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Inhibition of influenza virus infection by pine cone antitumor substances

Kyosuke Nagata[1], Hiroshi Sakagami[2], Hiroshi Harada[3], Meihan Nonoyama[4], Akira Ishihama[1] and Kunio Konno[2]

[1]*Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan,* [2]*First Department of Biochemistry, and* [3]*Second Department of Biochemistry, School of Medicine, Showa University, Shinagawa-ku, Tokyo 142, Japan and* [4]*Department of Virology, Showa University Research Institute for Biomedicine in Florida, St. Petersburg, FL 33716, U.S.A.*

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Summary

The anti-influenza virus activity of polysaccharides and other high molecular weight fractions from pine cone extract (PCE) of *Pinus parviflora* Sieb. et Zucc. was investigated. None of the fractions affected the growth of MDCK cells. The acidic PCE substances markedly suppressed the growth of the influenza virus in MDCK cells. Significant inhibition of both the viral protein synthesis in infected cells and virion-associated RNA-dependent RNA polymerase activity was observed with these acidic fractions. Although amantadine inhibited virus plaque formation as effectively as PCE fractions, it was less effective in inhibiting the RNA polymerase activity. These results suggest that PCE, which has been shown to contain antitumor substance(s), also contains anti-influenza virus substance(s).

Influenza virus; Pine cone extract; RNA dependent RNA polymerase

Introduction

Hot water extract of pine cones (PCE) of *Pinus parviflora* Sieb. et Zucc. has been used as a traditional medicine in certain areas of Japan to improve the conditions of patients, in particular those with gastric cancer. Recently, PCE has been shown to contain differentiation-inducing substance(s) for human leukemic cell lines (Sakagami et al., 1986) and antitumor substances against transplanted tumor cells

Correspondence to: Kyosuke Nagata, Department of Molecular Genetics, National Institute of Genetics, Mishima, Shizuoka 411, Japan.

(Sakagami et al., 1987). Acidic fractions of PCE stimulate the production of a differentiation-inducing factor(s) by macrophages (Sakagami et al., 1987), the iodination of cultured granulocytic cells (Unten et al., 1989) and the proliferation of isolated splenocytes (Kurakata et al., 1989). Treatment of mice with these fractions induces antimicrobial resistance (Harada et al., 1988). In addition to their immunopotentiating effects, these fractions are effective in inhibiting the replication of human immunodeficiency virus (HIV) (Lai et al., 1989) and herpes simplex virus (HSV) (Fukuchi et al., 1989). It is of interest to examine whether the antiviral activity of PCE extends to other viruses and to delineate the mechanism of their inhibitory effect on virus replication.

Influenza virus is one of the well-studied viruses with respect to both the molecular processes of replication cycle in vivo and the enzymatic mechanisms of genome RNA transcription and replication in vitro. Genetic and biochemical studies have revealed that the virion-associated RNA-dependent RNA polymerase and the nucleocapsid protein are needed for the synthesis of viral RNAs, i.e., mRNA, cRNA (complementary RNA to vRNA) and vRNA (Ishihama and Nagata, 1988). The ribonucleoprotein (RNP) cores consisting of the aforementioned proteins and vRNA have been isolated and characterized (Honda et al., 1987; Honda et al., 1988).

In this paper, we describe the inhibitory effect of PCE fractions on influenza virus replication. The molecular mechanism of this inhibitory effect is discussed.

Materials and Methods

Cell and virus

MDCK cells were maintained in MEM supplemented with 10% fetal calf serum. Influenza viruses A/PR/8/34 (H1N1), A/WSN/33 (H1N1), A/Urdon/72 (H3N2), A/Victoria/1/75 (H3N2) and B/Lee/40 were grown in allantoic sacs of 10 day-old embryonated eggs for 48 h at 35.5°C and purified as described previously (Kawakami et al., 1981). The purified virions were suspended in 10 mM Tris-HCl (pH 7.8 at 4°C) containing 1 mM DTT and 20% (w/v) glycerol and stored at −80°C until use.

