMP (specific activity, 62.2 mC/mM) were purchased from New England Nuclear Corp., Boston, Mass. Unlabeled nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo.

Virus. HSV-1 strain 17MP (WT) and the mutant $MMdU^r$-20 were propagated and assayed as described (26).

Preparation and assay of dTK and dTMP kinase. For the assay of dTK, both dTK^+ (BHK) and dTK^- (3T3 and L-Kit) cells were employed. dTK^- cells were grown without bromodeoxyuridine for one generation to eliminate the possibility of interference by residual bromodeoxyuridine. Cells in duplicate 5-cm plastic petri dishes were infected with WT virus or the drug-resistant mutant $MMdU^r$-20 at a multiplicity of infection of 5 to 10 PFU per cell. The cells were harvested 18 h after infection by scraping and were pelleted at 1,600 × g for 5 min at 4°C. The cell pellets were suspended in 0.25 to 0.5 ml lysis buffer at 4°C and were disrupted by sonic treatment for three 30-s intervals with a 10-kHz Raytheon Sonifier. The sonically treated material was then centrifuged for 1 h at 100,000 × g. The supernatant was removed and assayed as described below.

dTK was assayed as described by McGowan (Ph.D. thesis, University of Mississippi, Jackson, 1980) and Cheng (6). Briefly, 5 or 10 μl of enzyme extract was incubated with 45 or 90 μl of reaction mixture, respectively, at 37°C for 1 h (unless otherwise specified). Samples were spotted on disks of Whatman DE-81 filter paper. The filter disks were air dried, washed three times (10 min each) with distilled water and once with 95% ethanol, air dried again, and counted in a liquid scintillation counter. [^{14}C]araT was used as an alternative substrate in some experiments.

Extracts for dTMP kinase activity were assayed at 37°C by using [^{14}C]dTMP (1.2 μC/ml) as a substrate. The reaction buffer and the assay procedure were the same as for the dTK assay, except that dTMP was removed from the Whatman DE-81 filter paper by washing three times with 0.1 M sodium formate, pH 3.4. This procedure removed about almost all dTMP but essentially no dTDP or dTTP. TLC with polyethyleneimine-cellulose sheets was used to verify this procedure (data not shown). Preliminary experiments indicated that 80 to 90% of the dTMP kinase activity of the mock-infected (MI) cell extracts was lost when the extracts were exposed to 37°C for 30 min; this did not significantly alter the dTK activity of the WT viral enzyme. Extracts of MI cells and cells infected with WT virus and $MMdU^r$-20 were thus preincubated at 37°C for 30 min and then assayed for dTMP kinase activity.

Serum inactivation. Antiserum raised against purified dTK of HSV-1 strain KOS was a generous gift from Y.-C. Cheng (University of North Carolina, Chapel Hill, N.C.). Various amounts of antisera were thoroughly mixed with 10 μl of crude extract and incubated at 37°C for 1 h. The extracts were then assayed for dTK activity as described above. Lysis buffer was added to correct to a constant volume for the reaction mixture.

Thermal inactivation. Extracts of 3T3 cells infected with WT HSV-1 and $MMdU^r$-20 were incubated at 40 or 45°C in a water bath. Samples (10 μl) of the enzyme extract were removed at 0, 5, 10, and 20 min and were immediately put on ice. All the samples were assayed for dTK activity as described above.

In vitro inhibition of dTK by nucleoside analogs. Nucleoside analogs were prediluted in distilled water to 10, 1, and 0.1 mM, except for BVdU, which was diluted to 1, 0.1, and 0.01 mM. Next, 10 μl of nucleoside stock, 5 μl of $MMdU^r$-20- or WT-infected cell extract, and 85 μl of reaction mixture containing [^{14}C]dT were added, mixed, and incubated at 37°C for 1 h. Extracts without nucleoside analogs were used as controls. A sample was assayed for dTK activity. The percentage of residual dTK activity was calculated in each case, and the data were plotted.

