ANIMAL VIRUSES PROMOTE THE ENTRY OF POLYSACCHARIDES
WITH ANTIVIRAL ACTIVITY INTO CELLS

Ma Eugenia González and Luis Carrasco

Centro de Biología Molecular, Universidad Autónoma,
Cantoblanco, 28049 Madrid, Spain

Received June 15, 1987

Charged polysaccharides such as heparin and carrageenan show potent antiviral activity in cultured cells. Labelled [3 H]heparin binds to several virion particles as evidenced by Sepharose 6B chromatography. This binding is inhibited by carrageenan. The complex [3 H]heparin-HSV1 virions is able to enter cells. Thus, almost no entry of [3 H]heparin is observed in control HeLa cells, whereas in the presence of HSV1 or poliovirus the amount of radioactivity internalized is enhanced. This internalization is inhibited by carrageenan, suggesting that these two sulphated polysaccharides bind to the same sites on virion particles and may share a similar antiviral mechanism of action. © 1987

Academic Press, Inc.

Polysaccharides are a complex group of biological molecules that inhibit the growth of animal viruses (1,2). During titration of animal viruses by the standard plaque assay method, it was found that the formation of plaques by several picornaviruses such as poliovirus, encephalomyocarditis virus and coxsackie B4 virus, was reduced by the pre-

sence of sulphated polysaccharides in the agar (see review, 3). Heparin was also found to be a potent inhibitor of herpesvirus replication (4,5). The possibility that heparin was bound to virion particles and inhibited virus attachment and entry was suggested (3). In agreement with this idea we found that carrageenan when added together with herpes particles blocked the appearance of viral proteins (6). However, we observed that carrageenan blocked a step in viral replication subsequent to virus attachment and entry into HeLa cells (2). Those results suggested that sulphated polysaccharides prevented a step in virus growth subsequent to viral internalization but prior to the onset of viral translation.

Sulphated polysaccharides are negatively charged molecules that theoretically can interact with positive charges present in virion particles. However, no direct evidence has yet been obtained for this interaction, which is readily reversed by washing the virions with culture medium (4,5). In addition sulphated polysaccharides are thought not to pass through the cell membrane, since they are large heavily charged macromolecules (3). This idea was in apparent contradiction with our previous findings showing that virion particles enter into cells even in the presence of carrageenan. To know whether sulphated polysaccharides interact with virion particles and to study its passage into cells we have used [3 H]heparin and the results are presented in this work.

MATERIALS AND METHODS

Cells and viruses

Herpes simplex virus type 1 (HSV-1) was grown in Vero cells, poliovirus type 1 in HeLa cells and Semliki Forest virus (SFV) in BHK-21 cells, as described (6).

Source of polysaccharides

Iota-carrageenan was purchased from Sigma and [$^3\text{H}(6)$] heparin sodium salt, (0,34 mCi/mg) from NEN Research Products.

Purification of virion particles

Monolayer of cells grown on petri dishes were infected at a multiplicity of 0.1 pfu (plaque forming units) per cell. After 48 hours the infected cells and the culture medium were collected and centrifuged at 2,000 rpm, for 5 minutes, in the SS-34 Sorvall rotor. The pelleted cells were resuspended by a freezing and thawing cycle. The cellular extract was mixed with the first supernatant and centrifuged at 10,000 rpm in a SS-34 Sorvall rotor for 15 minutes. The supernatant was placed onto 6 ml of a 30% sucrose cushion and centrifuged at 38,000 rpm in a T865 Sorvall rotor for 3 hours. The resuspended pellet was placed on top of a 5-40% dextran gradient (HSV-1), 15-45% sucrose gradient (poliovirus) or in a 15-30% sucrose gradient (SFV) and centrifuged for 1 h (HSV-1), 4 h (poliovirus) or 2 h (SFV) at 18,000 rpm (HSV-1), 25,000 rpm (poliovirus and SFV), in an AH627 Sorvall rotor. The viral peak was collected and centrifuged in a T865 Sorvall rotor as described above. Virion particles were resuspended in one tenth of the original volume of Dulbecco's modified Eagle's medium.