Isolation of RNP cores and matrix protein

RNP cores and the matrix protein were isolated according to previous reports (Honda et al., 1987; Honda et al., 1988; Gregoriades, 1973).

RNA synthesis in vitro

RNA synthesis was carried out at 35°C for 1 h in a final volume of 25 μl, which contained 50 mM Hepes-NaOH (pH 7.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 2.5 mM DTT, 2.5% glycerol, 1 mM each of ATP, CTP and GTP, 25 μM UTP, 5 μCi [α-

^{32}P]UTP (specific activity, 1.5×10^7 dpm/nmol), 10 mM creatine phosphate, 120 μg/ml creatine kinase, 3000 units/ml of RNase inhibitor, 0.1% NP-40, 250 μM ApG as primer and 20–40 ng nucleoprotein equivalents of virions or isolated RNP cores. RNA synthesis was terminated by the addition of ice-cold trichloroacetic acid. The amount of [α-^{32}P]UMP incorporated was determined by collecting acid-insoluble materials onto a Whatman GF/C glass filter, and quantitating the radioactivity in a Beckman scintillation counter.

Plaque formation

Confluent monolayer cultures of MDCK cells in 60-mm plastic dishes were washed twice with MEM and exposed to influenza virus at the appropriate multiplicity of infection (MOI). After adsorption for 30 min at 37°C, cells were washed twice with MEM and then overlayed with MEM containing 0.8% agarose, 0.2% BSA and 4 μg/ml trypsin. After 2 to 3 days' incubation at 34°C, plaques were visualized by staining cells with amino black.

Preparation of PCE fractions

Polysaccharides and other acidic high molecular weight substances were fractionated from pine cones of *Pinus parviflora* Sieb. et Zucc. as described (Sakagami et al., 1987). Briefly, pine cones were extracted with boiling water. The hot-water extract was precipitated with 6 volumes of ethanol and subjected to DEAE-cellulose column chromatography. Stepwise elution was carried out with water (Fr. I), 0.5 M NaCl (Fr. II), 2 M NaCl (Fr. III) and 0.15 N NaOH (Frs. IV and V). Residue that was not extracted with boiling water was further extracted with 1% NaOH. The pH of the extract was adjusted to 5.0 with acetic acid. The precipitate was collected by centrifugation (Fr. VI) and the supernatant was successively precipitated with 1, 2 and 5 volumes of ethanol (Frs. VII, VIII and IX). All fractions were dialyzed against distilled water and lyophilized.

Results

Effect of PCE fractions on the growth of MDCK cells

Prior to the examination of the effect of the PCE fractions on influenza virus replication, we examined whether the PCE fractions affected the growth of MDCK cells, which are the host cells routinely used for influenza viruses. Neither the growth rate nor final cell density were affected by the PCE fractions I, II, V, VI, VII, VIII and IX at 100 μg/ml (Fig. 1A, and data not shown). A slight decrease in the cell growth rate was observed at 300 μg/ml (Fig. 1B). The cytotoxic effects of PCE fractions V, VI and VIII became apparent at a concentration of 1000 μg/ml (Fig. 1B). Thus, PCE fraction concentrations up to 100 μg/ml could be considered as essentially non-toxic to the host cells.