TLC. Plastic sheets precoated with polyethyleneimine-cellulose F (0.1 mm) were purchased from Sigma Chemical Co. All sheets were prewashed by ascending chromatography in 10% ammonium hydroxide, air dried, and stored at −20°C. Samples (1 to 10 μl) of the reaction mixture were spotted, air dried, and chromatographed by ascending chromatography with 0.1, 0.25, 0.5, and 2.0 M sodium formate,

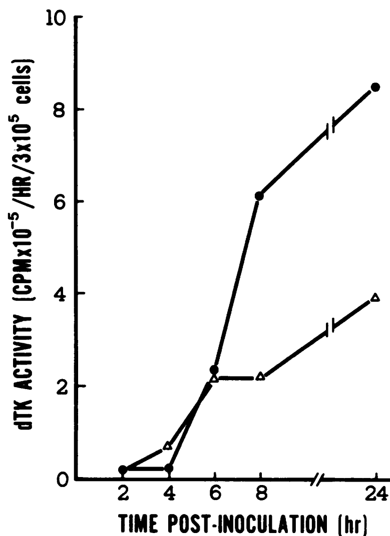

FIG. 1. Induction of dTK activity by MMdU^{r}-20 (△) and WT virus (●). Monolayers of dTK^{-} 3T3 cells were infected at a multiplicity of infection of 5 to 10 PFU per cell, cell extracts were prepared at the indicated times, and dTK activity was measured as described in the text.

pH 3.4. Each solvent front was run to a height of 3 cm except for the 2 M solvent, which usually was allowed to run 3 to 5 cm. After the samples were spotted, the TLC plates were washed in 0.001 M sodium formate, pH 3.4, to remove excess nucleoside. These plates were air dried, sprayed with En^{3}Hance (New England Nuclear Corp.), and again air dried. They were then exposed to X-ray film at −70°C. The En^{3}Hance allowed a marked reduction in the exposure time and also increased the sensitivity of the procedure.

RESULTS

Kinetics of induction of dTK activity. We reported earlier (26) that extracts of cells infected with MMdU^{r}-20 had only 50 to 70% of the dTK activity of extracts of cells infected with WT virus. This was observed in both cytosol dTK^{+} BHK cells and cytosol dTK^{-} 3T3 and L-Kit cells. To study this further, dTK^{-} 3T3 cells were grown in 24-well plates in the absence of bromodeoxyuridine and were infected at 5 to 10 PFU per cell with either WT virus or MMdU^{r}-20. After 2 h of infection, fresh medium was added, and one set of wells was harvested, lysed, and stored at −70°C. Similarly, after 4, 6, 8, and 24 h, replicate wells were harvested, and cell lysates were prepared and stored. All samples were assayed for dTK activity; the data are shown in Fig. 1. The MI cell extracts (dTK) served as the base line, and the total dTK activity per 3 × 10^{5} cells was calculated. The data show that dTK activity appeared at 2 h postinfection and continued to increase for 24 h. At any given time past 6 h postinfection, the level of dTK induced in cells infected with MMdU^{r}-20 was less than 60% of that induced in WT-infected cells, which confirms our previous observation (26). At 4 h, however, there seemed to be slightly more dTK in the cells infected with MMdU^{r}-20. We also scored for cytopathic effect during the course of these studies (data not shown) and found that cytopathic effect appeared in infected cells 30 min earlier in the case of MMdU^{r}-20 (around 6 h postinfection), indicating that the difference in the levels of dTK activities of WT and mutant viruses is not due to differences in the timing of the growth cycle of the virus.

