Antiviral Research 15 (1991) 41–50
Elsevier

AVR 00459

Possible involvement of lignin structure in anti-influenza virus activity

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(Received 2 May 1990; accepted 8 October 1990)

Summary

Commercial lignins suppressed the growth of influenza A virus infecting MDCK cells, and the RNA-dependent RNA synthesis, as efficiently as the high-molecular weight fractions extracted from pine cone of *Pinus parviflora* Sieb. et Zucc. The anti-influenza A virus activity of both pine cone extract and commercial alkali-lignin was considerably reduced by treatment with sodium chlorite, but was not affected by sulfuric acid or trifluoroacetic acid. The degraded components of lignin, various synthesized polyphenols unrelated to lignin, and natural and chemically modified glucans, were not appreciably inhibitory. The data suggest that the polymerized phenolic structure of lignified materials is responsible for the anti-influenza A virus activity.

Influenza virus; Lignin

Introduction

We have previously reported that high-molecular-weight fractions from pine cone extract (PCE) of *Pinus parviflora* Sieb. et Zucc. (Sakagami et al., 1987) markedly suppress the growth of the influenza virus infection in MDCK cells

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Abbreviations: HSV, herpes simplex virus; HIV, human immunodeficiency virus; ED_{50}, 50% effective dose.

without affecting the growth of the MDCK cells (Nagata et al., 1990). They inhibit both the viral protein synthesis in infected cells and virion-associated RNA-dependent RNA polymerase activity (Nagata et al., 1990). When the most active fraction (Fr. VI) of the pine cone extract was chemically analysed with IR, UV, or ESR spectroscopy, in addition to cellulose-TLC chromatography, this fraction was found to contain mainly lignin- related substances complexed with sugars or polysaccharides (Sakagami et al., 1989a). In the present study, to identify the active principle necessary for anti-influenza virus activity, Fr. VI was subjected to partial degradation in two ways: by chlorinated decomposition of the lignin portion, which is assumed to be a main component of lignified materials, and by acid hydrolysis of the polysaccharide portion. These preparations, in addition to a commercial alkali-lignin and its degradation products, were also tested for anti-influenza virus activity.

Materials and Methods

Materials

The cones of *Pinus parviflora* Sieb. et Zucc. were supplied by Mr. S. Matsuda. PSK, a protein-bound polysaccharide prepared from mycelia of a CM-101 strain of *Coriolus versicolor* (Tsukagoshi et al., 1984) was kindly provided by Kureha Chem. Ind. Co., Ltd., Tokyo, Japan. A carboxymethylglucan (CM-TAK) (Sasaki et al., 1979) was provided by Takeda Chem. Ind. Ltd., Osaka, Japan. Paramylon, a polysaccharide with an unbranched β-1,3-D-glycopyranoside structure (Clark and Stone, 1960) was extracted from *Euglena gracilis*, and its chemically modified derivative (paramylon sulfate) was synthesized as described previously (Sakagami et al., 1989b). Alkali-lignin, dealkali-lignin, lignin sulfonate, syringaldehyde, vanillin, and trifluoroacetic acid (TFA) were purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Coniferyl alcohol was purchased from Aldrich Chem. Co., Milv., WI. Sodium chlorite was obtained from Wako Pure Chemical Co., Ltd., Osaka, Japan. Bisphenol A and its isomeric dimers were synthesized as previously reported (Poroka and Cyrus, 1987).

Preparation of antiviral substance from pine cone

The acidic, high molecular weight substance was prepared from NaOH extract of cone of *Pinus parviflora* Sieb. et Zucc. (Sakagami et al., 1987). Briefly, the pine cones were washed successively with methanol, ethanol and then boiling water. The 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 and centrifuged for 20 min at 10000 $\times$ *g*. The precipitate was redissolved in a small volume of 1% NaOH, centrifuged to remove insoluble material, and the supernatant was then neutralized and dialyzed against distilled water and lyophilized (Fr. VI).

Preparation of hemicellulose

Hemicellulose was prepared from pine cone as previously described (Wise et al., 1946). In brief, the pine cone of *Pinus parviflora* Sieb. et Zucc. was extracted with methanol, ethanol and hot water. The residue was ground, treated with $NaClO_2$ as described below, and extracted with 1 N NaOH. The NaOH extract was dialyzed against distilled water and lyophilized.

