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Effect of Ribosome-Inactivating Proteins on Virus-Infected Cells. Inhibition of Virus Multiplication and of Protein Synthesis

By

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With 3 Figures

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Summary

HEp-2 cells were infected with herpes simplex virus-1 (HSV-1) or with poliovirus I in the presence of plant proteins which inactivate ribosomes in cell-free systems, while exerting scarce effect on whole cells. Ribosome-inactivating proteins used were gelonin, from the seeds of *Gelonium multiflorum*, an inhibitor from the seeds of *Momordica charantia*, dianthin 32, from the leaves of *Dianthus caryophyllus* (carnation), and PAP-S, from the seeds of *Phytolacca americana* (pokeweed). All proteins tested had the following effects: 1. They reduced viral yield; 2. They decreased HSV-1 plaque-forming efficiency; 3. They inhibited protein synthesis more in infected than in uninfected cells. These results strongly suggest that ribosome-inactivating proteins impair viral replication by inhibiting protein synthesis in virus-infected cells, in which presumably they enter more easily than in uninfected cells.

Introduction

A number of proteins isolated from plant materials inhibit protein synthesis by inactivating ribosomes in cell-free systems, whilst being much less effective on whole cells, in which they do not enter easily. These proteins resemble the A-chains of ricin and related toxins (reviewed in 12) and include the pokeweed antiviral protein (PAP) (10, 11), the wheat germ inhibitor (14), the *Momordica charantia* inhibitor (3), gelonin (16) and dianthins (17).

The pokeweed antiviral protein inhibits the transmission of plant viruses (18), thus accounting for the antiviral effect of the extracts of *Phytolacca americana* (pokeweed) leaves (20) and reduces also the multiplication of influenza virus (18),

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of poliovirus (19) and of herpes simplex virus (HSV-1) (1) in cell cultures. These effects were attributed to increased penetration of PAP into virus-infected cells, with subsequent inhibition of protein synthesis (13, 19). The other known ribosome-inactivating proteins also inhibit infection by tobacco mosaic virus, presumably acting in the same manner as PAP (15, 17).

We report now that gelonin, the *Momordica charantia* inhibitor, and dianthin 32, like PAP, all inhibit multiplication of HSV-1 and of poliovirus I in HEp-2 cells, and have a greater inhibitory effect by protein synthesis of cells infected with these viruses than by uninfected cells.

Materials and Methods

Ribosome-Inactivating Proteins

Momordica charantia inhibitor was purified as described by Barbieri *et al.* (3) and gelonin and dianthin 32 as described by Stirpe *et al.* (16, 17). The pokeweed antiviral protein from seeds (PAP-S), similar but not identical with PAP, was purified from the seeds of *Phytolacca americana* as described by Barbieri *et al.* (2).

Cells and Viruses

HEp-2 cells were grown in Eagle's minimum essential medium (MEM) containing 10 per cent of newborn calf serum (NCS) (Flow Laboratories, Irvine, Scotland). Maintenance medium for infected cells consisted of MEM containing 1 per cent NCS.

Viruses used were HSV-1 (F) (5), HSV-1 (MP) (9) and poliovirus I.

Determination of Virus Yield

Cell cultures, 24 hours-old, in 16 mm multiwell trays (Nunc, Roskilde, Denmark) containing $3.5—5 \times 10^5$ cells per well, were infected at an input MOI of 1 PFU/cell, in the presence of the indicated concentrations of ribosome-inactivating proteins, with quadruplicate cultures for each concentration. The inoculum was removed after a 90- or 60-minutes adsorption period for HSV-1 (F) or poliovirus I, respectively. Cells were rinsed three times with phosphate-buffered saline (PBS) and overlaid with maintenance medium containing, when appropriate, the ribosome-inactivating proteins. After incubation at 37° C in a humidified atmosphere with 5 per cent CO_2 for 48 hours [for HSV-1 (F)] or for 24 hours (for poliovirus I), infected cells were frozen and thawed three times. The media from quadruplicate cultures were pooled and viruses were titrated by plaque assay in HEp-2 cells.