Serum inactivation of dTK activity. The phosphorylating activities of extracts of 3T3 cells infected with WT virus and MMdU^{r}-20 were determined. WT-infected cell extract was diluted twofold with lysis buffer to equalize the phosphorylating activity with that of the mutant. The activities were again determined and found to be approximately the same. Constant amounts of extract were then preincubated with various quantities of antiserum or normal rabbit serum at 37°C for 1 h. Reaction mixtures containing [^{14}C]dT were then added, incubated for 1 h, and assayed for dTK activity. The residual dTK activity curves (Fig. 2) of both WT and mutant enzymes followed similar patterns of inhibition with the antiserum. In contrast, the dTK activity of the MI cell extract, although slight, was insensitive to inhibition by antiser-

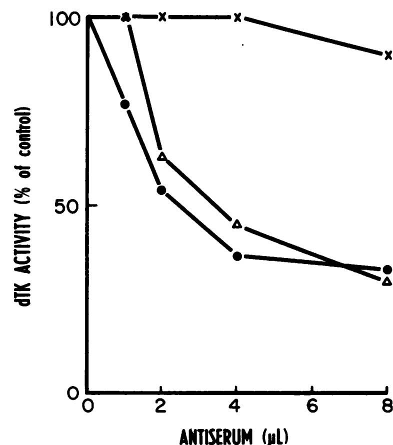

FIG. 2. Antiserum inhibition of dTK activity. Extracts of MI cells (×) and cells infected with MMdU^{r}-20 (△) and WT virus (●) were preincubated with increasing amounts of antiserum (raised against purified dTK of HSV-1 strain KOS) at 37°C for 1 h and then were assayed for dTK activity as described in the text. The percentage of residual activity was calculated with dT phosphorylated in the absence of antiserum as 100%.

um. Normal rabbit serum did not affect the dTK activity of any of the cell extracts (data not shown). These data indicate that the enzyme induced in cells infected with MMdU^r-20 was indeed virus specific.

Thermal inactivation of dTK activity. dTK$^-$ 3T3 cells were used to investigate the thermal stability of the dTK induced by WT virus and MMdU^r-20. Extracts of WT-infected 3T3 cells were diluted with lysis buffer to equalize the phosphorylating activity with those of MMdU^r-20-infected cells, and the extracts were incubated at 40 and 45°C in a water bath. Samples were removed to 0°C at various times. All samples were assayed simultaneously in the standard assay system. The results are presented in Fig. 3. The enzymes followed the same pattern of thermal inactivation, although the enzyme induced by WT virus was more rapidly inactivated than that induced by the mutant.

Kinetics of phosphorylation of [^{14}C]dT and [^{14}C]araT. In our previous studies (26), we reported that MMdU^r-20 was more resistant to MMdU, ACV, and araT than to BVdU. Because this mutant was as sensitive as the WT virus to phosphonoacetate, we suggested that the alteration was probably in the dTK rather than in the DNA polymerase. Our present studies support this hypothesis. Although we have not directly examined the possibility that MMdU^r-20 contains an additional mutation in the DNA polymerase, the data we have obtained do not appear to require such an additional mutation to explain the pattern of resistance of MMdU^r-20. This is substantiated by the recent study by Larder et al. (21) of three mutants of HSV-1 resistant in different degrees to ACV and BVdU. Each mutant specified a dTK, and the patterns of resistance correlated with the affinity of the analog for the dTK. The DNA polymerases of these mutants were also studied, and it was found that each was similar to that of the parental WT strain with respect to affinity for dTTP, dGTP, ACVTP, and BVdUTP. The authors concluded that the alterations in dTK substrate specificity were sufficient to explain the resistance observed, which is precisely our point in the present study.

Comparative studies on the phosphorylation of [^{14}C]dT and [^{14}C]araT were performed with extracts of MI cells and of cells infected with WT virus and with MMdU^r-20. Extracts with added substrates were incubated at 37°C, and samples were removed at various times and assayed in the standard dTK assay system for the amount of phosphorylated compound formed. The extract of cells infected with MMdU^r-20 contained about half the activity of the extract of WT-infected cells, and the reaction was linear to 40 min in both cases (Fig. 4). Further experiments (data not shown) confirmed linearity for 80 min, which is consistent with the observations of McGowan (Ph.D. thesis).