Degradation of Fr. VI and alkali-lignin

The decomposition of lignin structure was performed with sodium chlorite (Wise et al., 1946). Fr. VI or alkali-lignin (300 mg) was suspended in 15 ml of distilled water and the pH was adjusted to 4 with glacial acetic acid. After addition of 225 mg $NaClO_2$, the mixture was heated for 1 h at 75°C. To this mixture, 18 μl of acetic acid and 225 mg $NaClO_2$ were added and heated for 1 h at 75°C. This was repeated once more. The reaction mixture was dialyzed against distilled water and then lyophilized.

The decomposition of sugars or polysaccharides was performed with sulfuric acid or trifluoroacetic acid (TFA). Fr. VI or alkali-lignin (200 mg) was heated for 2 h at 20°C in 12 ml H_2SO_4 with occasional stirring. The concentration of H_2SO_4 was reduced to 3% by addition of 450 ml distilled water and heated for 4 h at 100°C. The precipitates (so-called Klason lignin) were filtered off with 0.45 μm of Durapore HV filter (Millipore) and washed thoroughly with distilled water. The precipitates were resuspended with distilled water, dialyzed against distilled water and lyophilized (H_2SO_4-ppt). The filtrates were then dialyzed against distilled water, and lyophilized (H_2SO_4-sup).

Fr. VI or alkali-lignin (200 mg) was heated for 4 h at 100°C in 20 ml 2 M TFA and the resultant precipitate (TFA-ppt) was separated from the supernatant (TFA-sup) by filtration through a Millipore filter as described above. The remaining TFA in these fractions was removed by evaporation under reduced pressure. The test samples were dissolved in a small volume of 0.1 N NaOH, and the pH was adjusted to 8.0 with HCl. When this solution was buffered with culture medium, the final pH was about 7.4 at which no precipitate was formed. This procedure overcame the difficulty of directly dissolving the material in neutral distilled H_2O.

Methylation of Fr. VI and alkali-lignin

Fifty mg Fr. VI or alkali-lignin was dissolved in 10 ml of 1 N NaOH and mixed with 1 ml methyl iodide. The mixture was vigorously stirred for 48 h at 25°C. After evaporating the remaining methanol and methyl iodide, the residue was dialysed against distilled water and then lyophilized.

Cells and virus

MDCK cells were maintained in MEM supplemented with 10% fetal calf serum.

Influenza virus A/PR/8/34 (H1N1) was 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 ml Tris-HCl (pH 7.8 at 4°C) containing 1 mM DTT and 20% (w/v) glycerol and stored at −80°C until used.

RNA synthesis in vitro

RNA dependent RNA transcriptase activity was measured, using the detergent-solubilized purified virion particles containing a fixed amount of nucleoproteins (Nagata et al., 1990). In brief, 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 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 RNase inhibitor, 0.1% NP-40, 250 μM ApG as primer, 20–40 ng nucleoprotein equivalents of virions and each test sample. 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 assessing the radioactivity with a Beckman scintillation counter.

Plaque formation

A confluent monolayer culture of MDCK cells in a 60 mm plastic dish was washed twice with MEM and exposed to influenza A virus at the appropriate multiplicity of infection (MOI). After adsorption for the indicated times at 37°C, cells were washed twice with MEM and then overlaid with MEM containing 0.8% agarose, 0.2% BSA, 4 μg/ml trypsin and each test sample. After 2–3 days of incubation at 34°C, plaques were visualized by staining the cells with amido black.

Results

Anti-influenza virus activity of Fr. VI and commercial lignin

As previously reported, Fr. VI, a NaOH extract of pine cone of *Pinus parviflora* Sieb. et Zucc., completely inhibited plaque formation of influenza virus in MDCK cells at doses over 30 μg/ml, as shown in Fig. 1. Since this fraction inhibited the plaque formation of five different influenza viruses (A/PR/8/34, A/WSN/33, A/Urdon/72, A/Victoria/1/75, B/Lee/40) (Nagata et al., 1990), the A/PR/8/34 virus was used in the following experiments. Alkali-lignin, dealkali-lignin, and lignin-sulfonate also inhibited the virus plaque formation, although slightly larger doses of these commercial lignins were required for inhibition equal to that by Fr. VI (Fig. 1). From the titration curves, the 50% effective doses (ED_{50}) of Fr. VI, alkali-lignin, dealkali-lignin, and lignin-sulfonate were determined to be 10, 16, 16, and 59 μg/ml, respectively.

