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Antiviral activity of sulfated polysaccharides against African swine fever virus

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Summary

The polyanionic substances lambda and kappa carrageenan, pentosan polysulfate, fucoidan, dextran sulfate and heparin were investigated for their inhibitory effect on the replication of African swine fever virus (ASFV) in vitro. Lambda carrageenan was the most efficacious with a selectivity index, as based on the ratio of the 50% cytotoxic concentration to the 50% antiviral effective concentration, of 120, followed by pentosan polysulfate with 30, kappa carrageenan 13.3 and fucoidan 10. Dextran sulfate and heparin were almost inactive. In general, the substances had low toxicity for Vero cells. The studies with radiolabeled ASF virions suggest that the sulfated polysaccharides inhibit virus adsorption. Inhibition of virus replication was found for all the polysaccharides only when the substances were present during virus adsorption, with the exception of lambda and kappa carrageenan, which were also inhibitory when added immediately after the adsorption period.

African swine fever virus; Sulfated polysaccharide; Lambda carrageenan; Kappa carrageenan; Pentosan polysulfate; Fucoidan; Dextran sulfate; Heparin

Introduction

African swine fever virus (ASFV), an important disease of domestic pigs, remains the subject of substantial attention because of its potential economic con-

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sequences. The sole controls so far practiced in the countries where the disease appears are the slaughter of all the exposed animals and the adoption of zoosanitary precautionary measures. One of the key features of ASFV, an icosahedral virus, is the inability of virus-specific antibodies from animals recovered from the illness to neutralize the virus (De Boer, 1967), although some resistance to reinfection is acquired (Ruiz Gonzalvo et al., 1986).

Prospects for developing a vaccine are poor and a chemotherapeutic approach is advisable. The replication of ASFV is inhibited by 5-iodo-2′-deoxyuridine (Haag et al., 1965; Gil-Fernández et al., 1979), rifampicin (Dardiri et al., 1971), phosphonoacetic acid (Moreno et al., 1978; Gil-Fernández et al., 1979) and lysosomotropic substances (Geraldes and Valdeira, 1985; Alcamí et al., 1989a). Also the fatty acids monoolein, monolinolein and γ-linolenyl alcohol (Sola et al., 1986a) and several adenosine analogues, which are targeted at S-adenosylhomocysteine hydrolase (De Clercq, 1987), proved to be the potent and selective inhibitors of ASFV replication (Gil-Fernández and De Clercq, 1987).

Several phosphonylmethoxyalkylpurine and -pyrimidine derivatives such as (*S*)-9-(3-hydroxy-2-phosphonylmethoxypropyl)adenine ((*S*)-HPMPA) and 9-(2-phosphonylmethoxyethyl)adenine (PMEA) have also proved to be selective inhibitors of ASFV (Gil-Fernández et al., 1987).

An alternative approach is to inhibit virus adsorption onto the cells, thus preventing virus replication. ASFV replication is inhibited by iota-carrageenan and suramin (Sola et al., 1986b). Other polyanionic substances have long been known to have antiviral activity (Vaheri, 1964). Furthermore, recent studies have emphasised the antiviral activity of a number of polysaccharides against several viruses (Gonzalez et al., 1987; Baba et al., 1988a), in particular human immunodeficiency virus (HIV) (De Clercq, 1989a,b). It seemed interesting, therefore, to investigate the inhibitory effect of these polysaccharides on ASFV replication.

Materials and Methods

Virus and cells

ASFV (Badajoz strain) adapted to grow in Vero cells, was kindly provided by E. Viñuela, Centro de Biología Molecular (Enjuanes et al., 1976). The virus was further propagated in Vero cells and the stock used in the present study was that obtained after 14 passages. Vero cells (African green monkey kidney cells) were grown in Dulbecco's modified Eagle's medium, supplemented with 10% newborn calf serum for the growth medium and 2% for the maintenance medium.

Compounds

Lambda and kappa carrageenan, heparin, pentosan polysulfate, fucoidan and dextran sulfate (MW 8000) were purchased from Sigma Chemical Co. (St. Louis, MO).