In Fig. 5, the phosphorylation of [^{14}C]araT by extracts of cells infected with MMdU^r-20 is shown to be considerably less than would be expected based on the relative amounts of [^{14}C]dT-phosphorylating activity in these and in extracts of WT-infected cells. This suggests that resistance of this mutant to araT was probably

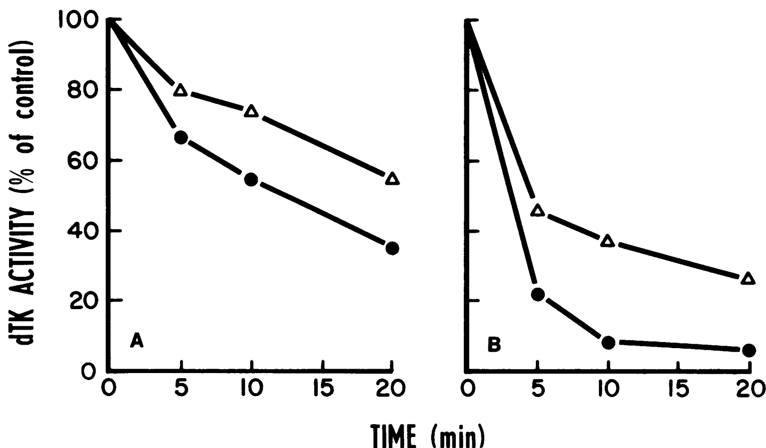

FIG. 3. Effect of heat on dTK activity. Extracts of dTK$^-$ 3T3 cells infected with MMdU^r-20 (△) and WT virus (●) were incubated at 40 (A) or 45°C (B) in a water bath. Samples were withdrawn at the indicated intervals and were assayed for dTK activity. The percentage of residual activity was calculated with the dT phosphorylated before the reaction mixtures were incubated taken as 100%. This 100% activity (in counts per minute per 10 μl of extract) was: for WT at 40°C, 6.4×10^4; at 45°C, 8.6×10^4; for MMdU^r-20 at 40°C, 7.6×10^4; at 45°C, 8.4×10^4.

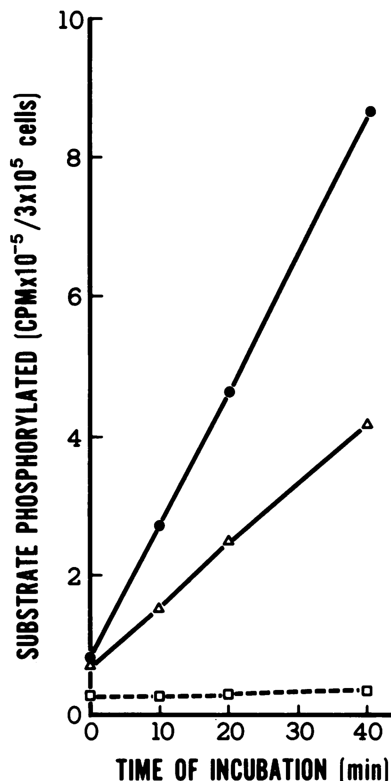

FIG. 4. Kinetics of phosphorylation of [^{14}C]dT. Extracts of cells infected with MMdU^{r}-20 (△) and WT virus (●) (16 to 18 h postinfection) were incubated at 37°C with reaction buffer containing [^{14}C]dT (1.0 μCi/ml, 0.02 mM). At the indicated times, samples were withdrawn and assayed for the amount of phosphorylated compound formed. □, MI cells.

due to a selective reduction in the ability of the MMdU^{r}-20 dTK to phosphorylate araT.

