Antiviral Activity of an Interferon-Inducing Synthetic Polymer (33897)

P. E. Came, M. Lieberman, A. Pascale, and G. Shimonaski
(Introduced by P. L. Perlman)
Department of Microbiology, Schering Corporation, Bloomfield, New Jersey 07003

Recently a wide variety of natural and synthetic anionic polymers have been shown to exhibit virus inhibitory capacities against a broad spectrum of viruses (1). One such polymer is polyvinyl sulfate (PVS), an anionic polymer of known structure which has been reported active in cell culture against herpes simplex (2, 3). These reports of antiviral activity of polysaccharides and sulfated polymers prompted this study to determine if polyvinyl sulfate has *in vivo* activity against influenze viruses and other viruses and to investigate the mode of action of the polymer.

Materials and Methods. Polyvinyl sulfate. Numerous preparations of polyvinyl sulfate, varying in molecular weight as well as other properties were kindly supplied by Dr. Walter Schlesinger, White Laboratories, Kenilworth, New Jersey. Lots of PVS were also obtained from Dr. Peter Daniels, Schering Corporation, Bloomfield, New Jersey and other commercial sources. The present studies were performed with the potassium salt of a single lot of PVS with an approximate molecular weight of 446,000, unless otherwise specified. Amantadine hydrochloride was obtained from E. I. DuPont de Nemours and Co., Wilmington, Delaware. The *N*-ethylisatin-β-thiosemicarbazone (NEIBT) was prepared by Schering Corporation.

Viruses. Influenza A/PR8, A_2/Jap. 305/57, and B/Great Lakes viruses were adapted to mice by repeated passage of homogenized infected lungs prior to propagation in eggs. Pneumonia virus of mice (PVM) was kindly supplied by Dr. Purnell Choppin, Rockefeller University. The WR strain of vaccinia virus obtained from the Public Health Laboratory of New York City and herpes simplex virus strain HF 378, were propagated in chorioallantoic membranes. Vesicular stomatitis virus (VSV) was obtained from Dr. S. Baron, NIH.

Cell cultures and assays. Plaque assays were performed on chick embryo fibroblasts (CEF) as described previously (4) but without pancreatin in the overlay medium. The VSV and interferon assays were performed on chick embryo cells or L929 cells according to Wagner (5). Inhibitor containing serum was separated, filtered and adjusted to pH 2.0 for 18–24 hr or employed directly after filtration without acidification.

Mice. Male CD-1 mice obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts were used for all studies except those performed with PVM. The PVM studies were done with mice supplied by Carworth, Inc., New City, New York. Mice were infected with influenza viruses by aerosolization by means of a model A4 aerosol apparatus, Tri-R Instruments Co., Rockville Center, New York. Intranasal inoculation of PVM was conducted as previously described (6). Vaccinia virus was injected by the ic (0.03 ml), ip (0.2 ml), or iv (0.2 ml) routes. With the latter route, focal lesions were produced on the tail and were evaluated according to Boyle *et al.* (7).

Results. In vivo *activity.* Mice were given subcutaneous injections of PVS, usually as three prophylactic doses; two on the day prior to infection and the last given 2 hr prior to infection.

Table I illustrates the protective effect of PVS against influenza virus A_2—Jap. 305/57. For comparative purposes amantadine was employed. It can be seen that treatment with 3.0 and 6.0 mg of PVS offered significant protection. Experiments with A/PR8 virus showed that similar protection was offered by PVS. No protection was observed when mice were infected with influenza B/Great Lakes virus or PVM.

The PVS was also administered prophylactically to mice infected with vaccinia virus. The effect of treatment on the production of

TABLE I. Activity of PVS against Aerosol Infection of Mice with Influenza Virus A_2/Jap. 305/57.[a]

Treatment	Total dose (mg)	Survivors/total	Survivors (%)
None	—	14/80	17.5
PVS	1.5[b]	6/30	20.0
	3.0[b]	21/40	52.5
	6.0[b]	24/39	61.5
Amantadine	4.0[c]	18/40	45.0

[a] Pooled results of four separate tests.
[b] Total of three equal doses.
[c] Single dose.

focal lesions on the tails of infected mice was studied. As shown in Table II, treatment with 4 mg of PVS reduced the number of tail lesions (87%) to the same extent observed with NEIBT (83%).

