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Inhibition of the Multiplication of Enveloped and Non-enveloped Viruses by Glucosamine

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Abstract—Glucosamine can inhibit the development of viral cytopathogenic effect and the production of infective viral particles of both enveloped and non-enveloped viruses. The extent of antiviral activity is dependent on drug concentration, composition of the culture medium and type of cell host.

Glucosamine (GN) possesses an antiviral activity both in-vitro and in-vivo (Scholtissek 1975; Floc'h & Werner 1976) and it is generally accepted that it inhibits the multiplication of enveloped viruses without affecting non-enveloped viruses (Kaluza et al 1972). This agrees with its action mechanism which is attributed to an impairment of the synthesis of glycoproteins of the viral envelope (Scholtissek 1975; Kaluza 1975; Knowles & Person 1976). However, the reproduction of non-enveloped viruses was also shown to be inhibited by sugar derivatives like *N*-carbobenzoxy-D-glucosamine and 2-deoxy-D-glucose (Halperen 1976; Shirobokov 1976).

Therefore, experiments have been carried out to study the influence of GN on the reproduction of infective viral particles of both enveloped and non-enveloped viruses to clarify its action mechanism.

Materials and Methods

Cell cultures

Vero cells were grown as monolayers using Tissue Culture Medium 199 (M199) (Wellcome, Beckenham, U.K.), supplemented with 7% newborn calf serum (Flow Inc. USA), 100 mg% sodium bicarbonate, pH 7·4, penicillin (100 iu mL^{-1}) and streptomycin (100 $\mu g\ mL^{-1}$) (Paul 1975).

Primary chick embryo cells were prepared from 11 days old embryos by a procedure involving trypsin treatment as described elsewhere (Schmidt 1969). These cells were grown as monolayers in M199 supplemented with 4% newborn calf serum, 2% chick embryo extract, 150 mg% succinic acid, 200 mg% sodium succinate, pH 7·2, penicillin 250 iu mL^{-1}, streptomycin 250 $\mu g\ mL^{-1}$ and nystatin 50 $\mu g\ mL^{-1}$.

To obtain cell monolayers, Vero cells suspended in growth medium were seeded at a cell density of 8×10^4 cells cm^{-2} in both 30 mL tissue culture flacks (Nunc, Denmark) and Cooke microtiterplates (Greiner, M 220–29 ART, Nurtingen, Germany). Correspondingly, primary chick embryo cells were seeded at a cell density of 5×10^5 cells cm^{-2}. All cells were incubated at 37 °C in a CO_2-incubator ($P\ CO_2 = 5\%$) until complete monolayers were formed.

Viruses

The virus strains used were obtained from the virus collection of the Laboratory of Tropical Virology (Institute for Tropical Medicine, Antwerp, Belgium) and the Laboratory of Microbiology (Faculty of Medicine, University of Antwerp, Belgium). Vesicular stomatitis virus, West Nile virus, measles virus, herpes simplex type 1 virus, and adenovirus type 5 were grown on Vero cells. Eastern equine encephalomyelitis virus, the virulent V13 and the avirulent A7 strains of Semliki Forest virus were orginally grown in 1 day old mice after intracerebral and intraperitoneal inoculation. Polio-virus type 1, strain Brunhilde ($d^+/1a/S_3$ line) and coxsackie B3 virus were grown in HeLa cell suspension (Vanden Berghe 1972).

Glucosamine stock-solution

D-(+)-Glucosamine hydrochloride (Merck), 2·5 g, was dissolved in 96 mL of NaCl-KCl-depleted serumless synthetic medium (Plaisner et al 1974; Hronovsky et al 1975) and adjusted to pH 7·4 by addition of 4 mL 1M NaOH. This stock solution showed a final osmolarity of 300 mosmol kg^{-1}. Only freshly prepared solutions were used.