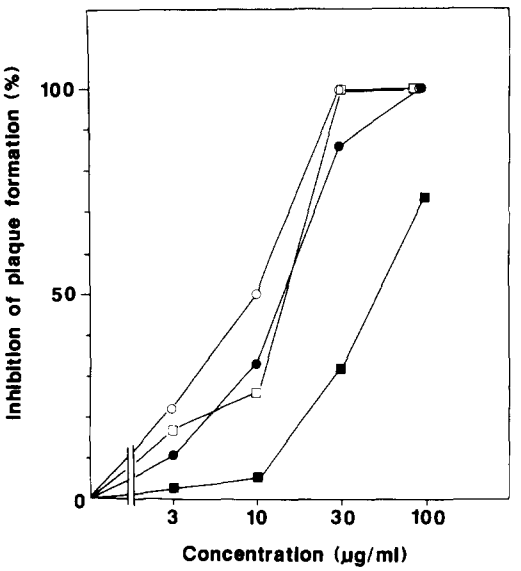

Fig. 1. Inhibition of influenza A virus plaque formation in MDCK cells by lignified materials. Thirty minutes after infection with the influenza virus strain, A/PR/8/34 (H1N1), MDCK cells were incubated with various concentrations of either Fr. VI (○), alkali-lignin (●), dealkali-lignin (□), or lignin sulfonate (■), and plaque formation was assayed as described in Materials and Methods. In the absence of test samples, 141 plaques were formed. Each value is the mean of duplicate assays.

All these preparations including Fr. VI also inhibited RNA synthesis in disrupted virion in vitro (Fig. 2). ED_{50} of Fr. VI was 19 μg/ml and those of alkali-lignin, dealkali-lignin, and lignin-sulfonate were slightly higher, being 25, 39 and 66 μg/ml respectively.

Anti-influenza activity of degradation products of Fr. VI and commercial alkali-lignin

When Fr. VI was treated with $NaClO_2$, the degradation product (Fr. VI ($NaClO_2$)) lost almost all capacity to inhibit either the plaque formation or virus RNA synthesis (Table 1). Alkali-lignin also lost its inhibiting capacity after treatment with $NaClO_2$ (Alkali-lignin ($NaClO_2$)). On the other hand, the hydrolyzed products of Fr. VI and alkali-lignin (Fr. VI (H_2SO_4-ppt) and alkali-lignin (H_2SO_4-ppt)), which were precipitates after treatment with sulfuric acid, displayed as much or more inhibition of the plaque formation and viral RNA synthesis. When trifluoroacetic acid (TFA) was used in place of sulfuric acid, the precipitate (alkali-lignin (TFA-ppt)) showed the same inhibiting capacity as the H_2SO_4-hydrolyzates. The supernatants obtained after the H_2SO_4 or TFA treatment (Fr. VI(H_2SO_4-sup) and alkali-lignin (TFA-sup)) showed little or no activity. Methylation of Fr. VI appeared to reduce its inhibition of RNA polymerase activity, whereas methylation of alkali-lignin abolished its activity (Table 1). These data suggest that the active ingredients of Fr. VI and

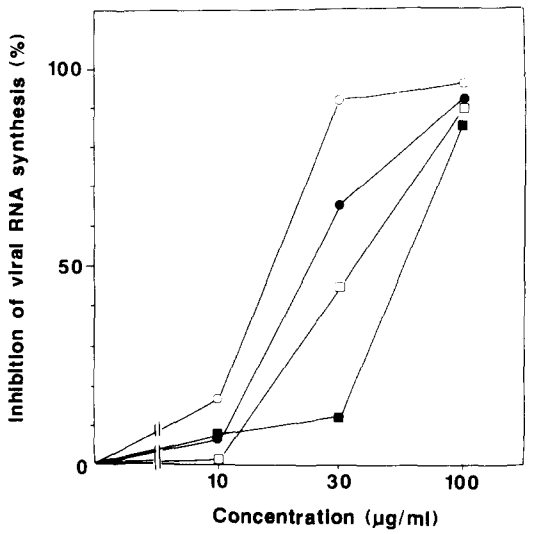

Fig. 2. Inhibition of viral RNA synthesis in disrupted virions by lignified materials. Viral RNA synthesis in vitro was carried out using disrupted virions as described in Materials and Methods in the presence of various concentrations of Fr. VI (○), alkali-lignin (●), dealkali-lignin (□) or lignin sulfonate (■). In the absence of test samples, 18.6 pmol of UMP was incorporated. Each value is the mean of duplicate assays.