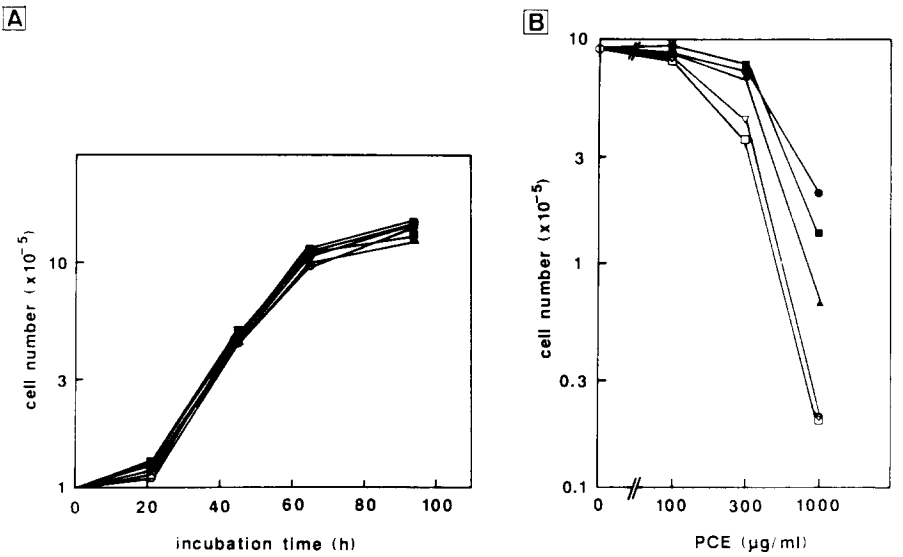

Fig. 1. Effect of PCE fractions on the growth of MDCK cells. (A) MDCK cells (1×10^5) were seeded in 22-mm plastic dishes and incubated at 37°C in the absence (○) or presence of 100 μg/ml of the PCE fractions V (□), VI (▽), VII (●), VIII (▲) and IX (■). After incubation for the indicated periods, the viable cell number was determined. (B) MDCK cells (1×10^5) were seeded in 22-mm plastic dishes and incubated at 37°C in the absence or presence of 100, 300 or 1000 μg/ml of the PCE fractions. After incubation for 60 h, the viable cell number was counted. Symbols are the same as in (A).

Inhibition of influenza virus growth by PCE fractions

The effect of PCE fractions on the production of infectious influenza virus was examined by the plaque assay. As shown in Fig. 2A, the formation of plaques was completely inhibited at a concentration of 100 μg/ml by PCE fractions V, VI, VIII and IX, whereas PCE fractions I and II at the same concentration had no effect. The level of inhibition with PCE fraction VII was lower than those with other fractions, although the plaques formed in the presence of fraction VII were smaller than those formed in the absence of the PCE fractions. Inhibition of plaque formation was proportional to increasing concentrations of the CPE fractions (Fig. 2B). Even when the plaque assay was carried out at higher multiplicity of infection than that used for Fig. 2, the level of inhibition was unchanged. Addition of PCE fractions either prior to or at the time of infection had no effect on virus production if infected cells were subsequently maintained in the absence of PCE fractions. Furthermore, the activity of the trypsin, which is known to activate influenza virus by cleavage of HA protein to HA1 and HA2, was not inhibited even in the presence of 25-fold excess amount of any PCE fraction. These observations thus suggest that the PCE fractions had no direct effect on virus adsorption to MDCK cells.