Efficiency of Plaquing

Duplicate cultures of 24 hours-old HEp-2 cells in 35 mm Falcon dishes were infected with 150 or 700 PFU of HSV-1 (F) or of HSV-1 (MP), in the presence of ribosome-inactivating proteins. After virus adsorption the inoculum was removed and cells were overlaid with maintenance medium containing the ribosome-inactivating proteins and 0.2 per cent human gamma globulin. After 56—60 hours monolayers were rinsed, fixed with methanol and stained with Giemsa for plaque scoring.

Effect of Ribosome-Inactivating Proteins on Virion Infectivity

Aliquots of 1.5 ml of HSV-1 (F) or of poliovirus I were incubated with ribosome-inactivating proteins (200 μg/ml) at 37° C in a shaking incubator. At the indicated times duplicate samples of 0.1 ml each were taken and immediately frozen. Infectious virus was estimated in each sample by plaque assay.

Determination of Protein Synthesis

Cultures, 24 hours-old, in 16 mm multiwell trays were infected in the presence of ribosome-inactivating proteins as described above, except that MOI used was 10 and 5 PFU/cell, respectively, for HSV-1 (F) and for poliovirus I, with quadruplicate cultures for each concentration of ribosome-inactivating proteins. Cells were pulse-labeled for 1 hour by adding to each well 0.5 or 1 μCi of [^{14}C] amino acid mixture (Chlorella hydrolysate, The Radiochemical Centre, Amersham, Bucks., U.K.), in 50 μl of MEM. Cells were labeled at 17 hours and at 7 hours after infection with HSV-1 (F) or with poliovirus I, respectively. At the end of the labeling period trays were put on ice, the medium was removed, and cells were frozen. Cells were solubilized with 1 ml of 0.1 N KOH. After 30 minutes at room temperature 1 ml of 10 per cent trichloroacetic acid was added to each well, and the precipitated material was collected and its radioactivity was determined as described previously (3). For the determination of the acid-soluble radioactivity, in some experiments cells were rinsed three times with PBS and twice with 1 ml of 5 per cent trichloroacetic acid, and finally were solubilized with KOH. Samples (0.4 ml) of the pooled trichloroacetic acid washings were transferred to counting vials and the radioactivity was determined as described above.

Results

Effects on Virus Multiplication

The yield of HSV-1 (F) was reduced when the infected cells were incubated in the presence of ribosome-inactivating proteins (Table 1). The effect of *M. charantia* inhibitor and of dianthin 32 was comparable to that of PAP-S, included as a positive control and for comparison, whereas the effect of gelonin was somewhat less marked.

Table 1. *Effect of ribosome-inactivating proteins on HSV-1 yield and efficiency of plaquing*

Expt. No.	Protein added	Concentration (μg/ml)	Yield (log PFU/ml)[a] HSV-1 (F)	Number of plaques HSV-1 (F)	Number of plaques HSV-1 (MP)
1	None		2.47	700	720
	Gelonin	200	1.70 (83)[b]	215 (70)	325 (55)
	Gelonin	100	2.31 (31)	280 (60)	440 (39)
	PAP-S	200	1.48 (90)	35 (95)	144 (80)
	PAP-S	100	1.74 (81)	180 (74)	296 (59)
2	None		3.63		
	Dianthin 32	200	2.82 (85)		
	Dianthin 32	100	3.00 (77)		
3	None		3.27	700	720
	M. charantia inhibitor	200	2.56 (81)	100 (86)	140 (81)
	M. charantia inhibitor	100	2.74 (70)	215 (69)	280 (61)

[a] Virus yields were obtained by subtracting the logarithm of the amount of virus adsorbed to cells from the logarithm of the virus titer after 48 hours of incubation. Virus titres were determined by plaque assay

[b] In parentheses, per cent inhibition

Ribosome-inactivating proteins affected also the plaque-forming efficiency of HSV-1 (F) and of HSV-1 (MP). The plaques formed in the presence of these substances were fewer (Table 1) and their size was smaller (Figs. 1 and 2) as compared with controls. Reduction both of the number of plaques and of their average size varied with the concentration of ribosome-inactivating proteins. Gelonin was the least effective substance in these experiments. It should be pointed out that, due to the tiny size of the plaques formed in the presence of ribosome-inactivating proteins, plaques had to be scored at times longer than usually practised. Under these conditions signs of cytotoxicity due to ribosome-inactivating proteins became evident, especially in cultures incubated in the presence of PAP-S (100 and 200 μg/ml) and of *M. charantia* inhibitor (200 μg/ml). Dianthin 32 was too toxic to cells, and plaque counting was not performed.

