Adsorption and Penetration of Enveloped Herpes Simplex Virus Particles Modified by Tunicamycin or 2-Deoxy-D-glucose

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SUMMARY

Tritium-labelled, purified herpes simplex virus (HSV) enveloped particles produced in the presence of tunicamycin (TM) or 2-deoxy-D-glucose (DG) adsorbed to GMK cells equally as well as standard virus. However, in the presence of TM or DG, there was a reduced transport of virus DNA to cell nuclei and an increased sensitivity of attached particles to proteinase K. The reduced infectivity of HSV produced in the presence of glycosylation inhibitors is therefore probably due to an impairment in the fusion of virus envelope with plasma membranes.

INTRODUCTION

Tunicamycin (TM) and 2-deoxy-D-glucose (DG) are both efficient inhibitors of protein glycosylation. TM inhibits formation of the lipid-linked intermediate in the glycosylation process by blocking synthesis of the N-acetylglucosaminylpyrophosphoryl dolichol (Heifetz et al., 1979). DG, behaving as an analogue of mannose, is incorporated as an antimetabolite into glycoproteins (Scholtissek, 1975). Recently, it has been suggested that DG may also act as an inhibitor of early steps in the formation of the dolichol oligosaccharide (Schwartz & Datema, 1980).

It has been observed with several enveloped viruses that inhibition of glycoprotein formation by TM and DG results in a defective production of virions (Gibson et al., 1978; Leavitt et al., 1977; Nakamura & Compans, 1978; Ogura et al., 1977; Schwartz et al., 1976). However, the biological consequences of this inhibition may differ depending on the virus studied. Thus, the unglycosylated envelope protein of fowl plague virus is rapidly digested by cellular proteases and the budding of viruses at the plasma membrane of the infected cells is arrested (Schwartz et al., 1976). The non-glycosylated G protein of vesicular stomatitis virus is not transported to the plasma membrane and therefore no budding of virus is observed (Gibson et al., 1978). With herpes simplex virus (HSV) and Rous sarcoma virus (RSV), no significant reduction in formation of enveloped virus particles is observed but the yields of infectious virions are markedly reduced (Courtney et al., 1973; Katz et al., 1980; Pizer et al., 1980; Stohrer & Hunter, 1979). The low yields of infectious RSV have been ascribed to the inadequate production of an essential envelope glycoprotein (Stohrer & Hunter, 1979) whereas the mechanism behind the reduction in infectivity of unglycosylated HSV is unknown. The present study shows that the adsorption of HSV particles produced in the presence of TM or DG is unaffected but that the penetration of these particles is impaired.

METHODS

Virus and cells. HSV type 1 strain F and an established line of green monkey kidney cells (GMK AH-1) were used throughout the study. Cells were cultured and HSV was passaged according to standard procedures (Lycke & Roos, 1972, Vahlne & Lycke, 1977).

A temperature-sensitive mutant, designated HSV-1 (HFEM) tsB5, was obtained from Dr A. Buchan, University of Birmingham, U.K. Virus stocks were produced at low input multiplicities (0.01 p.f.u./cell) in order to minimize the production of defective virus.
Inhibitors. Tunicamycin, lot 381-26-117, was a gift from Dr R. L. Hamill, Eli Lilly Research Laboratories, Indianapolis, In., U.S.A.; 2-deoxy-D-glucose was purchased from Sigma. TM was used at final concentrations of 1 to 2 μg/ml and DG was used at a concentration of 10 mg/ml.

Purification of virus. Roller cultures of GMK cells were infected at a multiplicity of infection of 10 and labelled with 1 mCi of [3H]thymidine or [32P]orthophosphate. TM or DG was present in the medium until the cells were harvested 18 h post-infection. Virus, obtained from culture media and from disrupted infected cells, was purified as previously described (Svennerholm et al., 1980).

Assay of virus attachment. Attachment of radiolabelled, purified HSV to GMK cells was assayed as described earlier (Vahlne & Lycke, 1978; Vahlne et al., 1979). Cells, suspended at a concentration of about 2 × 10^7 in 0-9 ml Eagle's minimal essential medium (MEM) in sterile plastic tubes, were infected by adding 0-1 ml of a virus suspension containing 10^6 p.f.u. or the corresponding amount of HSV particles purified from drug-treated cells. All steps were carried out at 4°C. The first sample was taken immediately after virus addition and further samples were taken at 10, 20, and 60 minutes. The samples (0-1 ml) were immediately diluted in 10 ml of cold medium, then the cells were pelleted, washed and assayed for radioactivity. Cell pellets were dissolved in 1% SDS and Insta-Gel was used as scintillation fluid.