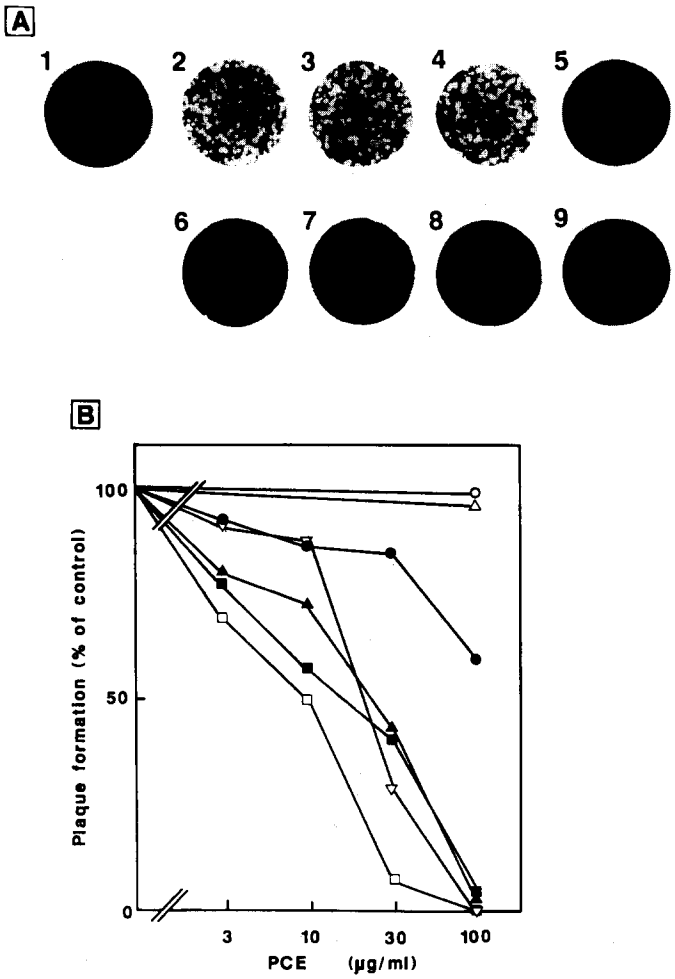

Fig. 2. Effect of PCE fractions on plaque formation. (A) Plaque formation assay was carried out as described in Materials and Methods. Panels: 1, no infection, no PCE fractions; 2, infection, no PCE fractions; 3, infection, PCE fraction I; 4, infection, PCE fraction II; 5, infection, PCE fraction V; 6, infection, PCE fraction VI; 7, infection, PCE fraction VII; 8, infection, PCE fraction VIII; 9, infection, PCE fraction IX. All PCE fractions were at a concentration of 100 μg/ml. (B) Plaque formation assay was carried out in the presence of increasing concentrations of PCE fractions I (○), II (△), V (□), VI (▽), VII (●), VIII (▲) and IX (■). Data are presented as percent of the plaque number (206 plaques) formed in the absence of the PCE fractions.

Effect of PCE fractions on viral RNA synthesis

To get insight into the molecular mechanism of inhibition of influenza virus replication by the PCE fractions, we first examined the effect of these fractions on viral RNA-dependent RNA polymerase activity. Using disrupted virions (Honda

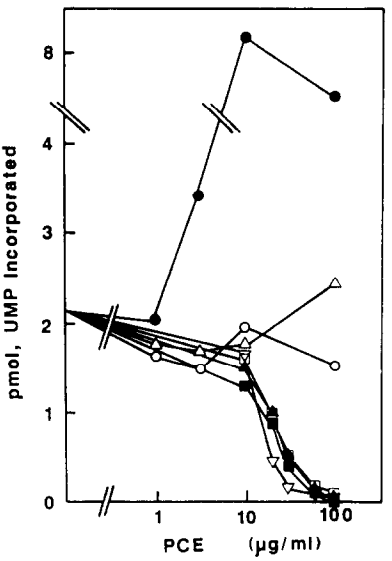

Fig. 3. Effect of PCE fractions on viral RNA synthesis in disrupted virions. Viral RNA synthesis in vitro was carried out using disrupted virions as described in Materials and Methods in the presence of increasing concentrations of PCE fractions I (○), II (△), V (□), VI (▽), VII (●), VIII (▲) and IX (■).

et al., 1987), the in vitro RNA synthesis was carried out in the presence of varying concentrations of PCE fractions and the incorporation of [α-^{32}P]UMP was measured (Fig. 3). PCE fractions I and II had no effect on viral RNA synthesis within the range of concentrations examined. In contrast, viral RNA synthesis was inhibited by increasing concentrations of PCE fractions V, VI, VIII and IX. When isolated RNP was used as an enzyme source, essentially the same result was obtained (Table 1). These results suggest that the inhibition of viral RNA synthesis by PCE fractions is due to their direct interaction with one of the RNP components.

Addition of PCE fraction VII to disrupted virions did not inhibit but rather enhanced viral RNA synthesis, whereas viral RNA synthesis by isolated RNP complexes was inhibited by the addition of PCE fraction VII. This could be explained

TABLE 1

Effect of PCE fractions on influenza viral RNA synthesis

PCE fraction	% of control, UMP incorporated						
	I	II	V	VI	VII	VIII	IX
Disrupted virions[a]	88.4	136.8	6.6	4.3	291.5	7.9	13.0
RNP complexes[b]	107.9	85.5	5.4	4.1	13.6	3.7	7.4

Synthesis of influenza viral RNA was carried out in the standard reaction mixture in the presence of 100 μg/ml of the indicated PCE fractions, using either disrupted virions[a] or isolated RNP complexes[b]. Data are presented as percent of the amount synthesized in the absence of the PCE fraction (1.88 pmol and 2.46 pmol for disrupted virions and RNP complexes, respectively).