Ribosome-inactivating proteins decreased also poliovirus I replication (Table 2). The extent of reduction induced by the various proteins was essentially similar, PAP-S being slightly less potent. The extent of reduction was similar to that described above for HSV-1 (F), and similar also to that exerted by PAP on poliovirus (19) and on HSV-1 (KOS) (1).

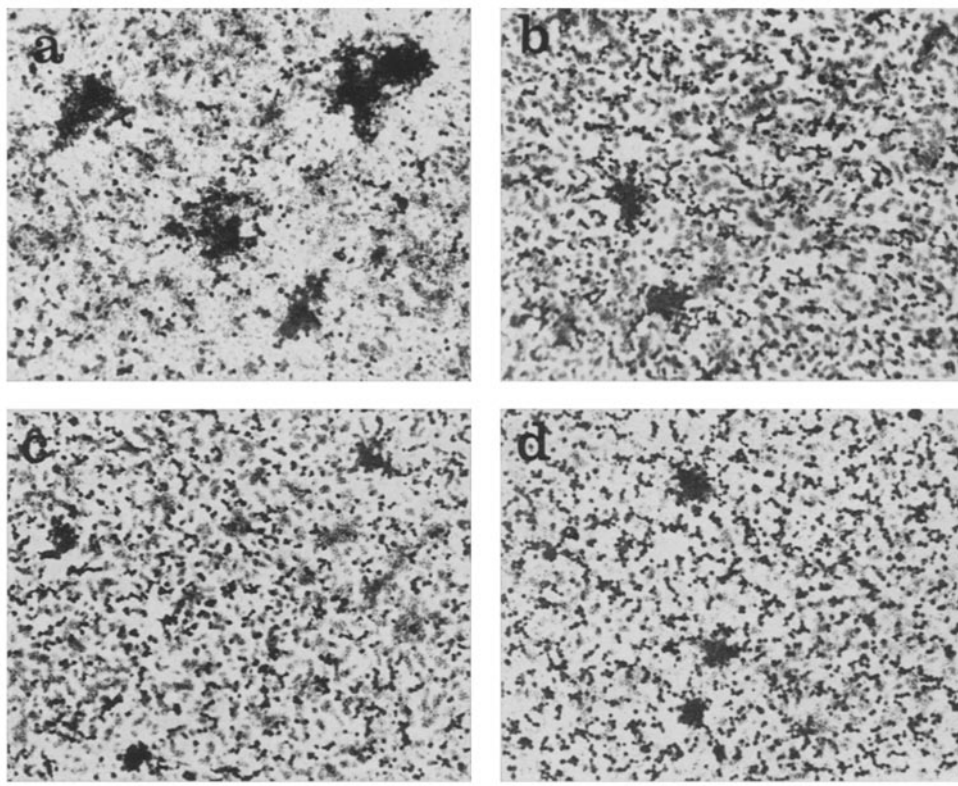

Fig. 1. Effect of ribosome-inactivating proteins on plaques induced by HSV-1(F) in HEp-2 cell monolayers. Cells were incubated without additions *(a)*, or in the presence of gelonin *(b)*, *Momordica charantia* inhibitor *(c)*, or PAP-S *(d)*, each added at a final concentration of 100 μg/ml. All micrographs were taken at the same magnification

Table 2. *Effect of ribosome-inactivating proteins on poliovirus I replication*

Protein added	Concentration (μg/ml)	Virus yield[a] (log PFU/ml)
None		4.44
Gelonin	200	3.21 (94)[b]
Gelonin	100	3.24 (94)
Dianthin 32	200	3.04 (96)
Dianthin 32	100	3.31 (93)
M. charantia inhibitor	200	2.84 (98)
M. charantia inhibitor	100	3.33 (92)
PAP-S	200	3.56 (87)
PAP-S	100	3.92 (70)

