Effect of herpes simplex virus type 1 infection on nucleoside transport in HeLa S3 cells

G. Palú, R. E. Handschumacher, C. Parolin, S. Stefanelli and P. Palatini

Institute of Microbiology, University of Padova Medical School, Via A. Gabelli 63, 35100 Padova, Department of Pharmacology, University of Padova Medical School, Largo Meneghetti 2, Padova, Italy and Department of Pharmacology, Yale University Medical School, 333 Cedar Street, New Haven, Connecticut, U.S.A.

The initial velocity of thymidine uptake was measured in HeLa S3 cells infected with herpes simplex virus type 1 (HSV-1). The rate of nucleoside influx into the cells was shown to increase from as early as 1 h post-infection (p.i.) up to 8 h p.i. This increased uptake was shown to be attributable to a progressively increasing contribution from passive diffusion superimposed upon normal transport. Thus, the specific nucleoside transport system was still operating with unaltered kinetic parameters 8 h after infection. Despite the inhibition of host cell protein synthesis and its replacement by the synthesis of virus-specified proteins, the numbers and affinity of the nucleoside transporters in cells 8 h after infection were virtually unchanged. The increased transport of thymidine in infected cultures was resistant to the nucleoside transport inhibitor dipyridamole, and was correlated with entry of a normally impermeant solute (sucrose) into infected cells. These data suggest that the system for the carrier-mediated facilitated diffusion of nucleosides remains intact in HSV-infected cells, but that progressively increasing passive diffusion takes place. Passive diffusion is the major process operating late after virus infection.

Introduction

The process of nucleoside influx into cells has been shown to be mediated by a mechanism of facilitated diffusion both in erythrocytes and cultured cells (Plagemann & Wohlhueter, 1980; Paterson et al., 1981; Young & Jarvis, 1983; Paul et al., 1975; Cass et al., 1974; Jarvis & Young, 1980; Lauzon et al., 1977). Recently, a concentrative carrier-mediated transport of nucleosides has also been found in cells directly isolated from normal tissues (Spector, 1980, 1982, 1985; Schwenk et al., 1984; Jakob & Paterson, 1986; Darnowski et al., 1987). Passive diffusion does not contribute significantly to nucleoside permeation under physiological conditions (Plagemann et al., 1988), an observation consistent with the hydrophilic nature of the nucleoside molecule. The properties of cell membranes can change quite drastically when cells are infected with viruses, leading to structural alterations of membranes themselves and to modifications in the cells' electric potential (Fritz & Nahmias, 1972; Carrasco & Esteban, 1982; Spear et al., 1970; Farnham & Epstein, 1963; Garry et al., 1979; Francoeur & Stanners, 1978). Moreover, the occurrence of membrane leakiness has been described to account for the entry of normally impermeant metabolites (Carrasco, 1977, 1978; Carrasco & Smith, 1976). Cell leakiness has a number of practical implications, particularly with respect to some aspects of drug metabolism under conditions of viral infection and the use of impermeant compounds as selective antiviral agents (Lacal & Carrasco, 1983; Benedetto et al., 1980). It is reasonable to assume that virus infection and leakiness, in particular, could also affect the uptake of those substances such as nucleosides, which normally only permeate through a transporter. Changes in the permeation of nucleosides could be relevant to the effectiveness of antiviral therapy, which is currently based on nucleoside analogues. The limited evidence that is available suggests a reduced nucleoside transport in cells infected with togaviruses or picornaviruses (Genty, 1975; Castrillo & Carrasco, 1986). However, the majority of these experiments were performed using techniques (filtration and washing) that do not permit measurement of the initial rates of solute influx. With these methods, the contributions from intracellular nucleoside metabolism and redistribution between intracellular and extracellular compartments are combined to prevent evaluation of effects on the uptake process. To gain further insight into the effects produced by viral infection on cell nucleoside permeation we have undertaken experiments on the transport of thymidine (TdR) by HeLa S3 cells infected with herpes simplex virus type 1 (HSV-1). TdR was used.
because most antiviral agents are related to it (Dolin, 1985) and HSV-1 was selected as the experimental model because of its sensitivity to nucleoside analogues (De Clercq, 1982, 1989).