TABLE 2

Anti-influenza activities of PCE fraction VI and amantadine

PCE Fr. VI (μg/ml)	Amantadine (μg/ml)	RNA synthesis[a]	Plaque formation[b]
		(% of control)	
10		91	ND
30		47	26
100		3	0
	10	98	68
	30	97	16
	100	94	0
	300	83	0
	1000	82	ND
	3000	77	ND

Viral RNA synthesis starting from disrupted virions[a] and plaque formation assay[b] were monitored as described in Materials and Methods in the presence of either PCE fraction VI or amantadine (purchased from Sigma). Data are presented as percent of the amount of UMP incorporated (2.22 pmol)[a] and the plaque number formed (77 plaques)[b] in the absence of drugs. ND, not determined.

if PCE fraction VII not only interacts with the RNP complexes but also with a viral envelope or membrane component(s) that suppresses viral RNA polymerase activity. Influenza virus matrix protein has been shown to inhibit viral RNA synthesis by interacting with RNP complexes (Zvonarjev and Ghendon, 1980; Ye et al., 1987). In a reconstitution experiment with RNP complexes and purified matrix protein, the matrix protein was found to inhibit viral RNA synthesis by the RNP complexes by 40–50%, whereas no inhibition was observed in the presence of PCE fraction VII (data not shown).

The inhibitory effect of the PCE fractions on viral RNA synthesis was compared with that of amantadine. The target for the anti-influenza virus activity of amantadine is the product(s) encoded by segment 7 of the viral genome (Hay et al., 1985; Lubeck et al., 1978; Zvonarjev and Ghendon, 1980). PCE fraction VI inhibited the production of infectious influenza virus as effectively as did amantadine. However, PCE fraction VI inhibited viral RNA synthesis much more strongly than did amantadine (Table 2). This suggests that the mechanism of the anti-influenza virus action of the PCE fraction is distinct from that of amantadine.

Effect of PCE fractions on viral protein synthesis

The effect of the PCE fractions on viral protein synthesis was also investigated. Infected cells were pulse-labeled with [^{35}S]methionine and labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 4). PCE fractions I, II and VII had no effect on viral protein synthesis, whereas PCE fractions VI and IX markedly inhibited viral protein synthesis at both the early and late stages of infection. Slight inhibition of viral protein synthesis was observed with PCE fractions V and VIII. The synthesis of late proteins, e.g. M and HA, was more sensitive to these PCE fractions than that of early proteins, e.g. P and NP.