The specificity of adsorption of labelled virus to cells was ascertained by blocking adsorption with a rabbit hyperimmune serum reacting type-specifically with HSV-1 envelope antigens. The antisera had a neutralization titre of 10^24 (Jeansson, 1975). Virus was preincubated with the serum (at a final concentration of 10^-1) for 15 min at 37°C before use in adsorption experiments. Cell-associated radioactivity after 1 h of adsorption at 37°C was compared to that obtained with treatment of virus with preimmune serum.

Assay of radiolabelled virus in fractions containing cell nuclei. The transport of virus to the nuclei of infected cells was determined as previously described (Vahlne & Lycke, 1978). Monolayer cultures of GMK cells were inoculated with about 10^6 cts/min of the [3H]thymidine-labelled virus preparation. After adsorption of virus for I h, the cells were washed five times in Hanks' balanced salt solution. The cells were then incubated for a further 4 h to allow transfer of attached virus to nuclei. All steps were carried out at 37°C. Thereafter, the cells were scraped off with a rubber policeman and nucleocyttoplasmic fractions were prepared by the method of Penman (1969). The radioactivity of the fractions was determined.

Assay for penetration of HSV by proteinase K treatment. Purified, tritium-labelled HSV was adsorbed for 1 h at 37°C to monolayers of GMK cells grown in 5 cm Petri dishes. After adsorption, the cells were washed five times with phosphate-buffered saline (PBS) and reincubated with Eagle's MEM, containing 2% newborn calf serum and antibiotics, at 37°C for 4 h. The overlay medium was then discarded and the cells were treated with proteinase K (Boehringer, Mannheim) as described by Helenius et al. (1980) but with minor modifications. The Petri dishes were shaken for 45 min with a 1 mg/ml solution of proteinase K in PBS containing 1 mM-phenylmethylsulphonyl fluoride and 30 mg/ml of bovine serum albumin. Cells were then scraped off and centrifuged at 800 g for 10 min at 4°C. Pellets, washed twice with PBS, and supernatants were counted for radioactivity.

Electron microscopy. Virus particles on carbon-coated copper grids were stained with potassium–phosphotungstic acid (2%, pH 6.8) and observed in a Philips 300 electron microscope.

RESULTS

Production of glycosylated virus particles

Physical particles isolated from HSV-infected GMK cells labelled with [3H]thymidine in the presence of TM or DG were characterized by their behaviour on centrifugation in Percoll gradients. Enveloped infectious HSV particles banding at a density of 1.06 to 1.07 g/ml can be clearly separated from the nucleocapsids (Svennerholm et al., 1980). The low density of those Percoll fractions containing virions is probably an effect of the exclusion of silica particles (Svennerholm et al., 1980; Vahlne & Blomberg, 1974).

The degree of inhibition of glycosylation was measured by SDS–acrylamide gel electrophoresis of glucosamine- or leucine-labelled extracts from HSV-infected cells (data not shown). The results, in agreement with those of other laboratories (Courtney et al., 1973; Knowles & Person, 1976; Pizer et al., 1980), indicated that the incorporation of glucosamine label into polypeptides was completely inhibited at 10 mg/ml of DG, while TM was an efficient inhibitor at a concentration of 0-1 μg/ml. Treatment with glycosylation inhibitors did not result in any detectable decrease in the incorporation of leucine label into virus polypeptides.

Cytoplasmic fractions of HSV-infected cells were first filtered on a Sepharose CL-2B gel column. All the infectivity was found in the void volume fractions. When virus preparations from DG- or TM-treated infected cells were filtered, the void fractions contained 100- to 1000-fold fewer infectious particles, respectively, than those obtained from infected cells cultured...
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Fig. 1. Adsorption of GMK AH-1 cells of [3H]thymidine-labelled, purified HSV particles produced in the presence of DG (\(\triangle\)), TM (\(\square\)), or without glycosylation inhibitor (\(\bigtriangleup\)). 100000 to 200000 ct/min of each virus preparation was added to the cell suspension. Each curve represents the mean of four adsorption experiments and bars indicate mean ± S.E.