Methods

Radioisotopes and compounds. [14C]Thymidine (>540 mCi/mmol), [3H]Thymidine (14-2 Ci/ml), tritiated water (1 Ci/ml) and inulin [3H]carboxyllic acid (2 to 10 mCi/mmol) were purchased from Amersham. [3H]NBMPR (4 Ci/mmol; 6-[(4-nitrobenzyl)thio]-9-β-D-ribofuranosylpurine) was purchased from Maravek Biochemicals. NBMPR and dipyridamole were from Aldrich and Sigma, respectively. All reagents used in our experiments were AnalyQ grade.

Cells and culture conditions. All experiments reported in this paper were performed using HeLa S3 cells, a cell line adapted to growth in suspension. These cells allowed the measurement of the initial rates of nucleoside influx, an approach not feasible with adherent cells. HeLa S3 cells were normally grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) foetal calf serum (FCS), 2 mM-glutamine, 20 mM-HEPES and antibiotics. Cells were cultured in 25 cm² diameter roller bottles at 3-5 r.p.m. in a moist atmosphere of air and 5% CO₂, starting with 5 x 10⁶ cells/ml.

Virus infection. HeLa S3 cells were infected with the wild-type (wt) strain of HSV-1 (Palú et al., 1988, 1989; Palú & Biasolo, 1988) at an m.o.i. of 20 p.f.u./cell. In some control experiments HeLa S3 cells were also infected with the HSV-1 thymidine kinase (TK)-deficient strains R100 (Palú et al., 1988, 1989) and CL 101-TK 43 (Summers et al., 1983), which multiplied at the same rate in this cell line, as shown by one-step growth measurements. During the adsorption period (1 h) bottles were rotated at 1-5 r.p.m. Cells were then sedimented and resuspended in DMEM with 2% FCS in new bottles, which were again rotated at 3-5 r.p.m. At various intervals after infection cells were harvested and used for studying TdR uptake, titration of NBMPR binding sites and protein synthesis. At the end of each experiment virus growth was measured by titrating the yields of infectious progeny by plaque titrations on susceptible vero cells (Palú et al., 1984).

Determination of protein synthesis inhibition. HeLa S3 cells were infected with the wt strain at an m.o.i. of 20 p.f.u./cell and labelled with L-[35S]methionine (>1000 Ci/mmol) for each successive 2 h interval during the period from 0 to 8 h post-infection (p.i.). Cultures were labelled in DMEM containing one-tenth the normal amount of L-methionine and 0.5% dialysed FCS. After the labelling period cells were lysed in Laemmli solution and the lysates separated by 12% SDS-PAGE. Following the electrophoretic run, gels were fixed in a 15% TCA and 30% methanol solution, dried and the gel films scanned with an AMBIS automated beta scanning system (Automated Microbiology Systems) to evaluate the relative amount of radioactivity incorporated in each protein band.

Determination of thymidine uptake. Initial rates of thymidine uptake were determined according to the method of Young & Jarvis (1983), as described in detail below. Solutions of the nucleoside were prepared that contained a mixture of unlabelled and radioactive TdR (in a 300 to 1 molar ratio). Equal volumes (100 µl) of TdR and cell suspension (5 x 10⁶ cells/ml) were simultaneously mixed, using a dual syringe (Young & Jarvis, 1983), and dispensed in a series of 500 µl microhaemocytometer (microfuge) tubes placed in an Eppendorf centrifuge 5414. These tubes contained 50 µl of a (20% w/v) solution of TCA overlaid with 100 µl of a mixture of silicone oil and paraffin (density 1.13 g/ml). After various periods of time (0 to 180 s) the cell suspensions were sedimented by centrifugation (12000 g for 30 s) through the oil-paraffin mixture into TCA. The microfuge tubes were frozen in dry ice and ethanol and the tube bottom containing the cell pellet in TCA was sliced off. The intracellular incorporation of TdR was measured in a liquid scintillation spectrometer (LKB 1214 RackBeta).