Infection procedure and virus titration

For screening purposes, serial ten-fold dilutions of virus suspension were made in serumless synthetic culture medium (SSM) with or without GN and inoculated immediately onto the cell monolayers (0·2 mL/well and four wells per dilution) in pre-emptied microtiterplates. All viruses tested were inoculated on Vero cells. Herpes virus, Semliki Forest virus and vesicular stomatitis virus were inoculated on primary chick embryo cells. The appearance of viral cytopathogenic effect was recorded on successive days after inoculation by means of an inverted microscope. Viral titres were calculated by the Reed-Muench method and expressed as TCD50 mL^{-1} (Reed & Muench 1938).

Virus growth rate

Cell monolayers (in 30 mL tissue culture flasks) were washed three times with serumless synthetic medium (SSM), and infected with virus suspended in SSM. After 1 h of adsorption at 37 °C, non-absorbed virus was removed by washing the cell monolayers three times with SSM. Vero cells and primary chick embryo cells were infected with either vesicular stomatitis or poliovirus at the multiplicity of infection of 1 to 5 PFU/cell and with eastern equine encephalomyelitis virus at a multiplicity of infection of 12 PFU mL^{-1}. GN was added to the culture fluid in two different ways: in type A addition, GN was added at the moment of infection, and in

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type B addition, two groups of cells (groups 1 and 2) were preincubated simultaneously in GN containing medium before infection. For the cells of group 1, the preincubation time was 4·75 h, after which the cells were infected by suspending the virus in their own culture medium (medium from cells of group 1). After the adsorption period and the removal of non-adsorbed virus, the GN-containing medium, which had been in contact with cells of group 2, was transferred to the infected cells (cells of group 1).

Samples for virus titration were harvested at different times after infection. Aliquots of culture fluid were harvested in order to determine the titre of the extracellular virus. For titration of the total virus production, cells in their own culture medium were freeze-thawed three times and this cell lysate was titrated. The intracellular virus was determined from the difference between total virus and extracellular virus. Before titration, samples were kept at −70 °C for one to three days. Samples were titrated by the plaque formation method. The plaque formation for vesicular stomatitis and eastern equine encephalomyelitis was carried out on primary chick embryo cells (Pattyn & De Vleeschauwer 1967). The agar overlay consisted of Minimal Essential Culture Medium, supplemented with 2% chick embryo extract, 1·5% glutamine, 5% neutral red, 0·9% agar, 150 mg% succinic acid, 200 mg% sodium succinate, pH 7·2, penicillin 250 iu mL^{-1}, streptomycin 250 μg mL^{-1}, and nystatin 50 μg mL. Poliovirus was plaqued on monkey kidney cells using the modified technique of Hiung and Melnick (Schmidt 1969).

Results

Influence of glucosamine on non-infected cells

Either Vero cells or primary chick embryo cells were maintained for six days at 37 °C in SSM containing different concentrations of GN. At doses of GN up to 2 mmol, Vero cells did not show any apparent morphological alteration. At 3 and 9 mmol GN, about 40 and 80% of Vero cells, respectively, became round and detached from the glass surface on the second day of culture. The remaining attached cells showed normal morphology during six days of observation.

At doses of GN up to 12 mmol primary chick embryo cells did not show any apparent change in morphology. At 24 mmol GN about 80% of cells became round only at the sixth day of culture.

Effect of glucosamine in the virus titre

Effect of glucosamine on enveloped viruses in Vero cells. Herpes simplex virus type 1, the avirulent strain of Semliki Forest virus, eastern equine encephalomyelitis virus, measles virus, and vesicular stomatitis virus were titrated (ten-fold dilutions in SSM) on Vero cells in the presence of 3 mmol GN. The virus titre (TCD50 mL^{-1}) was determined every day for five days. As shown in Fig. 1, GN significantly lowered the titre of all viruses tested.