Inhibition of ASFV replication

The procedure for measuring anti-ASFV activity in Vero cells has been described previously (Gil-Fernández and De Clercq, 1987). Briefly, monolayers of Vero cells (growing in 24-well plates) were infected with ASFV at a multiplicity of infection (moi) of 0.5. In the present experiments, the compounds were added at various concentrations during or after virus adsorption. When the controls without drug showed complete destruction, the cells were removed from the wells and total virus yield was determined by plaque formation.

Virus purification

150-mm plates were inoculated with ASFV at 2 plaque-forming units (pfu)/cell for 2 h. Following removal of the inoculum, medium supplemented with 15 μCi/ml of [^{35}S]methionine was added and the cells were further incubated at 37°C for 48 h. Virus was harvested and disrupted by sonication. The cellular extracts were centrifuged at 2000 rpm for 5 min. The supernatants were layered on top of a 3.5 ml cushion of 35% sucrose with 1 M NaCl, 10 mM Tris pH 8 and 1 mM EDTA pH 8 and centrifuged in a Beckman SW 50.1 rotor at 20000 rpm for 1 h at 4°C. Pellets were suspended in 1 ml of phosphate-buffered saline (PBS). The final virus titer was to 4.5×10^6 pfu/ml and 5×10^6 cpm/ml.

Binding of radiolabeled virus to cells

Confluent monolayers in 96-well plates were pretreated during 15 min at 37°C with PBS containing 1% fetal bovine serum, 0.1% glucose and bovine serum albumin (5 mg/ml) in order to block nonspecific adsorption. Then the cells were inoculated with purified radiolabeled virions (2.7×10^5 pfu/ml or 3×10^5 cpm/ml) in 50 μl of PBS supplemented with serum albumin (1 mg/ml) with or without compounds to be tested. After 1 h adsorption at 37°C, the virus inoculum was removed and the cells were rinsed three times with PBS. Cells with bound or penetrated virus were lysed in PBS with 1% sodium dodecyl sulfate (SDS) and 1% Triton X-100. The lysates were counted in an LKB Wallac 1219 Rackbeta liquid scintillation spectrometer.

Estimation of the cytopathic effect and cytotoxicity

Cells were seeded in 96-well plates (10^4 cells/well). When the cell monolayers were confluent, wells were inoculated with the virus at moi equal to 1 in the presence of the inhibitors. Between the rows of infected cells, rows of uninfected cells with the same final concentration of the inhibitors were included to study the cytotoxicity of the compounds. When the virus controls without drug were completely destroyed, cytotoxicity and cytopathic effect were evaluated by (a) microscopic evaluation of cell morphology, (b) staining of the cells with 1% crystal violet in ethanol as described previously (Gil-Fernández and De Clercq, 1987), and (c) inhi-

bition of protein synthesis as described by Sola et al. (1986).

Results

The cytotoxicity of the six sulfated polysaccharides, i.e., kappa carrageenan, lambda carrageenan, dextran sulfate (MW 8000), heparin, pentosan polysulfate and fucoidan was measured by three parameters, namely (i) alteration of normal morphology, (ii) dye (crystal violet) uptake method and (iii) inhibition of host cell macromolecule (protein) synthesis on the basis of the incorporation of [^{35}S]methionine. When the cytotoxicity was assayed with the dye uptake method, kappa carrageenan showed a 50% cytotoxicity concentration (CC_{50}) of 2000 μg/ml. Among the other polysaccharides the CC_{50} proved higher than the greatest concentration tested (3000 μg/ml). On the basis of the inhibition of the incorporation of [^{35}S]methionine, the CC_{50} of the six compounds tested was above 3000 μg/ml.

The 50% antiviral effective concentration (EC_{50}) and CC_{50} for mock-infected cells and the selectivity index (SI) with the different compounds are presented in Table 1. All the results were obtained with the inhibitor present during virus adsorption and also thereafter, when virus and drug were removed and fresh medium with the compounds was added.

In Figs. 1 and 2 the dose-response inhibition curves for the six compounds are presented. Virus yields were obtained three days after inoculation on Vero cells. In the case of lambda and kappa carrageenans (Fig. 1) two curves are presented for each compound: (A) shows the inhibition when the drug is present only during virus adsorption and (B) when the compound is added after virus adsorption. Higher inhibition was found when the compounds were present during adsorption. In Fig. 2 the dose response curves of pentosan polysulfate, fucoidan, dextran sulfate and heparin are shown. The compounds were present only during virus adsorption. No inhibition was found when the compounds were added after adsorption, except for lambda and kappa carrageenans.