For inhibition experiments with dipyridamole HeLa S3 cells were preincubated at 21 °C for 20 min with the inhibitor.

Sucrose uptake and calculation of intracellular and extracellular space. HeLa S3 cells (5 x 10⁶ cells/ml) were incubated for 2 min with increasing concentrations of [14C]sucrose (from 0-1 to 1 µCi), tritiated water (from 0-1 to 1 µCi) and [3H]cinulin (from 0-1 to 1 µCi). At the end of the incubation cells were centrifuged through the oil-paraffin mixture, as previously described. Intracellular volume was calculated by subtracting the inulin space from the water space (Belt, 1983). Sucrose permeation was considered to have occurred when the space occupied by this molecule exceeded the extracellular space, as measured by radioactive inulin.

Titrations of NBMPR binding sites. HeLa S3 cells were incubated at 21 °C for 20 min with an increasing concentration of [3H]NBMPR (from 0-1 to 20 nM). After this time cells were centrifuged, as above, and radioactivity was measured in the cell pellets. Specific binding was estimated as the difference between [3H]NBMPR binding in the presence and absence of a 1000-fold excess of unlabelled NBMPR.

Results

Inhibition of protein synthesis

After virus infection, synthesis of cellular polypeptides declined progressively from 2 h onwards, as depicted in Fig. 1 and confirmed by a computerized radiometric analysis of radioactive polyacrylamide gels (not shown). A drastic inhibition of the host cell protein synthesis in HSV-1-infected cells began from 4 h p.i., at which time cells were still intact, as judged by trypan blue exclusion. Although there was an almost 100% reduction in the synthesis of some cellular proteins, complete inhibition was not produced, even at 8 h p.i. Actin seemed to be the least affected of the whole set of cellular proteins.

Transport studies

Zero-trans flux experiments were performed at 1, 4, 8 and 24 h p.i. At 24 h p.i. the majority of cells could not be sedimented through the silicone oil mixture, but remained at the interface between the medium and the lipophilic solvent. The uptake process was therefore only measured at intervals from 0 to 8 h p.i. In both infected and uninfected HeLa S3 cells the process of TdR influx increased linearly in the period from 2 to 20 s after addition of the nucleoside to the incubation medium. Thereafter the rate of uptake declined, reaching a plateau after about 40 s (Fig. 2a). Addition of a large excess of unlabelled TdR abolished the linear uptake that occurred from 2 s onwards, but did not prevent the very rapid increase in radioactivity observed within the first
Nucleoside uptake in HSV-1-infected cells

2 s. Therefore, it seems likely that a non-specific TdR association with the plasma membrane, rather than a carrier-mediated uptake, was responsible for the increase in radioactivity measured during the initial 2 s. Accordingly, initial rates of uptake were determined as the difference between the uptake at 15 and 5 s. The rates of TdR influx are expressed as the change in the intracellular concentration of nucleoside (molarity) per s. As shown in Fig. 2(b) a threefold increase in the influx rate occurred after 8 h of viral infection, although the increase in TdR influx was already evident as early as 1 h p.i.

Influx of sucrose

The entry of sucrose was measured in order to determine whether infected cells became leaky to this normally impermeant molecule. In contrast to the negligible uptake of sucrose by uninfected cells (<0.1 μmol/5 × 10⁵ cells), sucrose uptake in cells at 8 h p.i. corresponded to 1.5 μmol per 5 × 10⁵ cells (0.2 μl sucrose per μl intracellular space, Fig. 2b). The increased uptake of sucrose clearly paralleled the increased uptake of TdR by infected cultures and clearly indicates a change in the permeability of infected cells.