Effect of glucosamine on non-enveloped viruses in Vero cells. GN, 3 mmol, significantly reduced the titre (TCD50 mL^{-1}) of poliovirus type 1, adenovirus type 5 and coxsackie B3 virus (Fig. 2) on Vero cells.

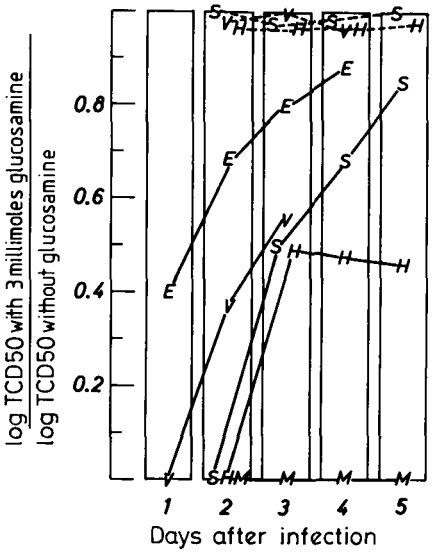

FIG. 1. Influence of 3 mmol glucosamine on the viral cytopathogenic effect. Titration (microtitre plate) of enveloped viruses on Vero cells (——) and on primary chick embryo cells (----) in serumless synthetic culture medium. E, eastern equine encephalomyelitis; H, herpes simplex virus type 1; M, measles virus; S, Semliki Forest virus; V, vesicular stomatitis virus.

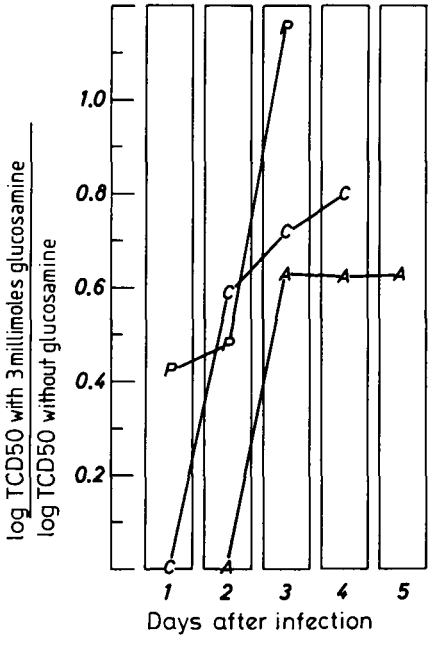

FIG. 2. Influence of 3 mmol glucosamine on the viral cytopathogenic effect. Titration (microtiterplate) of non-enveloped viruses on Vero cells in serumless synthetic medium. A, adenovirus type 5; coxsackie B_3 virus; P, poliovirus type 1.

Kinetics of the activity of glucosamine. In the presence of GN, virus titre on Vero cells in SSM depends on the time elapsed after inoculation, so that generally the greatest effect on titre was observed on the first day of infection, while the effect decreased progressively during the following days (Fig. 1). This effect may be due either to a decrease of the actual GN concentration by normal cell metabolism or to the emergence of viral strains resistent to GN. This point will be analysed below.

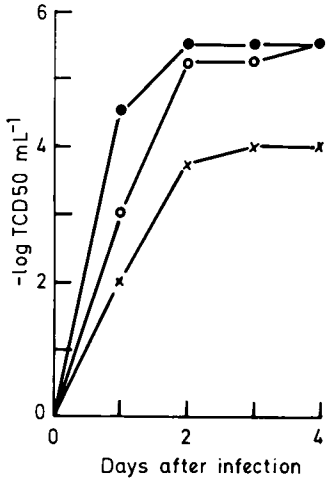

FIG. 3. The counteracting effect of glucose. Vero cells infected with vesicular stomatitis virus in serumless synthetic medium (SSM). Symbols: ●——●, without glucosamine, without glucose; ×——×, 3 mmol glucosamine, without glucose; O——O, 3 mmol glucosamine, 11 mmol glucose.