[a] Virus yields were calculated as described in the legend to Table 1. Infected cells were incubated for 24 hours. Virus titres were determined by neutral red plaque assay

[b] In parentheses, per cent inhibition

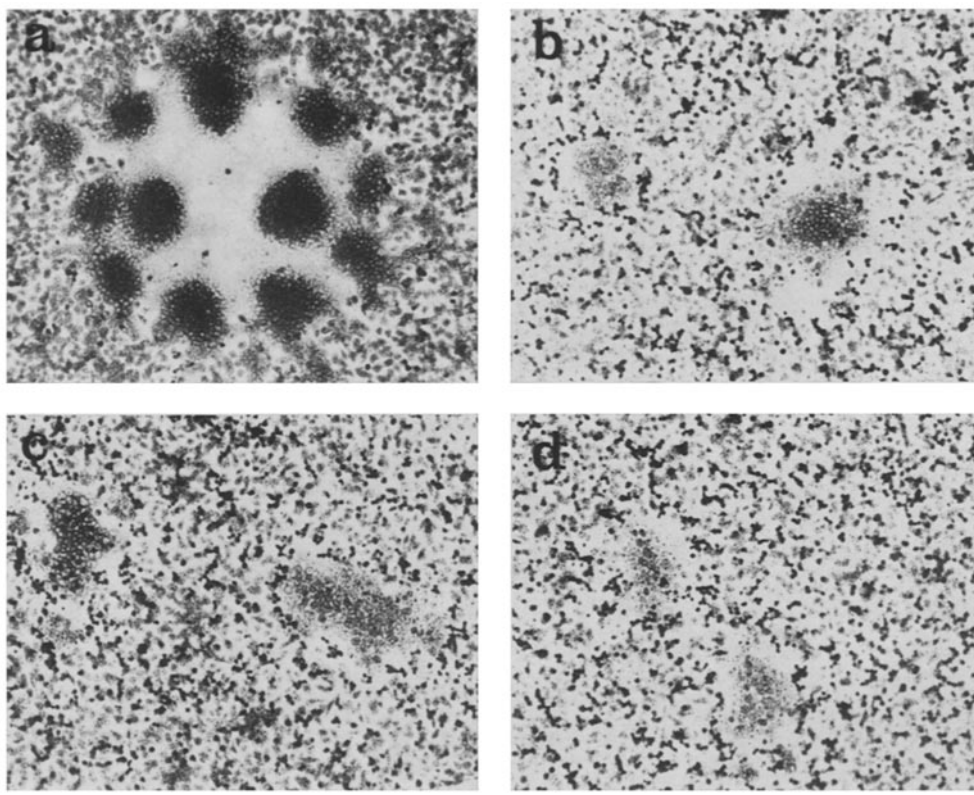

Fig. 2. Effect of ribosome-inactivating proteins on plaques induced by HSV-1(MP) in HEp-2 cell monolayers. Details were as described in the legend to Fig. 1

The yield of both HSV-1 (F) and poliovirus I was not affected if ribosome-inactivating proteins were present only during virus adsorption (Table 3).

Gelonin, PAP-S and *M. charantia* inhibitor, at the concentration of 200 μg/ml, did not modify the loss of infectivity of HSV-1 (F) virions during a 48-hour incubation at 37° C, whereas diantihn 32 slightly increased the inactivation of HSV-1 (F) infectivity. Poliovirions were thermostable during a 24-hour incubation period either in the absence or in the presence of the ribosome-inactivating proteins (results not shown). This rules out that the reduction of virus yield in the presence of the inhibitory proteins was due to inactivation of infectivity of progeny virions.