Titration of NBMPR binding sites

The results of NBMPR binding to uninfected HeLa cells and cells 8 h after virus infection are presented in Fig. 3(a) and (b) as Scatchard plots. Virtually the same maximal binding occurred in mock-infected and infected cells. This indicates that, even after 8 h, virus infection did not cause a reduction in the number of those nucleoside transporters capable of binding NBMPR (9.5
Fig. 3. Scatchard plots of the binding of [3H]NBMPR to mock-infected (a) and infected (b) HeLa S3 cells. Binding to 5 x 10^5 cells was measured at concentrations of NBMPR from 0.1 to 20 nM at 24 °C. Computer-generated fits gave a K_d value for mock-infected cells (high affinity site) of 1.3 x 10^{-10} M and 1.4 x 10^{-10} M for the infected cells. Numbers of binding sites per cell were 9.5 x 10^5 and 8.9 x 10^5 for mock-infected and uninfected cells, respectively.

Fig. 4. TdR uptake by HeLa S3 cells as a function of TdR concentration (a). Numbers in the figure (h p.i.) represent the time of cell exposure to HSV-1 before uptake determination, which was measured between 5 and 15 s, as described in the text. Within this time the uptake was linear at all concentrations used. (b) Double-reciprocal plot of the data shown in panel (a).

preliminary experiments had indicated that the maximal inhibition of TdR uptake by NBMPR was 65%, an observation that is consistent with previous results (Plagemann et al., 1988). The curvature of the binding isotherms suggests the presence of at least two independent binding sites with different affinities for NBMPR. Dissociation constants for NBMPR, evaluated from binding isotherms by computer fitting, as previously described (Wohlhueter et al., 1983), indicated that the overall affinity of NBMPR for the transporter was not changed significantly by viral infection (K_d for the high affinity site was 1.3 x 10^{-10} and 1.4 x 10^{-10} M in uninfected and infected cells, respectively).

Saturation kinetics experiments
Fig. 4 shows the kinetics of TdR uptake by infected and
uninfected cells as a function of TdR concentration. Consistent with the exclusive presence of a carrier-mediated influx, the uptake process reached a plateau in uninfected cells. In infected cells the transport kinetics underwent a dramatic change and the saturation curve did not reach a plateau. This alteration in the uptake process was evident as early as 1 h p.i. and was the major uptake process by 8 h p.i. Kinetic parameters of the influx process were determined by computer fitting (Roos & Pfleger, 1972) of the experimental data to the following equation: 

\[ Y = \frac{V_m \times [S]}{K_m + [S]} + k \times [S], \]

where \( Y \) is TdR uptake, \([S]\) is TdR concentration, \( V_m \) and \( K_m \) are maximum uptake and Michaelis constant of the saturable component, respectively, and \( k \) is the first-order rate constant for passive permeation. This analysis showed that \( k \) increased dramatically with the infection time, whereas \( K_m \) and \( V_m \) showed little variation. In particular, these values before and after 8 h of cell exposure to HSV-1 were 203 ± 18 and 185 ± 25 μM for \( K_m \), 0.10 ± 0.01 and 0.11 ± 0.01 μM per second for \( V_m \) and \( 0.10 \times 10^{-4} ± 0.002 \times 10^{-4} \) and \( 2.1 \times 10^{-4} ± 0.2 \times 10^{-4} \) per second for \( k \), respectively (means ± s.d. of four determinations). The same data are represented in Fig. 4(b) as double-reciprocal plots. It can be seen that the plot is linear, as expected from pure Michaelis-Menten kinetics, only for uninfected cells. After infection the plots curved downwards, a result consistent with the presence of a passive diffusion component (Roos & Pfleger, 1972).