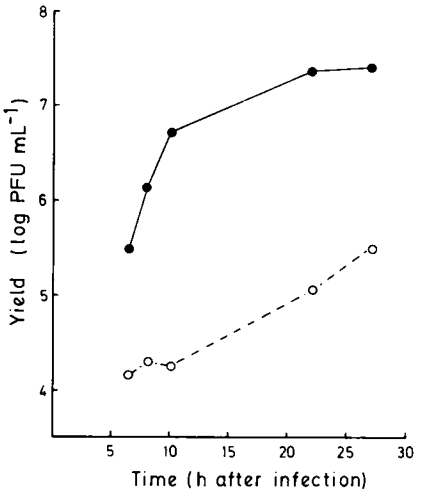

FIG. 4. Effect of glucosamine on the total yield of poliovirus in Vero cells. Symbols: ●——●, without glucosamine; O----O, 3 mmol glucosamine.

The counteracting effect of glucose. The effect of 3 mmol GN was also studied in the presence of 11 mmol glucose. Vesicular stomatitis virus was titrated (ten-fold dilutions) on Vero cells in a microtiterplate and virus titre (TCD50 mL^{-1}) was determined every day for four days. Analysis of the results showed that the inhibitory effect of GN on Vero cells, infected with vesicular stomatitis virus, is partially reversed by glucose on the first day of infection, while, on the following days, glucose completely reversed the inhibitory effect of GN (Fig. 3).

Effect of glucosamine on enveloped viruses in chick embryo cells. Herpes simplex virus type 1, vesicular stomatitis virus and the avirulent strain of Semliki Forest virus were titrated (ten-fold diutions in SSM) on primary chick embryo cells in microtiterplates. The virus titre (TCD50 mL^{-1}) was determined daily up to four days for vesicular stomatitis virus and up to five days for herpes simplex virus and Semliki Forest virus. The results showed that concentrations up to 24 mmol of GN did not significantly reduce the titer of the viruses tested (Fig. 1).

Influence of glucosamine on the production of infective viral particles

The effect of glucosamine on the virus yield in Vero cells. The yield of infective viral particles in Vero cells in the presence of GN was determined (plaque formation method). To avoid variations of the latent period during the replication cycle in different experiments, a delay interval (DI) was defined as the difference in time (h) between the latent period in the presence of GN (LP_{gn}) and the latent period without GN (LP_o): $DI = LP_{gn} - LP_o$.

The total viral yield of poliovirus (m.o.i. = 5 PFU/cell) was drastically decreased by 3 mmol GN at least until 27 h after infection (Fig. 4). The yield of vesicular stomatitis virus (m.o.i. = 5 PFU/cell) was drastically decreased by 0·3 mmol GN during at least 9 h after infection (Fig. 5). In this case, a DI of about 2 h was observed. These results allow the

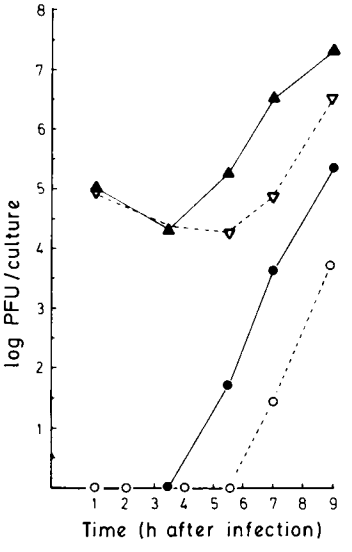

FIG. 5. Effect of glucosamine on the yield of vesicular stomatitis virus in Vero cells. Symbols: ▲——▲ intracellular virus titre without glucosamine; ▽----▽ intracellular virus titre at 0·3 mmol glucosamine, ●——● extracellular virus titre without glucosamine; O----O extracellular titre at 0·3 mmol glucosamine.

conclusion that GN inhibits the production of both enveloped and non-enveloped infective viral particles in Vero cells.