Detection of mono-, di-, and triphosphates of dT and araT. We determined the kinetics of phosphorylation of dT to dTMP, dTDP, and dTTP using TLC and high-pressure liquid chromatography and found detectable levels of the di- and triphosphates of dT by both techniques (manuscript in preparation). The possibility of formation of araTMP, araTDP, and araTTP was also investigated; it was necessary to increase the incubation time because of the relatively lower specific activity of the [^{14}C]araT. Extracts (10 μl) of MI 3T3 cells and cells infected with WT virus and MMdU^{r}-20 were incubated at 37°C with 90 μl of reaction buffer containing [^{14}C]dT or [^{14}C]araT. After 60 min, the reaction was terminated (in the case of [^{14}C]dT) by freezing. The reaction with [^{14}C]araT was terminated after 8 h of incubation, when the sample was freeze-dried and suspended in 10 μl of distilled water. A 3-μl sample of [^{14}C]dT-enzyme reaction mixture was spotted on a polyethyleneimine-cellulose F TLC plate; in the case of [^{14}C]araT, the total reaction mixture was spotted. The plates were then processed as described.

The results (Fig. 6) show several important features. First, both enzyme extracts phosphorylated [^{14}C]dT to dTMP, dTDP, and dTTP, but the amount of dTTP formed by MMdU^{r}-20 was somewhat less than that formed by WT virus. Second, and more important, the mutant enzyme phosphorylated [^{14}C]araT to araTMP only to a slight extent as compared with the WT virus, and no araTDP or araTTP could be detected. The WT enzyme extract, however, phosphorylated araT to araTMP, araTDP, and araTTP. It therefore appears that the mutation(s) in MMdU^{r}-20 affect araTK activity. Whether araTMPK activity was affected cannot be determined from these data, but it remains a possibility. No nucleotide spots were detected with the MI cell extract. These results agree with the data on the relative phosphorylation of [^{14}C]araT (Fig. 5) and further support the notion that resistance of the mutant to araT was due to a loss of phosphorylating ability.

Kinetics of phosphorylation of [^{14}C]dTMP. To investigate possible differences (suggested by the previous experiments) in the dTMPK activity of the WT and MMdU^{r}-20 enzymes, [^{14}C]dTMP was used as a substrate. Because host dTMPK, in contrast to HSV dTMPK, is very sensitive to heat (3, 19), we used freshly

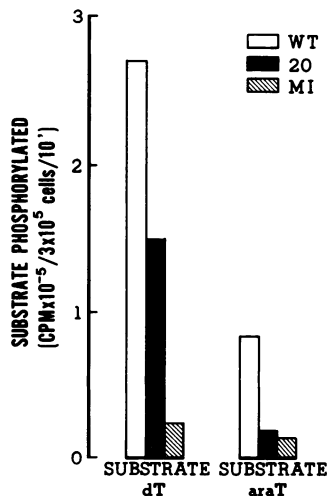

FIG. 5. Relative phosphorylation of [^{14}C]araT by extracts of MI cells and cells infected with MMdU^{r}-20 and WT virus. Experiments were performed like those shown in Fig. 4, except that [^{14}C]araT (17 μCi/ml, 1.7 × 10^{-3} M) was used. Phosphorylation after 10 min is shown.