The effect of PVS injection on the multiplication of vaccinia virus in the spleens and livers of mice was studied following ip inoculation with a nonlethal infection of vaccinia virus. The spleens and livers were harvested 48 hr postinfection and the virus content was determined by plaque assay. Table III shows that treatment with PVS causes a significant reduction in the quantity of infectious virus recoverable from the spleens and livers of infected animals. For comparative purposes a sodium salt of PVS with an estimated mol. wt. 200,000–300,000 was also tested. In this test the sodium salt protected as well as the potassium salt. PVS showed the same approximate magnitude of activity as NEIBT. PVS did not protect against vaccinia virus when the virus was injected by the ic route.

TABLE II. Effect of PVS on the Formation of Tail Lesions Induced in Mice[a] by Vaccinia Virus.[b]

Treatment (total dose; mg)	No. of lesions: Per mouse	Average	Reduction (%)
None	3,3,7,9,24,19, 4,12,20	11.2	—
PVS, 3[c]	3,4,3,12,4,8,7, 5,0,3,0,1	4.1	63
PVS, 4[c]	0,1,2,0,5,2,0,2	1.5	87
NEIBT, 2[d]	1,1,0,4,6,2,1,0	1.9	83

[a] Thirteen g male Swiss (CD-1).
[b] Intravenous injection.
[c] Total of three equal doses.
[d] Single dose 2 hr after infection.

Induction of circulating inhibitors. The antiviral activity against both RNA and DNA containing viruses prompted speculation as to the mode of action of PVS. To investigate if interferon was involved, the following studies were performed. A single protective dose of PVS (3 mg) was administered subcutaneously to mice, and blood was collected at various time intervals after injection and assayed for interferon content. As shown in Table IV an inhibitor was detectable at approximately 16 hr postinoculation and could be demonstrated up to 30 hr after injection. In numerous tests the highest titer ever obtained was 1:32.

TABLE III. Effect of PVS on Multiplication of Vaccinia Virus[a] in Mice.[b]

Treatment (total dose; mg)	pfu/0.2 ml of homogenate		Reduction (%)
None	Liver	610	—
	Spleen	1600	—
PVS • K, 3[c]	Liver	70	89
	Spleen	<100	>94
PVS • K, 4.5[c]	Liver	110	82
	Spleen	800	50
PVS • Na, 3[c]	Liver	160	74
	Spleen	400	75
PVS • Na, 4.5[c]	Liver	60	90
	Spleen	200	87
NEIBT, 2.0[d]	Liver	190	69
	Spleen	600	63

[a] Intraperitoneal injection.
[b] Twenty–22 g male Swiss (CD-1).
[c] Total of three equal doses.
[d] Single dose 2 hr after infection.

Characterization of the inhibitor was performed on serum obtained 18–21 hr postinjection. Table V illustrates some of the properties of the inhibitor. The results of these tests indicated that the inhibitor was interferon.

Two experiments were designed to show that the observed interferon activity was due to interferon and not to residual injected

TABLE IV. Kinetics of Appearance of Inhibitor in Mice Injected with PVS.

After injection, 3 mg (hr)	Titer/2.0 ml
4	<8
8	<8
12	<8
16	8
24	32
30	20
36	<4
48	<4

PVS contained in the serum employed for interferon assay.

The first, involved a test to determine if PVS had inhibitory activity against VSV. Incorporation of PVS into the overlay medium of L cells did not cause a significant reduction of VSV plaque formation at concentrations ranging from 60 to 2000 μg/ml.

TABLE V. Characterization of Serum Inhibitor from PVS Injected Mice.

Property tested	Result
Acid-stable (pH 2.0/24 hr)	+
Dialyzable	—
Trypsin-sensitive (200 μg/60 min)	+
Inactivation of virus by contact	—
Removable from cells by 5 washings	—
Active on mouse cells (L929)	+
Active on chick embryo cells	—
Active on unrelated virus	+
Activity prevented by actinomycin	+

The second experiment was designed to show that PVS is not capable of inducing VSV plaque inhibition in cells of the assay system. Various dilutions of PVS were added to monolayers of CEF or L929 cells and incubated at 37° overnight, the time employed for assay of serum interferon. The PVS was removed and monolayers were washed with PBS prior to challenge with VSV. It was found that concentrations of PVS, much higher (1000 μg/ml and greater) than expected to be found in the serum of PVS-treated animals, did not induce plaque inhibition. Moreover, fluids concentrated fivefold from CEF cultures which were treated with PVS contained no inhibitors to VSV.

The demonstration of interferon in the serum, however, did not rule out the possibility that other factors could also contribute to the protective effect of PVS. Preliminary studies had shown PVS to exert *in vitro* activity against several viruses in chick cells, even though the chick cells did not produce interferon as a result of PVS treatment.