Fig. 2. Penetration and transport to nuclei of [3H]thymidine-labelled, purified HSV particles produced in the presence of TM or without glycosylation inhibitor. Following adsorption at 4 °C for 2 h, the relative amounts of radioactivity in the nuclei were determined immediately and again after 4 h of penetration at 37 °C for standard virus (\(\square\)) and for virus produced in the presence of TM (\(\square\)). The penetration of standard virus at 4 °C was also measured (\(\bigtriangleup\)).

Table 1. Characteristics of HSV produced in cultures containing 1% 2-deoxy-D-glucose or 2 μg/ml tunicamycin

<table>
<thead>
<tr>
<th></th>
<th>Standard</th>
<th>DG-modified</th>
<th>TM-modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infectivity (p.f.u./ml)(\dagger)</td>
<td>2.0 ± 0.98 (\times 10^7)</td>
<td>3.2 ± 0.74 (\times 10^5)</td>
<td>2.5 ± 0.97 (\times 10^4)</td>
</tr>
<tr>
<td>[3H]Thymidine incorporation (ct/min/ml)(\dagger)</td>
<td>56200 ± 26870</td>
<td>49140 ± 21240</td>
<td>39410 ± 15910</td>
</tr>
<tr>
<td>Density in Percoll gradients (g/ml)</td>
<td>1.064 ± 0.004</td>
<td>1.061 ± 0.004</td>
<td>1.064 ± 0.005</td>
</tr>
</tbody>
</table>

\(\dagger\) The values given are the means of five experiments ± S.E.

without any inhibitor. In contrast, only a minor reduction of radioactivity in these void volume fractions was observed (Table 1). Subsequent centrifugation of these fractions in Percoll gradients, whether from inhibitor-treated or untreated cells, gave one major band at density 1.061 to 1.064 g/ml containing all infectivity and radioactivity (Table 1), indicating a very high proportion of enveloped particles in all preparations studied (Svennerholm et al., 1980). This result was confirmed by electron microscopy. These results are in close agreement with the observations of Courtney et al. (1973) and Katz et al. (1980), showing no major influence of glycosylation inhibitors on the proportion of enveloped particles.

Adsorption of virus particles

The kinetics of attachment of HSV particles from TM- or DG-treated and untreated GMK cells to cellular receptors was studied after mixing radiolabelled, purified virus particles with a GMK cell suspension. Samples were withdrawn at various times post-infection and the cell-bound virus radioactivity was determined.

The results of such experiments are presented in Fig. 1. It is seen that the rate of attachment of virus particles produced in cultures containing TM or DG was not significantly lower than that of unmodified virus. After 60 min of adsorption, 16, 20 and 21% of TM-modified, DG-modified and standard virus, respectively, was recovered in the cell-bound fractions. The differences in the amounts of TM-modified and standard virus adsorbed to the cells were not statistically...
Table 2. Penetration and transport of HSV produced in the presence or absence of glycosylation inhibitors

<table>
<thead>
<tr>
<th>Standard</th>
<th>TM-modified</th>
<th>DG-modified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-associated ct/min</td>
<td>3170 ± 180</td>
<td>3040 ± 450</td>
</tr>
<tr>
<td>Nuclear fraction (% total)</td>
<td>24.5 ± 0.5</td>
<td>12.1 ± 2.1</td>
</tr>
</tbody>
</table>

*GMK cells (5 × 10^6) were inoculated with 10^5 ct/min of radiolabelled HSV. Recovery of radioactivity, expressed as cell-associated (ct/min) and that in nucleus fractions (per cent of total radioactivity), was at 4 h post-infection. The values given are the means of 9 experiments ± s.e.

The specificity of the adsorption of labelled virus to cells was tested with a rabbit hyperimmune serum against HSV-1 envelope antigens. Adding antibody to the reaction mixtures reduced the TM, DG and untreated virus adsorbing in 1 h by 71, 68 and 67%, respectively. A rabbit hyperimmune serum, type-specifically reacting against HSV-2, did not influence the adsorption rates.