TABLE 1

Comparative potency, cytotoxicity and selectivity of antiviral compounds as inhibitors of ASFV in vitro

Compound	CC_{50}[a] (μg/ml)	EC_{50}[b] (μg/ml)	Selectivity[c] index
λ-carrageenan	3000	25	120
κ-carrageenan	2000	150	13.3
Pentosan polysulfate	3000	100	30
Fucoidan	3000	300	10
Heparin	3000	2000	1.5
Dextran sulfate	3000	2000	1.5

[a]Cytotoxic concentration affecting 50% of the cells, as assessed colorimetrically by the dye uptake method.
[b]Antivirally effective concentration required to effect a 50% reduction in virus yield.
[c]Ratio of CC_{50} to EC_{50}.

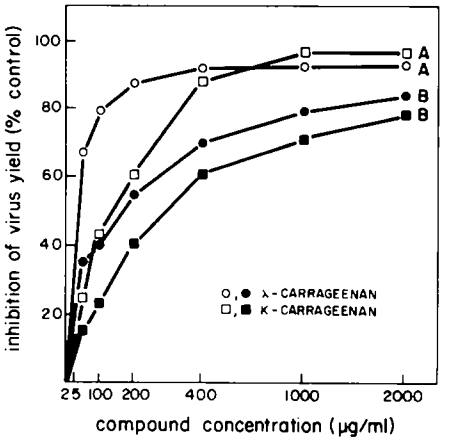

Fig. 1. Inhibitory effects of lambda and kappa carrageenans on the replication of ASFV in Vero cells. The compounds were added during (A) and after (B) virus adsorption (moi: 0.5). Virus yield (based on plaque formation) was measured when the virus control cultures showed complete destruction (normally three days after virus inoculation). Virus plaques were counted on days 7 or 8 after removing the agar coat and staining the cell monolayers with 1% crystal violet in alcohol. Compounds: lambda carrageenan (○, ●) and kappa carrageenan (□, ■).

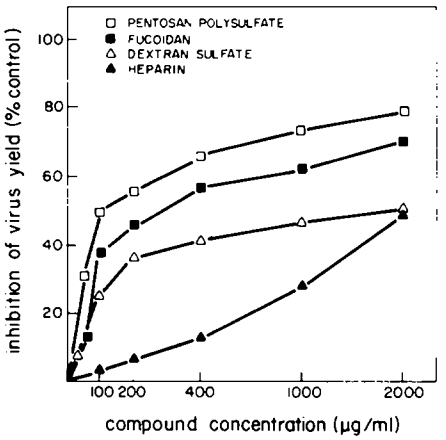

Fig. 2. Inhibitory effects of the test compounds on the replication of ASFV in Vero cells. The compounds were added during virus adsorption (moi: 0.5). Virus yield was measured as for Fig. 1. Test compounds: pentosan polysulfate (□), fucoidan (■), heparin (▲), dextran sulfate (△).

At a compound concentration of 50 μg/ml, the highest inhibition of ASFV replication was produced by lambda carrageenan (68% inhibition) and pentosan polysulfate (30% inhibition), while at 400 μg/ml the strongest inhibitors were lambda (90%) and kappa (88%) carrageenan.

From the experiments of incorporation of [^{35}S]methionine into protein (Fig. 3) following two days of incubation of uninfected cells with the polysaccharides, dextran sulfate and heparin proved to be the least cytotoxic, while pentosan polysul-