Sephacrose 6B chromatography

A column (1x10 cm) was filled with 10 ml of Sepharose 6B (Sigma). After washing the column with phosphate buffered saline, a total of 100 μl of sample was applied to the column and fractions of 400 μl were collected at a rate of 1 ml/min. Radioactivity of each fraction was measured in an Intertechnique liquid scintillation counter. Infectivity of fractions was monitored by the plaque assay technique on HeLa cell monolayers.

Estimation of radioactivity incorporated into cells

HeLa cell monolayers were incubated with [^3H]heparin in 24-well plates. At the indicated times, the cells were fixed with 5% trichloroacetic acid and then washed twice with ethanol. Dried precipitated cells were suspended in 0.2 ml of 0.1 N NaOH, 1% SDS and the radioactivity was measured in an Intertechnique liquid scintillation counter.

Radiolabelling of virions

HeLa cells were grown on 100 mm diameter dishes. The cell monolayer was infected with HSV-1 at a multiplicity of 10 in Dulbecco's modified Eagle's medium supplemented with 2% newborn calf serum. At 9 h postinfection (h.p.i.) the medium was replaced for 2 ml of fresh methionine-free medium and 100 μCi of [^{35}S]methionine were added. At 24 h.p.i. the cells were harvested and virions were purified as described.

Measurement of the entry of virus particles into cells

HeLa cells grown on 35 mm petri dishes were infected with HSV-1 labelled with [^{35}S]methionine, purified as described above, and incubated at 37°C. At the indicated times cell monolayers were washed with saline phosphate buffer saline and treated with 1 ml phosphate-buffered saline containing 50 $\mu\text{g/ml}$ proteinase K (Merck) for 15 min. at 4°C. Then, cells were washed with saline phosphate buffer and pellets precipitated with 5% trichloroacetic acid. Radioac-

tivity retained in GF/C filters was determined in a liquid scintillation counter (Intertechnique).

RESULTS

Binding of labelled [3 H]heparin to several virion particles was assayed by Sepharose 6B chromatography. Figure 1 shows that no radioactivity is observed in the void volume

Figure 1. Competitive binding of carrageenan and [3 H]heparin to several virion particles. A concentrate of purified virion particles was incubated with the polysaccharides and applied to a Sepharose 6B column (10 $\times$ 1 cm). Radioactivity of fractions was measured in: Control [3 H]heparin sample (O); virion particles incubated with 4 μ Ci of [3 H]heparin for 90 minutes (●) and virion particles incubated with 4 μ Ci of [3 H]heparin for 90 minutes, after being incubated with 500 μ g/ml of carrageenan for 30 minutes (▲). Infectivity of fractions (pfu) was measured in control virion particles incubated for 90 minutes in absence of polysaccharide (Δ). Panel A : Herpes simplex virus type 1. Panel B : Poliovirus type 1. Panel C : Semliki Forest virus.

when [^3H]heparin is added alone to the column, whereas a clear peak of radioactivity appears when virus particles were incubated together with [^3H]heparin. This peak of radioactivity in the void volume is coincident with the peak of infectivity present in the different fractions. Carrageenan, a sulphated polysaccharide with antiviral activity similar to heparin competed with the binding of [^3H]heparin to virions (Figure 1), suggesting that carrageenan also interacts with viral particles.

Once we established that [^3H]heparin is able to form complexes with virions, we analyzed the entry of [^3H]heparin into cells. Almost no entry of [^3H]heparin is observed in control cells, in agreement with the idea that this polysaccharide does not pass the plasma membrane. However, a significant increase in the internalization of [^3H]heparin is evident when HSV1 are present (Figure 2). The increased internalization of [^3H]heparin promoted by HSV1 is inhibited by cold carrageenan, suggesting that the increased entry of polysaccharides is mediated by prior binding to virion particles. These results are in good agreement with our previous findings that virion particles enter into cells even in the presence of carrageenan (2). The time course of this internalization follows a kinetic similar to the entry of labelled HSV1 particles into cells (Figure 3). On the other hand, we also tested cell debris obtained from uninfected cells and they did not promote [^3H]heparin internalization, adding more support to the idea that polysaccharide entry is mediated by virion particles.