The nature of virus grown under the influence of glucosamine. The yield of infective viral particles is completely inhibited on Vero cells after a single dose of GN, although only for a limited period of time; after that, infective viral particles are synthesized. Then the question arises whether the formation of these new viral particles is the result of the emergence of viral particles resistent to GN. To answer this question the following experiment was carried out: the virus, grown on Vero cells under the influence of GN, was cloned by isolating viral plaques formed under agar on primary chick embryo cells and monkey kidney cells, for vesicular stomatitis virus

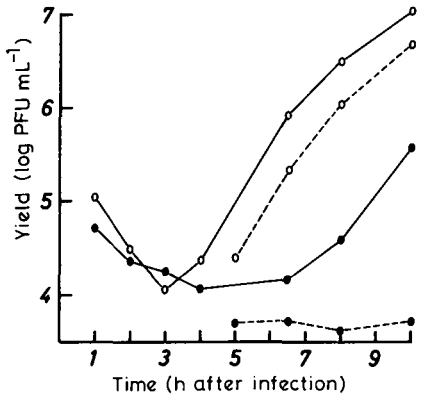

FIG. 6. Total virus yield on Vero cells of a virus stock obtained by plaque formation of a virus previously grown on Vero cells in the presence of 3 mmol glucosamine. Symbols: O——O, vesicular stomatitis virus stock without glucosamine; ●——● vesicular stomatitis virus stock with 0·5 mmol glucosamine; O----O, poliovirus stock without glucosamine; ●----●, poliovirus stock with 3 mmol glucosamine.

and poliovirus, respectively. Virus stocks were prepared from the clone-purified virus after one passage without GN, either on Vero cells (poliovirus) or on primary chick embryo cells (vesicular stomatitis virus). The yield of infective viral particles produced by Vero cells infected with these cloned virus stocks was again drastically reduced by GN (Fig. 6). Therefore, virus growth in the presence of GN is not due to the formation of viral particles resistent to GN. Another explanation could be a decrease of the concentration of GN by cellular metabolism allowing for a virus to resume its growth.

That GN is metabolized by cells in tissue cultures is a well established fact (Keppler et al 1970; Plagemann & Erbe 1973; Schlotissek 1975; Koch et al 1979). However, under the experimental conditions used here, it was not known whether Vero cells could still metabolize GN at the concentration that shows an antiviral activity. In the next experiment it was assumed that, if GN was metabolized, its anti-viral activity would decrease.

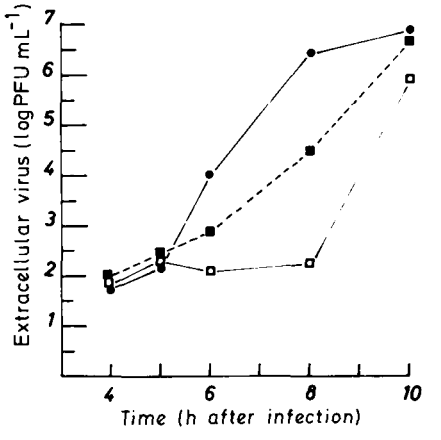

FIG. 7. Yield of eastern equine encephalomyelitis virus on Vero cells in serumless synthetic culture medium (SSM). Symbols: ●——●, without glucosamine; □——□ 3 mmol glucosamine added at the moment of infection; ■——■, two groups of cells (group 1 and 2) were preincubated in SSM with 3 mmol glucosamine. After 4·75 h min of incubation, cells of group 1 were infected with eastern equine encephalomyelitis virus, which was resuspended in the preincubation medium from group 1 cells. After virus adsorption and removal of the non-adsorbed virus, the preincubation medium from group 2 cells was transferred to cells of the group 1.