In vitro *activity*. Preliminary *in vitro* tests employing plaque inhibition test showed that PVS was active against influenza A/PR8, A_2/Jap. 305/57, herpes simplex, and vaccinia viruses. Plaque formation by West Nile, reovirus, Type 3, and vesicular stomatitis virus was not inhibited. The 50%inhibitory concentration of PVS against influenza A_2/Jap. 305/57 and vaccinia virus was determined by incorporating various concentrations of polymer into the overlay medium applied to monolayers of CEF inoculated with 100–300 pfu. The effect of direct inactivation by the polymer was determined by adding virus to PVS at a final concentration of 1000 μg/ml and incubating at 37° for 60 min prior to determination of virus concentration by plaque assay. Table VI shows the results of these studies. It is evident that PVS is capable of inhibiting the infectivity and multiplication of influenza virus A_2/Jap. 305/57 and vaccinia viruses in the absence of interferon. The mode of action of PVS was not investigated beyond these observations.

TABLE VI. *In Vitro* Antiviral Activity of PVS.

Virus	Concentration of PVS in overlay required for 50% pfu reduction (μg/ml)	Virus reduction after 60 min at 37° with PVS (1000 μg/ml)		
		Initial titer (pfu/ml)	Titer after 60 min ($\times 10^4$ pfu/ml)	Reduction (%)
Influenza A_2/Jap. 305/57	100–200	2.20×10^4	1.05	48
Vaccinia	50–100	1.25×10^5	7.50	40

Discussion. Although these studies show PVS protected mice infected with several viruses the data do not permit quantitation of protection afforded by: (i) direct inactivation of virus by contact, (ii) inhibition of virus multiplication by PVS, or (iii) inhibition due to PVS-induced interferon. It is also conceivable that mechanisms other than those considered above are responsible in part for the protective action of the polymer. Merigan and Finkelstein (8) and DeSomer *et al.* (9) have recently described the protection of mice by injection of interferon inducing synthetic polymers. These workers were also unable to elucidate completely the mechanism of action. In the same report DeSomer and his colleagues failed to detect interferon in the serum of mice injected with PVS. This finding is at variance with the present study but two possibilities could account for the discrepancy. First, the lowest dilution of serum employed by DeSomer and co-workers was 1:25. A 1:32 titer was the highest titer obtained in many tests conducted in the present report and usually it was only 1:16. Secondly, several preparations of PVS from different sources failed to induce detectable interferon even when assayed at dilutions as low as 1:4. Furthermore, we have observed alterations of the physical properties of a lot of PVS on storage as powder, accompanied by loss of *in vivo* protective activity and interferon inducing capacity.

Studies with isotopically labeled PVS would enable investigation of PVS distribution in the animal and may aid in interpreting to what degree virucidal activity plays a role in protection. Protection experiments with viruses completely resistant to PVS *in vitro* may also be of value in the elucidation of the mode of action of PVS and related substances. The present studies suggest, however, that a compound may exert dual modes of action against certain viruses *in vivo*.

Summary. A synthetic anionic polymer, polyvinyl sulfate (PVS) showed *in vitro* and *in vivo* antiviral activity. The polymer did not induce interferon *in vitro* in L929 or chick embryo cells but interferon could be detected in the circulation of mice treated with polyvinyl sulfate. The mechanism of protection of PVS has not been completely elucidated and the possibility that it exerts multiple modes of activity cannot be excluded.

1. Takemoto, K. K. and Spicer, S. S., Ann. N. Y. Acad. Sci. **130**, 365 (1965).

2. Nahmias, A. J., Kibrick, S., and Bernfield, P., Proc. Soc. Exptl. Biol. Med. **115**, 993 (1964).

3. Silver, G. H. and Smith, J. E., Bacteriol. Proc. **1967**, 163.

4. Came, P. E., Pascale, A., and Shimonaski, G., Arch. Ges. Virusforsch. **23**, 346 (1968).

5. Wagner, R. R., Bacteriol. Rev. **24**, 151 (1960).

6. Harter, D. H. and Choppin, P. W., J. Exptl. Med. **126**, 251 (1967).

7. Boyle, J. J., Haff, R. F., and Stewart, R. C., Antimicrobial Agents Chemotherapy **1966**, 536.

8. Merigan, T. C. and Finkelstein, M. S., Virology **35**, 363 (1968).

9. DeSomer, P., DeClercq, E., Billiau, A., Schonne, E., and Clasen, M., J. Virol. **2**, 878 (1968).

Received Dec. 20, 1968. P.S.E.B.M., 1969, Vol. 131.