Table 3. *Effect on virus yield of ribosome-inactivating proteins present either during adsorption or during adsorption and incubation*[a]

Protein during	HSV-1(F) yield (log PFU/ml)[b]		Poliovirus I yield (log PFU/ml)[b]	
	Adsorption	Adsorption and incubation	Adsorption	Adsorption and incubation
None	2.45	2.45	4.24	4.24
Gelonin	2.93	1.00 (97)[c]	4.60	3.05 (94)
Dianthin 32	2.70	1.60 (86)	4.56	2.96 (95)
M. charantia inhibitor	3.02	N.D.[d]	4.12	2.81 (96)
PAP-S	2.60	1.64 (84)	4.30	3.40 (86)

[a] HEp-2 cell monolayers were infected with HSV-1(F) or poliovirus I. Adsorption was for 90 or 60 minutes for HSV-1(F) or poliovirus I, respectively, in the presence of 200 μg/ml of each ribosome-inactivating protein. At the end of virus adsorption, inoculum was removed; monolayers were rinsed three times with PBS and overlaid with medium containing ribosome-inactivating proteins, when appropriate. Incubation was for 48 or 24 hours for HSV-1(F) or poliovirus I, respectively

[b] Virus yields were calculated as described in the legend to Table 1

[c] In parentheses, per cent inhibition

[d] Not determined

Effect on Protein Synthesis

The time-interval of exposure to virus (and to the ribosome-inactivating proteins) and MOI used were designed so that cells infected with HSV-1 (F) or with poliovirus I were modified to a comparable extent, as assessed by microscope examination. Thus protein synthesis was measured at 17 hours after infection with HSV-1 (F), and at 7 hours after infection with poliovirus I, since the latter virus has a more rapid replication cycle and is more cytopathogenic.

All ribosome-inactivating proteins inhibited amino acid incorporation into proteins in HSV-1 (F)-infected cells more than in uninfected cells (Fig. 3). The difference between infected and uninfected cells was particularly evident in the case of the *M. charantia* inhibitor and of gelonin, which at the highest concentration tested (200 μg/ml) had no significant effect on uninfected cells, consistently with previous observations (3, 16), whereas dianthin 32 and PAP-S exerted some inhibition on control cells.

Similar effects were observed on cells infected with poliovirus I (Fig. 3). In these experiments the difference between the action of dianthin 32 and of PAP-S