DISCUSSION

Although the possibility that sulphated polysaccharides can interact with viral particles was advanced several

Figure 2. Competition of carrageenan and [³H]heparin for entry in HSV-1 infected cells. Monolayers of HeLa cells were infected with HSV-1 at a m.o.i. of 100, in absence (○) or presence of 1 (▽), 10 (□) or 100 (Δ) μg/ml of carrageenan. After 1 h of adsorption at 4°C the indicated concentrations of [³H]heparin were added and cell monolayers were incubated for 2 h at 37°C. Black symbols represent mock-infected cells pretreated with corresponding concentrations of carrageenan.

years ago (7) a direct proof of such interaction was still lacking. Our findings indicate that this is so. Moreover, [³H]heparin is able to interact with all virion particles we tested, but still this interaction is not sufficient to inhibit viral growth. A likely interpretation is that the strength of this interaction is different for each type of viral particle, or alternatively, that some viral particles are able to decapsidate and/or replicate even if heparin was bound to them.

Another finding of interest is the enhanced internalization of [³H]heparin that occurs when viral particles are present. This finding is in accord with the idea that viral adsorption is not the step blocked by sulphated polysaccharides, in contrast with current ideas on the antiviral mechanism of action of these compounds (1,3). It still

Figure 3. Kinetics of $[^3\text{H}]\text{heparin}$ entry in HSV-1 infected cells and HSV-1 internalization in cells. Panel A: Kinetics of HSV-1 entry into HeLa cells. HSV-1 was radiolabelled and purified as described in Materials and Methods. 40,000 cpm of the virion concentrate was added to HeLa cells in the presence of 100 $\mu\text{g/ml}$ carrageenan (Δ) or in the absence of the polysaccharide ($\blacktriangle$). The virus entry at 37°C was measured as described in Materials and Methods. Background of entry at 4°C was determined in treated cells ($\circ$) and in untreated cells ($\bullet$).

Panel B: Kinetics of $[^3\text{H}]\text{heparin}$ entry in HSV-1 infected cells. HeLa cells were infected at a m.o.i. of 100 in the presence of 1,25 $\mu\text{Ci/ml}$ of $[^3\text{H}]\text{heparin}$. At the indicated times the radioactivity incorporated in HSV-1 infected cells ($\circ$) and mock-infected cells ($\bullet$) were determined as described previously.

remains to be determined what is the ultimate fate of these polysaccharide-virion complexes. As we indicated previously we do not yet know if they are able to escape the pinocytotic vesicles. Indirect evidence obtained from our findings on permeabilization of viruses to alpha-sarcin and other molecules (8,9), suggests that this may be so. We do not yet know if virions in these complexes are still able to deacapsidate. Sulphated polysaccharides interact with nucleic acids and inhibit genome expression (10). Therefore, it is possible that the expression and/or replication of the HSV1

DNA is the target blocked by these agents. Experiments are under way to distinguish between these possibilities.

ACKNOWLEDGEMENTS

The expert technical assistance of Ms M.A. Ramos is acknowledged. FISS, CAICYT and Plan Concertado 41/82, Anti-bióticos S.A. are acknowledged for financial support.

REFERENCES

1. Shanon, W.M. (1984). In "Antiviral agents and viral diseases of man". (Ed. G. J. Galasso, T.C. Merigan and R.A. Buchanan). Raven Press. New York.
2. González, M.E., Alarcón, B. and Carrasco, L. Ant. Agents. Chemoth. (Submitted for publication).
3. Takemoto, K.K. (1966). Progr. Med. Virol. 8, 314-348.
4. Vaheri, A. and Castell, K. (1963). Virology 21, 661-662.
5. Nahmias, A.J. and Kibrick, S. (1964). J. Bacteriol. 87, 1060-1066.
6. Castrillo, J.L. Vanden Berghe, D. and Carrasco, L. (1986). Virology 152, 219-227.
7. Campbell, J.B. and Colter, J.S. (1965). Virology 25, 608-619.
8. Fernández-Puentes, C. and Carrasco, L. (1980). Cell 20, 769-775.
9. Otero, M.J. and Carrasco, L. (1986). Biochim. Biophys. Res. Commun. 134, 453-460.
10. Lewin, B. (1985). Genes II. John Wiley and Sons, New York.