Penetration and transport of virus particles

By assaying the radioactivity of DNA associated with the cytoplasmic and nuclear fractions of cells infected with HSV, the processes of penetration and transport of HSV nucleocapsids were followed. Monolayer cell cultures were infected with radioactivity labelled purified virus, which was allowed to adsorb to the cells for 1 h. The cells were then washed and incubated at 37 °C. After 4 h of incubation, the cells were harvested and the radioactivity of nuclear and cytoplasmic fractions was assayed. Results obtained with TM-modified, DG-modified and standard virus are listed in Table 2.

No differences in cell-bound radioactivity were found when equal amounts of radiolabelled modified or standard virus were added to the system. However, the fraction of cell-associated radioactivity recovered from nuclei was significantly lower when using virus particles produced in the presence of TM and DG than that obtained with standard virus (P < 0.01), suggesting an impaired transport of attached virus.

As assayed, the decreased transmission to the nucleus of virus produced in the presence of TM or DG was not enough to explain a 2- to 3-log fall in HSV infectivity. This could be due to the transport assay system employed. Two control experiments, where transmission of virus to the nucleus was not to be expected, were performed in order to see if this could be the case.

The effect of lowering the temperature to 4 °C on the transport of standard virus was studied and compared to that of TM-modified HSV at 37 °C. As shown in Fig. 2, the restriction of virus transport observed at 4 °C was of the same order as that of TM-modified HSV.

Virus particles of an HSV-1 temperature-sensitive mutant (HFEM) tsB5 produced at non-permissive temperature (39 °C) lack the envelope glycoprotein gB. This protein is necessary for the fusion of the virus envelope with the cellular plasma membrane (Sarmiento et al., 1979) and the infection of cells by particles produced at non-permissive temperature is therefore arrested at the penetration level.

The infectivity of the (HFEM) tsB5 produced at 39 °C was reduced 4 logs compared to the yields of mutant virus at permissive temperature (34 °C). To minimize any effects of differences in experimental conditions, virus preparations from cells incubated at 34 or 39 °C or TM-treated were tested in parallel by double-isotope experiments for transport to the nuclei of the same population of cells. An impaired penetration rate of (HFEM) tsB5 produced at non-permissive temperature was observed. The decrease in transport of tsB5 grown at 39 °C to the nuclei was again of the same order of magnitude as that found with TM virus (Table 3).

Release of cell-associated HSV by proteinase K

The fate of adsorbed HSV particles was further characterized by treatment of infected cells with proteinase K. This enzyme has been reported to digest only external membrane proteins and has been shown to discriminate between Semliki Forest virus associated with the cell surface and intracellular virus (Helenius et al., 1980).
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Table 3. **Penetration of (HFEM) tsB5 and TM virus**

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Virus</th>
<th>Cell-associated radioactivity in the nucleus (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>tsB5 at 34 °C</td>
<td>38-0</td>
</tr>
<tr>
<td></td>
<td>tsB5 at 39 °C</td>
<td>13-1</td>
</tr>
<tr>
<td>2</td>
<td>tsB5 at 34 °C</td>
<td>28-6</td>
</tr>
<tr>
<td></td>
<td>F-TM</td>
<td>9-0</td>
</tr>
</tbody>
</table>

* In double-isotope experiments, penetration and transport to the nuclei was studied by using purified 3H-labelled tsB5 produced at permissive temperature (34 °C) and 32P-labelled tsB5 produced at non-permissive temperature (39 °C). Purified HSV F-TM was labelled with 32P. After adsorption at 37 °C for 1 h, cells were washed four times and re-incubated at 37 °C for 4 h. Then, 2 × 10⁷ cells were processed and counted for radioactivity; the proportion associated with the nuclear fraction was calculated.

Table 4. **Release of cell-associated HSV after proteinase K treatment**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Cell-associated virus (% of total) after adsorption</th>
<th>Radioactivity (ct/min) after enzyme treatment</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Supernatant</td>
<td>Cell pellet</td>
<td></td>
</tr>
<tr>
<td>−</td>
<td>4000 ± 101†</td>
<td>4160 ± 42</td>
<td>0-96</td>
</tr>
<tr>
<td>TM</td>
<td>4626 ± 172</td>
<td>1236 ± 127</td>
<td>3-7</td>
</tr>
<tr>
<td>DG</td>
<td>1716 ± 72</td>
<td>758 ± 5</td>
<td>2-3</td>
</tr>
</tbody>
</table>

* 3H-labelled, purified HSV produced in the presence or absence of glycosylation inhibitors was adsorbed to GMK cell monolayer cultures for 1 h at 37 °C. The cells were washed four times and reincubated in maintenance medium for 4 h. Thereafter they were treated with proteinase K as described in Methods.
† Mean ± s.E. of five samples.