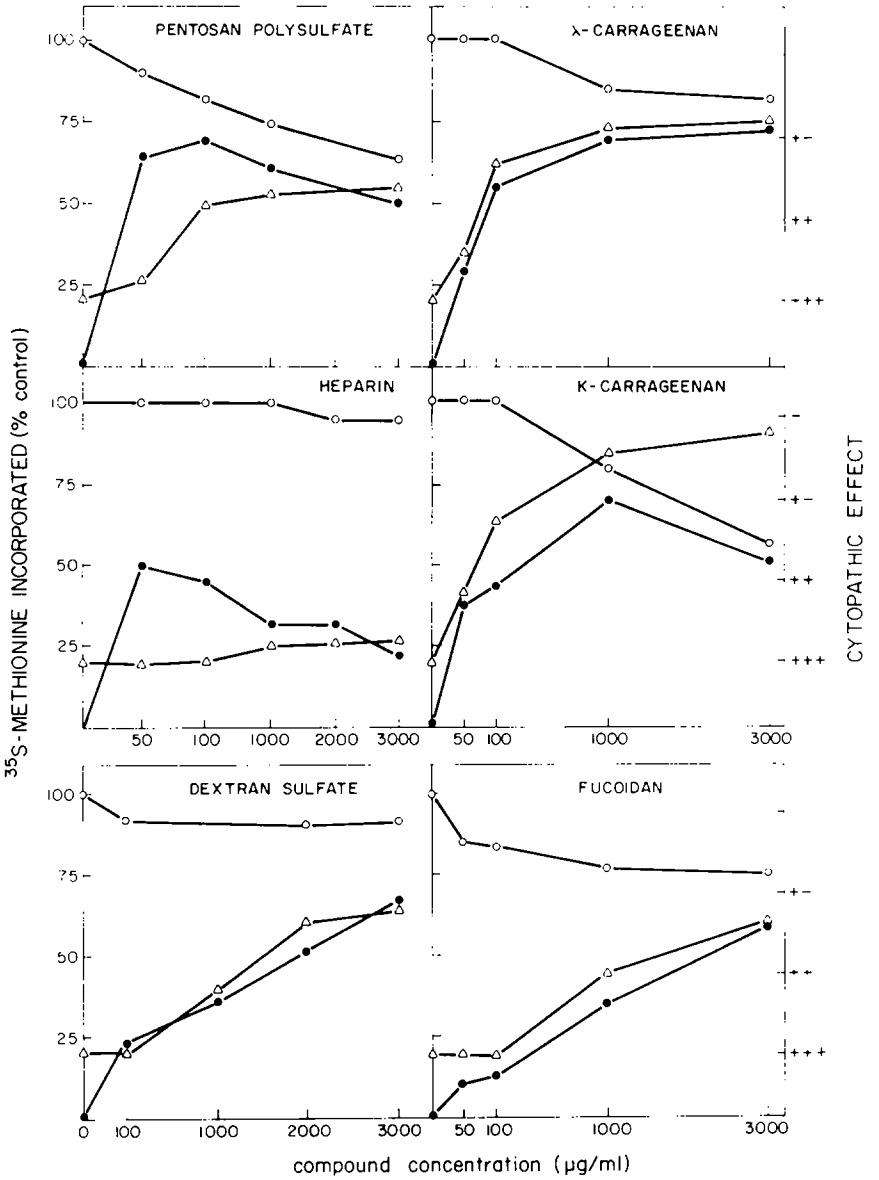

Fig. 3. Cytopathic effect (CPE) of ASFV in Vero cells. Moi: 1. CPE was recorded at 48 h p.i. (∠). −, +−, ++ and +++ represent CPE on a linear scale; (−) no CPE; (+++) maximum CPE. Protein synthesis was measured as indicated in Materials and Methods for ASFV-infected cells (•) and uninfected control cells (○) in the presence of pentosan polysulfate, lambda carrageenan, heparin, kappa carrageenan, dextran sulfate and fucoidan during and after the virus adsorption period.

fate, kappa carrageenan and fucoidan inhibited incorporation by 25%. The CC_{50} of all the polysaccharides was above 3000 μg/ml.

The incorporation of [^{35}S]methionine in infected cells treated with dextran sul-

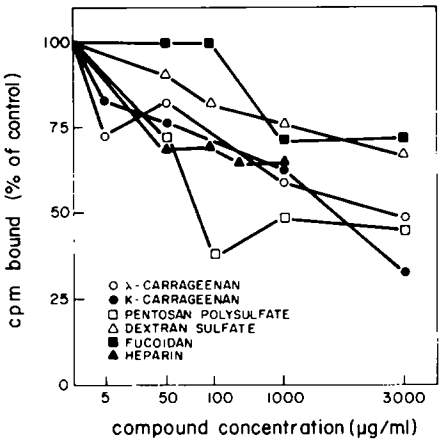

Fig. 4. Effect of sulfated polysaccharides on adsorption of purified [^{35}S]methionine-labeled ASFV particles to Vero cells. Vero monolayers were infected with radiolabeled ASFV (moi: 2) in the presence of 5, 50, 100, 1000 and 3000 µg/ml of lambda or kappa carrageenan or pentosan polysulfate, or 50, 100, 200, 300, 1000 and 3000 µg/ml of dextran sulfate, fucoidan or heparin. After 1 h of virus adsorption, the cells were washed three times with phosphate-buffered saline to remove unadsorbed virus. Cells were lysed with 1% SDS and 1% Triton X-100. The lysates were counted (LKB Wallac 1219 Rackbeta liquid scintillation spectrometer). Each point represents the mean value of two cultures.