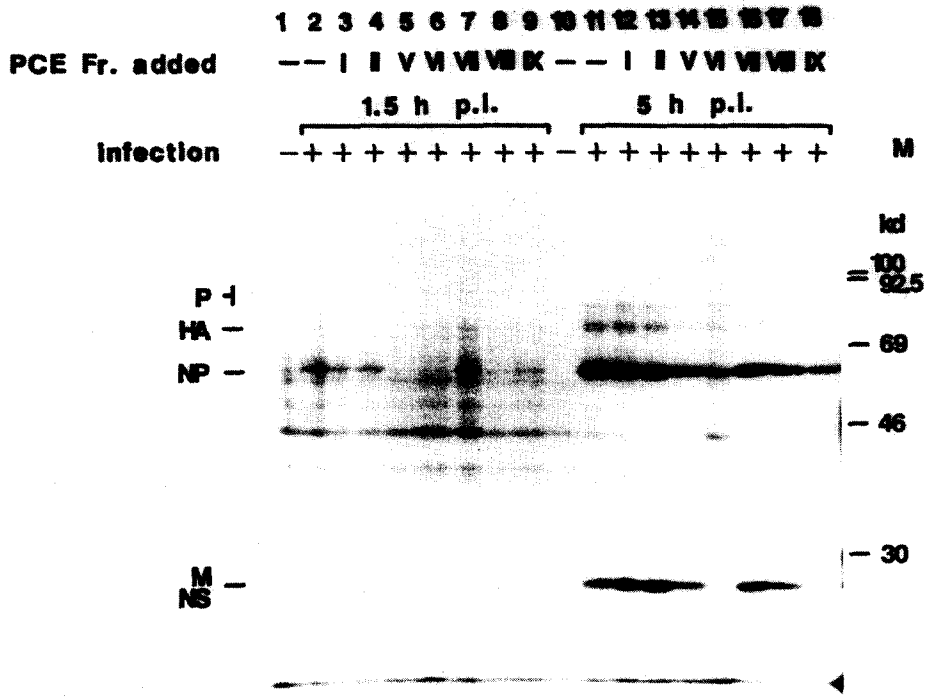

Fig. 4. Effect of PCE fractions on viral protein synthesis. Monolayer culture of MDCK cells (1×10^6) was infected (lanes 2–9 and 11–18) or mock-infected (lanes 1 and 10) with influenza virus at an MOI of 50. After incubation for 1.5 h (lanes 1–9) and 5 h (lanes 10–18) in the absence (lanes 1, 2, 10 and 11) or presence of PCE fractions I (lanes 3 and 12), II (lanes 4 and 13), V (lanes 5 and 14), VI (lanes 6 and 15), VII (lanes 7 and 16), VIII (lanes 8 and 17) and IX (lanes 9 and 18), cells were pulse-labeled with [^{35}S]methionine for 30 min in MEM supplemented with the respective PCE fractions. Cells were then washed twice with phosphate-buffered saline and lysed in SDS sample buffer. Labeled proteins were analyzed by electrophoresis on 12.5% polyacrylamide gel in the presence of 0.1% SDS. Gels were exposed to X-ray films at −80°C. Migration positions of viral proteins are indicated on the left side of the gel: P, RNA polymerase subunits PB2, PB1 and PA; HA, hemagglutinin; NP, nucleoprotein; M, matrix protein; NS, nonstructural protein. The positions of the molecular weight standards ($\times 10^{-3}$) are indicated on the right side of the gel.

Susceptibility of various influenza viruses to PCE fractions

We finally examined the effect of the PCE fractions on the growth of a variety of influenza viruses. The formation of plaques by A/WSN/33, A/Urdon/72, A/Victoria/1/75 and B/Lee/40 strains as well as A/PR/8/34 was inhibited by the PCE fractions, albeit to different extents (Table 3). A/WSN/33 showed the same sensitivity as A/PR/8/34 to all PCE fractions and amantadine. In contrast, A/Urdon/72, A/Victoria/1/75 and B/Lee/40 strains were less sensitive than A/PR/8/34 or A/WSN/33 to PCE fractions V, VI, VIII and IX.

TABLE 3

Plaque formation by influenza viruses in the presence of PCE fractions and amantadine

Drugs (μg/ml)	% of control									
	Fraction V		Fraction VI		Fraction VII	Fraction VIII	Fraction IX		Amantadine	
	30	100	30	100	100	100	30	100	30	100
Viruses										
A/PR/8/34	18*	0	14*	0	84	2.3	21*	6.8*	24*	0
A/WSN/33	2.0	2.0	6.1	0	76	4.0	6.1	0	45*	0
A/Urdon/72	49*	20*	40*	19*	77	44	46	22*	0	0
A/Victoria/1/75	48	20	53	18	87	32*	57	33	18	3.0
B/Lee/40	41	20	62	35	84	72	69	28	28	14*

Plaque formation assay was carried out as described in Materials and Methods with either 30 μg/ml or 100 μg/ml of the drugs. Data are presented as percent of the plaque number formed in the absence of the drugs. Asterisks indicate minute plaques.