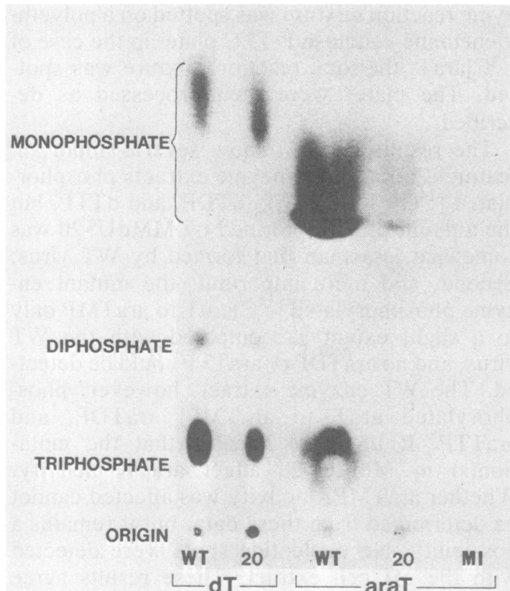

FIG. 6. Detection of di- and triphosphates of [^{14}C]dT and [^{14}C]araT by TLC. Extracts of MI cells and cells infected with MMdU^r-20 and WT virus were incubated at 37°C (without added dT) with reaction buffer containing either [^{14}C]dT (1.0 μCi/ml, 0.02 mM) or [^{14}C]araT (4.5 μCi/ml, 0.45 mM). The reaction was terminated after 1 h in the case of [^{14}C]dT (8 h in the case of [^{14}C]araT), and the total reaction mixture (100 μl) was freeze-dried, suspended in 10 μl of distilled water, and spotted on polyethyleneimine-cellulose F TLC plates. A 3-μl sample of the [^{14}C]dT reaction mixture was spotted. TLC plates were air dried and processed as described.

prepared enzyme extracts, preincubated at 37°C for 30 min, to eliminate host dTMPK activity. [^{14}C]dTMP was added, and the samples were further incubated at 37°C. At various times, samples were removed and assayed for the formation of dTDP and dTTP. The data (Fig. 7) suggest that dTMP kinase activity was linear for at least 20 min in the case of the WT enzyme. There was, however, no significant dTMPK activity with the extracts of MMdU^r-20-infected or MI cells. A similar experiment with the extract of WT cells heated at 45°C for 30 min indicated a substantial loss of dTMPK activity (data not shown).

Inhibition of dT phosphorylation by araT, MMdU, and BVdU. The abilities of BVdU, MMdU, and araT to inhibit the phosphorylation of [^{14}C]dT were next compared. Extracts of cells infected with WT and mutant virus were incubated with these nucleoside analogs at various concentrations and with [^{14}C]dT at a constant concentration. After 1 h of incubation at 37°C, the reaction mixture was assayed for the phosphorylated [^{14}C]dT product. The data (Fig. 8) complement the data obtained in the [^{14}C]araT phosphorylation studies (see Fig. 5 and 6). AraT and MMdU significantly inhibited the phosphorylation of [^{14}C]dT by the WT enzyme only. The reduced inhibition by araT and MMdU with the mutant enzyme further indicates an altered substrate specificity of this enzyme. BVdU had somewhat less effect on the mutant dTK than did araT and MMdU; this agrees with our earlier observation (26) that MMdU^r-20 is only partially resistant to BVdU.

DISCUSSION

Previous studies (26) showed that MMdU^r-20 induced dTK activity both in dTK$^-$ (3T3 and L-Kit) and in dTK$^+$ (BHK) cells, but only to a level 50 to 70% as high as that of WT virus. The present studies confirm that observation (Fig. 1).

Antiserum raised against purified dTK of HSV strain KOS specifically inhibited both MMdU^r-20 and WT dTK activities, confirming the viral specificity of the induced dTK activities (Fig. 2).