fate and fucoidan showed a quasi linear dose-dependence. With lambda carrageenan and pentosan polysulfate, the incorporation of [^{35}S]methionine in infected cells levelled off at concentrations of 100 µg/ml and higher (Fig. 3).

To elucidate the mechanism of the inhibitory effect of the sulfated oligo- and polysaccharides, studies using radiolabeled ASFV were undertaken to test whether the compounds inhibit early events in ASFV infection, and in particular virus adsorption.

Fig. 4 shows the inhibition of the adsorption of ASFV at different concentrations of the six compounds which were incubated with the cells during the 1 h exposure period of the cells to the virus, as indicated in Materials and Methods. At 50 µg/ml, all the compounds, with the exception of fucoidan and dextran sulfate, inhibited virus adsorption by about 25%. Pentosan polysulfate reached the maximum inhibition at a concentration of 100 µg/ml. Lambda carrageenan was more inhibitory than kappa carrageenan at 5 µg/ml, but at 3000 µg/ml the inhibition was higher for kappa than lambda carrageenan.

Discussion

The initial step for the entry of ASFV in Vero cells has been investigated recently (Alcamí et al., 1989a). Saturable binding sites on the plasma membrane of Vero cells but not in virus-resistant L cells, have been demonstrated (Alcamí et al., 1989b). We have found that the competition between labeled and unlabeled ASFV

during adsorption for saturable binding sites reduces the adsorption of labeled virions to one half at an moi of two. We did not observe a reduction in adsorption when herpes simplex virus was used for competition experiments. This suggests that these binding sites are specific for ASFV (results not shown). This fact prompted us to search for compounds which could specifically impede the adsorption of ASFV to the cells.

For the reasons explained in the introduction we have chosen several polysaccharides. The mechanism of action of sulfated polysaccharides has been proposed to be at the level of virus adsorption. In our experiments with pentosan polysulfate, fucoidan, heparin and dextran sulfate (MW 8000) we confirmed that they are inhibitory to ASFV replication only when present during virus adsorption. With lambda and kappa carrageenans, however, we found that they are also inhibitory when added immediately after virus adsorption (Fig. 2). This indicates that with these polysaccharides two different mechanisms of action may be involved. Gonzalez et al. (1987) have also found that iota-carrageenan inhibits an early step of herpes simplex virus (HSV) replication.

Among all the polysaccharides studied, the carrageenans and pentosan polysulfate showed the best activity both in the experiments of virus yield reduction and virus adsorption inhibition.

While heparin and dextran sulfate have proved to be markedly inhibitory to HSV (Vaheri, 1964) and HIV-1 (Ito et al., 1987; Ueno and Kuno, 1987; Mitsuya et al., 1988; Baba et al., 1988a,b), they have little effect on ASFV. Lambda carrageenan, which is the most active against ASFV, inhibits the adsorption of radiolabeled virus at 5 μg/ml by 30%, while a concentration of 3000 μg/ml is needed to reach 70% inhibition with kappa carrageenan. The sulfated polysaccharides are inhibitory to HIV-1 at concentrations of 0.1–1 μg/ml (De Clercq, 1989a,b). At 8 μg/ml heparin blocks the attachment of HSV to the cell surface heparan sulfate which serves as the initial receptor for the virus (Dunn and Spear, 1989).

Our results demonstrate that the sulfated polysaccharides show little toxicity for the cells and inhibit virus adsorption. This may be due to the interaction with non saturable sites. The inhibition found at the highest concentration (3000 μg/ml) could be explained by an increase in the viscosity of the medium, which may impede contact of the virus with the cell surface.

The antiviral action of the carrageenans cannot be attributed solely to inhibition of virus adsorption. Some additional action following virus internalization may occur for these compounds.

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