Discussion

It has been reported that the pine cone extract (PCE) has immunopotentiating effects in mice (Sakagami et al., 1987; Harada et al., 1988). The PCE fractions also have been shown to inhibit HIV replication in human T cells (Lai et al., 1989) and HSV replication in African green monkey CV-1 kidney cells (Fukuchi et al., 1989). The results presented here showed that the PCE fractions are also effective in inhibiting influenza virus replication. Neutral (fraction I) and uronic acid-rich polysaccharide fractions (fraction II) had no effect on influenza virus replication. The acidic fractions V, VI, VIII and IX contain substances that suppress virus replication.

IR, UV and ESR spectroscopy revealed that PCE fractions V, VI, VII, VIII and IX contain oxidizable phenolic groups as sole donors of acidity (Sakagami et al., 1989). Other acidic groups, such as sulfate, uronic acid and sialic acid were not detected. We previously reported that PCE fractions I and II as well as PCE fractions V, VI, VII, VIII and IX have four different neutral sugars (glucose, galactose, mannose and arabinose or fucose) in common (Sakagami et al., 1987). Further purification and chemical analysis of the active components are in progress.

Studies presented here support the idea that PCE may contain several components that inhibit influenza virus replication. PCE fractions were not toxic to MDCK cells at concentrations up to 100 μg/ml (Fig. 1 and Fig. 2A).

PCE fractions VI and IX markedly inhibited viral RNA synthesis in vitro and viral protein synthesis in infected cells. The latter completely depends on primary transcription. Viral RNA synthesis by isolated RNP complexes is as sensitive as disrupted virions. These observations suggest that the molecular target for the antiviral action of these PCE fractions is the viral RNA polymerase.

PCE fractions V and VIII inhibited viral RNA synthesis in vitro, but synthesis of late viral proteins in infected cells was inhibited to a lesser extent. Synthesis of late proteins is directed mainly by secondary transcription which is dependent on

newly synthesized vRNA from cRNA (Ishihama and Nagata, 1988). It has been reported that in infected cells the RNA polymerase involved in replication is structurally different from that involved in transcription (Beaton and Krug, 1986). RNA polymerase involved in the synthesis of cRNA and vRNA may be sensitive to PCE fractions V and VIII, but alternatively or additionally, post-transcriptional process(es) specific for the expression of late proteins may also be a site for the action of the PCE fractions V and VIII.

Amantadine inhibits the formation of infectious virus by interacting with products encoded on segment 7 (Hay et al., 1985; Lubeck et al., 1978; Zvonarjev and Ghendon, 1980). PCE fractions V, VI, VIII and IX inhibit virus production as effectively as amantadine does, whereas these PCE fractions are much more inhibitory to RNA synthesis than amantadine (Table 2, Figs. 2 and 3). From these observations, it is assumed that the PCE fractions do not interact with a protein encoded from segment 7, or that even if they interact with a protein from segment 7, their mode of action is distinct from that of amantadine.

PCE fraction VII was found to enhance viral RNA synthesis in disrupted virions, whereas it proved inhibitory when RNP complexes were used (Table 1). However, viral protein synthesis in infected cells was not affected by this fraction (Fig. 4). These observations could be explained by the interaction of this fraction with the matrix protein that is capable of inhibiting viral RNA synthesis by RNP complexes (Zvonarjev and Ghendon, 1980; Ye et al., 1987). Inhibition of plaque formation by PCE fraction VII (Fig. 2) may be due to the modification of the matrix protein during virus encapsidation.

The PCE fractions inhibited plaque formation by various influenza strains, i.e. A/PR/8/34, A/WSN/33, A/Urdon/72, A/Victoria/1/75 and B/Lee/40. A/PR/8/34 and A/WSN/33 were more sensitive than the other strains to PCE fractions V, VI, VIII and IX. The reason for the interstrain differences in susceptibility to the CPE fractions remains to be elucidated. It may be possible to determine genetically the target(s) for each PCE fraction by reassortments between more sensitive and less sensitive virus strains.

Knowledge of the molecular mechanism of action of the PCE fractions should not only increase our insight in influenza virus replication, but also help in defining molecular targets for antiviral agents in general and anti-influenza agents in particular.

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