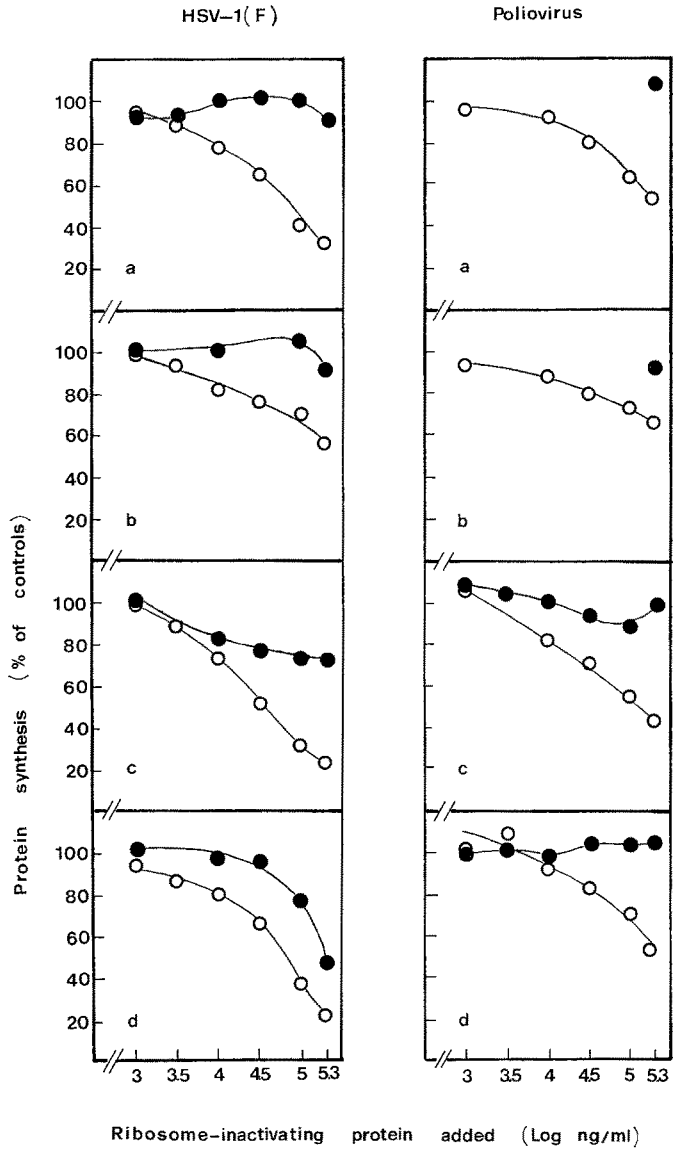

Fig. 3. Effect of ribosome-inactivating proteins on protein synthesis by normal and by virus-infected HEp-2 cells. Cells were infected with HSV-1(F) or with poliovirus I in the absence or in the presence of gelonin *(a)*, *Momordica charantia* inhibitor *(b)*, dianthin 32 *(c)* and PAP-S *(d)*, and were pulse-labelled with [^{14}C] amino acids (protein hydrolysate) at 17 hours and at 7 hours, respectively, after infection with HSV-1(F) or with poliovirus. The radioactivity incorporated was measured as described in the text. Results are expressed as per cent of controls without ribosome-inactivating proteins (12,000 to 20,000 dpm, approximately, for uninfected and for HSV-1(F)-infected cells, 8500 to 17,000 dpm for poliovirus-infected cells). Filled symbols: uninfected cells; empty symbols: infected cells

on infected and control cells was more evident, since these ribosome-inactivating proteins had almost no effect on uninfected cells, presumably due to the shorter period of exposure.

The acid-soluble radioactivity was not changed in HSV-1 (F)-infected cells treated with PAP-S, gelonin or *M. charantia* inhibitor, each at the concentration of 200 μg/ml, and was actually increased in poliovirus-infected cells treated with the same proteins (results not shown). The acid-soluble radioactivity was slightly decreased in both HSV-1 (F)- and poliovirus-infected cells treated with dianthin 32. These results rule out the possibility that the reduced amino acid incorporation observed in the presence of the ribosome-inactivating proteins is due to decreased amino acid uptake by the cells.

Discussion

Our results demonstrate that the ribosome-inactivating proteins gelonin, *M. charantia* inhibitor and dianthin 32 have effects on virus-infected cells which are comparable to those of PAP. Thus they reduce the multiplication of HSV-1 (F) and of poliovirus in a comparable manner to PAP (1, 19), and consistently with the reduction of lesions by tobacco mosaic virus exerted by PAP (10) and by the other substances used in present experiments (15, 17). It was demonstrated that the reduced multiplication of poliovirus was not due to reduced penetration of the virus into cells, but rather to an inhibition of protein synthesis in infected cells (19). A comparable inhibition was exerted by PAP, by the A-chain of abrin and by other inhibitors on cells infected by another picornavirus (6). Most probably this is the mechanism underlying the action of the ribosome-inactivating proteins examined in present work. This is indicated i) by the observation that these proteins do not modify the stability of virion infectivity nor virus adsorption, thus suggesting they act during the intracellular reproductive cycle, and ii) by the effect of these substances on protein synthesis, which is inhibited more markedly in virus-infected than in control cells. This effect on protein synthesis in cells is not exactly related to the potency of ribosome-inactivating proteins in cell-free systems. In the latter, PAP-S, *M. charantia* inhibitor, dianthin 32 and gelonin act in a decreasing order of potency, whereas in both uninfected and virus-infected HEp-2 cells PAP-S and dianthin are consistently more potent than the *M. charantia* inhibitor and gelonin. It is likely that other factors, such as the rate of penetration or degradation of inhibitory proteins, are involved in the case of cells.

These results, together with those quoted above (1, 6, 18, 20) and other (21) indicate a facilitated entry into virus-infected cells of macromolecules retaining their biological activity, probably as a consequence of an alteration of cell membrane following infection, as postulated by Carrasco (4). This author suggested that this increased permeability could be exploited for antiviral therapy, and Aron and Irvin (1) considered the possibility of a clinical significance of PAP as a wide-spectrum antiviral agent. Should this be the case, it would apply as well to the ribosome-inactivating proteins used in present experiments, and probably to other similar substances which may be available in the future. There is evidence for the presence in plants of several ribosome-inactivating proteins

(7, 8), the availability of which would be most useful to overcome the consequences arising from immune response, should these proteins be administered to whole animals.

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