After adsorption of [3H]thymidine-labelled, purified virus to GMK cells for 1 h and subsequent incubation at 37 °C for 4 h, the cells were treated with proteinase K. As shown in Table 4, cell-associated HSV particles produced in the presence of glycosylation inhibitors were significantly more accessible to proteinase K treatment than virus produced in the absence of inhibitor. No evidence for different adsorption rates between standard, TM- and DG-modified virus was found. However, with TM- and DG-modified virus, the amount of radioactively-labelled virus released into the supernatants after proteinase K treatment exceeded the amount of cell pellet-associated virus by a factor of 3-7 and 2-3 respectively, in contrast to a ratio with standard virus of 0-96.

**DISCUSSION**

In the presence of TM and DG, which are efficient inhibitors of protein glycosylation, non-infectious enveloped HSV particles are produced (Courtney et al., 1973; Katz et al., 1980). The relevance of the results obtained in the present study to binding and transport of TM- and DG-modified HSV particles hinges entirely on the nature of the radioactive DNA-containing virus preparations employed. For instance, radioactively labelled virus or naked virus DNA could be trapped in cellular membraneous material. However, in our experience, virus preparations are freed from most such trapped radioactivity by sedimentation at 1000 g (Vahlne & Blomberg, 1974) because cellular membranes band at a lower density than virions in silica gradients (Vahlne & Blomberg, 1974). In the present study, it was shown that all the radioactivity, in purified virus preparations from HSV-1 infected cells treated with glycosylation inhibitors, was associated with particles which were not precipitable at 15000 g but were excluded from Sepharose CL-2B. Furthermore, these particles banded after isopycnic centrifugation in colloidal silica at exactly the same density as infectious HSV. Lastly, no particles other than enveloped virus could be detected by electron microscopic examinations of purified virus preparations and the binding of the particle-associated radioactivity to cells was significantly
reduced by HSV antisera to an extent comparable to that with standard virus. These characteristics of the radiolabelled particles strongly suggested that the radioactivity of the purified virus preparations entirely corresponded to enveloped HSV particles.

There may be several reasons why inhibition of glycosylation should result in changes of biological activities of glycoproteins. The oligosaccharide moiety of the glycoprotein may itself be biologically active or, according to the prevailing concept, responsible for the formation and maintenance of the tertiary structure of the peptide part, necessary for the proper functioning of the protein, necessary for transport or responsible for the protection of the protein from proteolytic degradation (Gibson et al., 1980). It has recently been reported that the presence of TM results in a drastic decrease in abundance of at least three (gA, gB and gC) of the glycoproteins coded for by HSV in the cytoplasm of infected cells (Pizer et al., 1980). However, this does not exclude the possibility that the non-glycosylated precursor polypeptides may be found in trace amounts in other compartments of infected cells (P. Spear, personal communication).

Because the occurrence of virus antigens in the cytoplasmic membrane of HSV-infected cells correlates well with those found in the envelope of virus particles produced (Spear, 1976), one might assume that inhibition of glycosylation would result in the production of HSV particles devoid of gA, gB and gC. The presence of gC in the virion envelope does not seem to be necessary for the maintenance of infectivity (Spear, 1976). However, glycoprotein gB is probably involved in the process of virus penetration (Sarmiento et al., 1979) and is essential for the formation of syncitia (Manservigi et al., 1977). Accordingly, Knowles & Person (1976) reported that DG will inhibit HSV-induced cell fusion. We suggest that the infection of cells with HSV particles produced in the presence of TM or DG is blocked at the level of penetration. This assumption is based on the results of experiments assaying transport of virus DNA to cell nuclei and on the availability of attached virus to proteinase K. Any glycoprotein responsible for virus attachment must resist proteolytic activity and be incorporated in sufficient amounts into the virus envelope; HSV adsorption apparently does not require the presence of N-linked oligosaccharides associated with the virus adsorption protein.

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REFERENCES


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