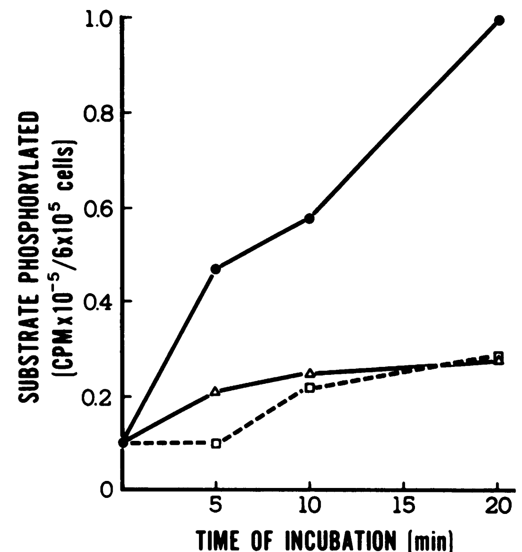

FIG. 7. Kinetics of phosphorylation of [^{14}C]dTMP. Extracts of MI cells (□) and cells infected with MMdU^r-20 (△) and WT virus (●) were preincubated at 37°C for 30 min. Reaction mixtures containing [^{14}C]dTMP (1.2 μCi/ml, 0.024 mM) were added and again incubated at 37°C. At the indicated intervals, samples were withdrawn, spotted on DE 81 filter paper, and washed in 0.1 M sodium formate (pH 3.4) to remove dTMP. The amount of bound nucleoside di- and triphosphates was determined by liquid scintillation counting. Results are plotted as counts per minute of [^{14}C]dTMP phosphorylated against time.

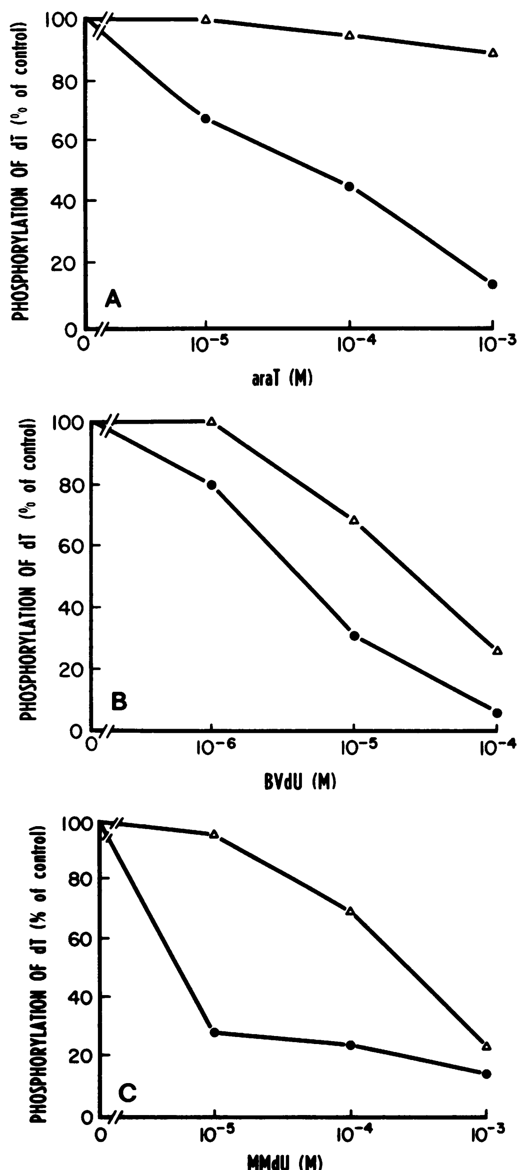

FIG. 8. Effect of araT (A), BVdU (B), and MMdU (C), on the phosphorylation of [^{14}C]dT. Cells infected with MMdU^r-20 (△) and WT virus (●) were lysed in buffer containing 0.05 mM dT, and lysates were assayed for dTK in the presence of various concentration of nucleoside analog. The percentage of residual activity was calculated with dT phosphorylated when no nucleoside analog was added to the reaction mixture.

Thermal inactivation studies showed that the mutant enzyme was more thermostable than the WT enzyme at both 40 and 45°C (Fig. 3). In contrast, Larder and Darby (20) recently showed that the dTK of a dTK$^+$ mutant unable to phosphorylate ACV was more thermolabile than the dTK of the WT virus.