The viral yield after type A addition of GN was compared with the viral yield after type B addition on Vero cells infected with eastern equine encephalomyelitis virus. The results (Fig. 7) showed that the viral yield after GN type B addition was decreased less drastically than after type A addition. This experiment shows that the effective antiviral concentration of GN decreased progressively, probably due to its consumption during cellular metabolism. This statement is valid for mock-infected cells as well as for virus-infected cells. In the latter, the virus may be able to grow only after GN has been metabolized by cells.

Effect of the concentration of glucosamine on virus yield in Vero cells. The effect of increasing doses after type A addition of GN was studied on Vero cells infected with either vesicular stomatitis virus (m.o.i. = 1 PFU/cell) or eastern equine encephalomyelitis virus (m.o.i. = 12 PFU/cell). The results showed that by increasing the dose of GN, the viral yield decreases, while the delay interval increases (Table 1).

According to the degree of viral inhibition, three groups of GN concentrations could be observed in the case of vesicular stomatitis virus: group 1 (0·07 and 0·14 mM GN) showed short delay intervals and viral yields slightly higher or about equal as in controls after a few hours of GN addition. Group 2 (0·72 mM GN) was characterized by delay intervals longer than in group 1 and viral yields slightly lower than in controls, until about 24 h after GN addition. In group 3 (1·5 mM to 6 mM GN) the delay interval was longer than in group 2 and the viral yields remained significantly lower than in controls, until at least 50 h of GN addition (Table 1).

The influence of glucosamine on virus yield in chick embryo cells. Primary chick embryo cells were infected with vesicular stomatitis virus (m.o.i. = 2 PFU/cell). 1 h after infection, GN was added at the final concentration of 24 mmol. The pattern of virus yield under the influence of GN was different from that in Vero cells. In chick embryo cells up to 5 h p.i. no significant effect on the virus yield was observed (Fig. 8). However, during the time between 6 and 20 h postinfection, there was a slight and progressive decrease of the viral yield. In comparison, on Vero cells 1·5 mmol GN inhibited the yield of VSV until at least 8 h after infection.

Table 1. Influence of the glucosamine (GN) concentration on viral yield in Vero cells.

GN (mmol)	Delay interval VSV*	Delay interval EEE*	Log PFU mL⁻¹ with GN / PFU mL⁻¹ without GN VSV		Log PFU mL⁻¹ with GN / PFU mL⁻¹ without GN EEE	
0·07	1 h	ND**	1·05	(4 h p.i.)	ND	
0·14	3 h	1 h	1·25	(4·5 h p.i.)	0·90	(4 h p.i.)
0·72	5 h	ND	0·95	(24 h p.i.)	ND	
1·50	>7 h	ND	0·70	(50 h p.i.)	ND	
3·00	>7 h	3 h	0·70	(50 h p.i.)	0·93	(24 h p.i.)
6·00	>7 h	ND	0·53	(50 h p.i.)	ND	

* VSV = vesicular stomatitis virus; EEE = Eastern equine encephalomyelitis virus

** ND = not done

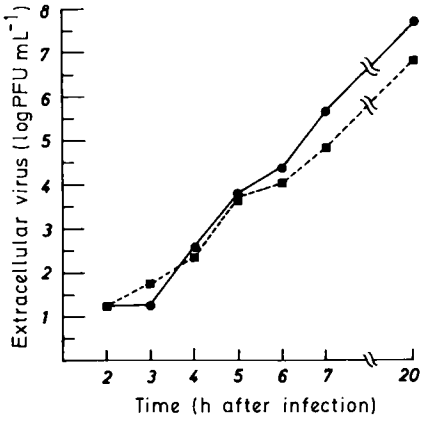

FIG. 8. Effect of glucosamine on the yield of vesicular stomatitis virus in primary chick embryo cells. Symbols: ●——●, with glucosamine; ×---×, 24 mmol glucosamine.