**Inhibition studies**

Further evidence for development of a carrier-independent entry of TdR was obtained by inhibition experiments with dipyridamole. This inhibitor specifically prevents the carrier-mediated uptake of nucleosides (Cass et al., 1981; Paterson et al., 1980; Eilam & Cabantchik, 1977; Aaronow et al., 1986), whereas the passive diffusion component should be unaffected. Dipyridamole was preferred to NBMPR for these experiments, since HeLa cells proved to be more sensitive to the former inhibitor (maximal inhibition was 90% at 1 μM-dipyridamole, as previously observed; Paterson et al., 1980). Fig. 5(a) shows that 100 nm-dipyridamole produced 67% inhibition of nucleoside uptake in uninfected cells. At progressively later times after virus infection, the proportion of uptake that was inhibited by dipyridamole decreased, indicating that the dipyridamole-sensitive component of the total uptake was becoming less and less important as the viral infection progressed. Fig. 5(b) is a representative experiment of TdR uptake in the presence and absence of 1 μM-dipyridamole at 8 h p.i. In the presence of a saturating concentration of the inhibitor the nucleoside uptake was relatively resistant to the inhibitor and residual uptake was almost linear, again indicating the presence of a major contribution from a passive diffusion process.

**Discussion**

The process of virus infection produces gradual alterations of the permeability and transport properties of the
cell membrane. For example, certain molecules that do not enter the cell under normal conditions can cross the membrane barrier after viral infection. Conversely, some molecules that are actively transported into normal cells are no longer accumulated in infected cells (Lacal & Carrasco, 1983). The exact consequences of viral infection on the entry of nucleosides into cells have not yet been elucidated. In the present paper we have shown that the transport of TdR in HeLa S3 cells is greatly affected by infection with HSV-1. The first important observation is the increase in the net rate of TdR uptake that occurs after infection. As the initial rates of TdR influx were always measured, this phenomenon is probably a direct effect of modifications to the properties of the membrane and not secondary to intracellular changes in the activity of enzymes encoded by either the cell or the virus, such as nucleoside kinases, phosphorolases or phosphatases. Similar results were obtained using the HSV-1 TK-deficient strains R100 and CL 101-TK 43, or after depleting the cells of ATP by arsenate treatment (Paterson et al., 1981; Wohlhueter et al., 1979) (not shown).

After infection, as illustrated in Fig. 4, passive diffusion was gradually superimposed upon facilitated transport. Accompanying the increase of passive diffusion of TdR is the entry of sucrose, a normally impermeant molecule with approximately the same molecular size as the nucleoside. Inulin (6000K), a much larger polymer, is still excluded by the intracellular compartment under the same conditions. The increase in passive diffusion caused by HSV-1 infection correlates temporally with the virus-induced inhibition of cell protein synthesis.

Saturation kinetics experiments provided evidence that the affinity of the nucleoside transporter for thymidine is not significantly changed by viral infection and that the maximum velocity of the carrier-mediated transport process is unaltered. This latter result is consistent with the observation that the total number of transporter molecules, as deduced from the maximum value of NBMPR binding, is not decreased in infected cells. These results suggest that the half-life of the transporter is quite long (certainly more than 8 h) and that the facilitated diffusion route is still operating in HSV-infected cells.

Our results on the transport of TdR in HSV-1-infected HeLa S3 cells are somewhat at variance with data previously reported by other authors (Genty, 1975; Castrillo & Carrasco, 1986), showing a decrease in total nucleoside uptake after virus infection. However, these studies were carried out with different viruses (vesicular stomatitis virus and encephalomyocarditis virus). In addition, the experimental conditions were such that actual differences in transport could not be measured, since uptake was assessed from data on incorporation and variation of nucleoside pools at times longer than 1 min.

Influx of normally impermeant substances, especially low Mr aminoglycosides, has previously been reported in virus-infected cells (Lacal & Carrasco, 1983; Benedetto et al., 1980). This property led to the suggestion of a new approach to an antiviral chemotherapy with substances that are made permeant by virus infection. For those antiviral nucleosides which are transported via the facilitated diffusion system, it could be suggested that prior blockade with dipyridamole would prevent entry of cytotoxic agents into uninfected cells, but allow entry into the infected cell population.

Further investigation into the nature of the damage produced by HSV-1 infection upon cell structures that are directly or indirectly involved in transport functions is certainly warranted. Such information may bear a potential relationship to viral pathogenicity and may also contribute to dissection of some functional properties of carriers whose structure has not been defined.

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References


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