Recent in vivo studies of MMdU^r mutants revealed differential sensitivities to various nucleoside analogs (Veerisetty and Gentry, unpublished results). In an earlier study, the mutant MMdU^r-20 was slightly resistant to BVdU, as compared with all other mutants (26). The present studies on the phosphorylation of [^{14}C]araT and the inhibition of [^{14}C]dT phosphorylation by araT, MMdU, and BVdU suggest altered substrate recognition by the mutant enzyme (Fig. 4–6, 8). The WT dTK efficiently phosphorylated both dT and araT, whereas the MMdU^r-20 dTK failed to phosphorylate araT appreciably (Fig. 5 and 6). Longer incubation periods and alternative methods of detection (TLC coupled with the use of radioactive enhancer to compensate for the reduced specific activity of the araT) also showed negligible phosphorylation of araTMP by the MMdU^r-20 enzyme (Fig. 6) as well as a failure of formation of araTTP. In contrast, significant amounts of araTMP, araTDP, and araTTP were formed by the WT enzyme. This suggests that the mutant may have acquired its resistance to nucleoside analogs because of its relative inability to phosphorylate them. The results showing inhibition of dT phosphorylation by MMdU, BVdU, and araT (Fig. 8) substantiate this and agree with the differences in levels of resistance reported earlier (26).

An effort was made to establish a relationship between the thermostability of the dTK and its ability to phosphorylate dTMP because it has been suggested (3–5) that a virus-specific heat-resistant dTMPK activity is present in HSV-infected cells.

The data in Fig. 7 show that there is dTMPK activity in extracts of WT-infected cells and, furthermore, that this activity is relatively heat stable. In contrast, no significant activity was seen in extracts of cells infected with MMdU^r-20. This lack of activity suggests that either MMdU^r-20 dTK does not have dTMPK activity at all, or that if it does, the dTMPK activity is relatively more heat labile than the corresponding activity seen in the extracts of WT-infected cells. In any case, it seems clear that both dTK and dTMPK activities are altered in the case of the mutant MMdU^r-20. This provides genetic evidence to support the suggestion of Chen, Prusoff, and co-workers (3–5) that both activities are present on the same polypeptide. If this is true, and if the dTK of MMdU^r-20 differs from that of the WT virus by a single mutation (which seems likely because the mutant was originally selected by a single step), then it could be argued further that the active sites, if not identical, should at least be located close together. An alternative possibility is that the mutation could be remote from the active site and yet still affect folding of the polypeptide with similar results.

Resolution of this question must await further study.

Two classes of dTK mutants have been described by Summers et al. (24), one resulting from a nonsense mutation and the other from a missense mutation. The former would be devoid of enzyme activity, whereas the latter might include considerable variation in the physicochemical properties of the enzyme. We conclude that the mutant MMdU^{r}-20 dTK belongs to the missense type, as is evident from the various altered properties that are described above.

Recently, several mutants of HSV-1 resistant to ACV, BVdU, or both and having an altered dTK substrate specificity with no loss in virulence to mice have been isolated (9, 21). These mutants were further shown by Darby and coworkers to have decreased affinity for several other nucleosides as compared with the parental strain (20, 21). The mutant virus described in the present communication seems to behave somewhat like these mutants in at least in two respects: (i) it induces a virus-specific dTK, and (ii) the dTK has altered substrate specificity for certain nucleoside analogs. The fact that dTK^{+} drug-resistant mutants could be isolated in vitro with ACV (9), BVdU (21), and MMdU (26) suggests that similar mutants may be obtained with other nucleoside analogs that require HSV dTK specifically for their activation. Since such dTK^{+} mutants are virulent, greater understanding of them may help us develop new and effective drugs.

ACKNOWLEDGMENTS

This study was supported by grants from the National Institute for Dental Research (Public Health Service grant DE-05089) and the American Cancer Society (CH-74).

It is a pleasure to acknowledge the technical assistance of Nancy Lawrence, L. Devine, M. Minyard, and M. Meek. We thank Y.-C. Cheng for his generous gift of HSV-1 antiserum.

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