Discussion

Doses of GN up to 2 mmol in SSM, in which glucose is replaced by galactose, did not cause any apparent morphological alteration on Vero cells, as proved by an examination under light microscopy. At 3 and 6 mmol GN, the gross morphological cell changes were rather characteristic, because only a part of the cell population was affected by GN, leaving the rest of the cells without any apparent morphological alteration. In virus-infected Vero cells with 3 and 6 mmol GN it was possible to differentiate between the viral cytopathogenic effect induced on non-detached cells and the cytotoxic effect caused by GN which was manifested by the rounding and detachment of cells.

The GN pattern of inhibition of the cytopathogenic effect was the same for all viruses tested on Vero cells. There was a transitory complete inhibition of the cytopathogenic effect at each virus dilution tested. This finding may rule out a non-specific antiviral effect of GN, because a viral-induced cytopathogenic effect is still able to develop after a well defined period of incubation. In the case of enveloped viruses the inhibition of the cytopathogenic effect caused by GN could be explained, as currently suggested, by an impairment of the synthesis of the envelope glycoproteins (Scholtissek 1975; Kaluza 1975; Knowles & Person 1976). In the case of adenovirus, which is not an enveloped virus, it may be argued that GN may impair the synthesis of the capsidial glycoprotein. However, in the case of poliovirus, which is thought to contain no structural glycoproteins (Scraba 1979), the action of GN can not be explained yet. On Vero cells, glucose counteracted the antiviral effect of GN. This antagonism has previously been reported for enveloped viruses in other cell systems (Scholtissek 1975).

GN added to virus-infected primary chick embryo cells at even higher concentrations than in Vero cells, did not inhibit the development of viral cytopathogenic effect.

From the above, it can be concluded that GN exerts a well defined antiviral activity, which depends on both the cell type tested and on the chemical composition of the culture medium.

Since the pattern of GN inhibition was the same for all viruses tested, more detailed experiments were carried out with two enveloped viruses (vesicular stomatitis virus and eastern equine encephalomyelitis virus) and one-enveloped virus (poliovirus).

On Vero cell, GN significantly impaired the production of infective viral particles of vesicular stomatitis virus, which confirms a report from other authors (Marnell & Wertz 1979). On Vero cells the production of infective viral particles of a non-enveloped virus (poliovirus) was also impaired by GN. This is comparable with the characteristics of another sugar derivative, 2-deoxy-D-glucose, which was reported to inhibit the reproduction of enterovirus in Hep-2 human oral carcinoma cell culture (Shirobokov 1976). On Vero cells, the infective viral particles of both poliovirus and vesicular stomatitis virus, which were grown in the presence of GN after a delay interval, were isolated and passed, respectively, to new Vero cells containing GN. In that case the production of infective viral particles was again impaired by GN. Hence, the possible presence of infective viral particles resistent to GN was excluded. This fact may be of importance if GN is regarded as a potential antiviral drug, because viruses resistent to the most currently used anti-viral drugs have already been detected (Crumpacker 1983; De Clercq 1984).

Experiments measuring the viral yield after type B addition of GN, lead to the conclusion that the actual concentration of GN decreases in function of time. This is very likely due to a consumption of GN during cell metabolism. This could explain both the delayed growth of viruses after the addition of a single GN dose, and the fact that the delay interval depends on the GN concentration; because the higher its concentration, the longer the time needed for its metabolization. This holds true for GN concentrations up to 0·7 mM on Vero cells under the experimental conditions here used. However, at higher GN concentrations (3 to 6 mM) another factor may be added, which is the differential cytotoxic effect of GN on a cell subpopulation, resulting in viral yields, which will remain lower than in controls.

In contrast with Vero cells, the yield of infective viral particles of vesicular stomatitis virus was only slightly decreased by 24 mmol GN.

In summary, virus-infected Vero cells maintained in serumless synthetic medium, represent a suitable model in which it is possible to study the kinetics of the activity of GN against enveloped and non-enveloped viruses under well-controlled conditions.

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