TABLE 1

Effect of degradation products of Fr. VI and alkali-lignin on influenza A virus plaque formation and RNA-dependent RNA polymerase activity

Treatment	Plaque[a] formation (% of inhibition)	Viral[b] RNA synthesis (% of inhibition)
Control (None)	–	–
Fr. VI	84.9	93.2
Fr. VI($NaClO_2$)	7.1	0
Fr. VI(H_2SO_4-sup)	4.8	2.1
Fr. VI(H_2SO_4-ppt)	100	92.2
Fr. VI(methylated)	N.D.	58.0
Alkali-lignin	57.1	64.7
Alkali-lignin($NaClO_2$)	0	0
Alkali-lignin(H_2SO_4-ppt)	100	73.3
Alkali-lignin(TFA-sup)	25.0	0
Alkali-lignin(TFA-ppt)	100	84.0
Alkali-lignin(methylated)	N.D.	0

[a]Ninety min after infection with influenza virus strain A/PR/8/34 (H1N1), MDCK cells were incubated without (control) or with 30 μg/ml each test sample, and plaque formation was assayed. The number of plaques in the control cultures was 126.

[b]Viral RNA synthesis in vitro was carried out using disrupted virions without (control) or with 50 μg/ml each test sample. Control RNA synthesis: 3.45 pmol UMP incorporated. N.D.: not determined.

alkali-lignin are both lignin structures, and not hydrolyzable polysaccharide portions attached to or contaminating the lignin skeleton.

Inhibition capacity of other substances

The ultimate degradation products of lignin (vanillin, syringaldehyde) and its component unit (coniferyl alcohol), did not appreciably inhibit plaque formation or viral RNA synthesis (data not shown). Synthetic polyphenolic compounds (Bisphenol A (MW 228), two isomeric dimers (MW 454)), protein-bound antitumor polysaccharide (PSK (50–200 kD)), chemically modified glucans (CM-TAK (MW 97 kD), paramylon sulfate (162 kD)), and hemicellulose obtained from pine cone extract, caused scarcely any inhibition even at 100 μg/ml (data not shown).

Discussion

Anti-influenza virus activity was demonstrated by the pine cone extract Fr. VI and all the commercial lignins examined. Also, both Fr. VI and alkali-lignin were deprived of anti-influenza activity following $NaClO_2$ treatment, which chemically modifies the lignin portion of lignified materials, but does not markedly affect polysaccharide portions such as hemicelluloses. The anti-influenza activity of Fr. VI and the alkali-lignin was either not affected, or was increased in most cases, after the severe acid treatment which is an established analytical procedure to isolate the lignin component from lignified materials (Wise et al., 1946). These results strongly indicate that anti-influenza virus activity, in terms of ability to inhibit plaque formation and virus RNA synthesis, is a characteristic of the lignin structure, regardless of its natural origin. It is unlikely that the hemicellulose portions attached to or contaminating the lignin skeleton might be involved in the anti-influenza activity. Since neither the degradation products nor the component unit of lignin were active, a polymerized structure must be required. A similar structure-activity relationship of intact versus degraded lignified materials was also demonstrated with regard to inhibition of purified poly (ADP-ribose) glycohydrolase activity (Tanuma et al., 1989) and stimulation of the iodination of human peripheral blood polymorphonuclear cells (Sakagami et al., 1990).

We have previously reported that the Fr. VI and commercial lignins inhibit the adsorption of herpes simplex virus (HSV-1, HSV-2) to CV-1 African green monkey kidney cells (Fukuchi et al., 1989). The present study reveals that these substances effectively inhibit plaque formation by influenza virus in MDCK cells, even if they are added 90 min after virus infection (Table 1). Another interesting finding is that the extent of inhibition of plaque formation was correlated to the ability to inhibit viral RNA synthesis in vitro (Figs. 1, 2). It remains to be investigated whether the compounds can be taken up into MDCK cells, and if so, whether they remain intact or are processed to further metabolites.

Lignins, constituting 30% by weight of land plants, have not been studied for their potential utility (Yamafuji and Murakami, 1968; Ikeda et al., 1982) and are regarded as waste from the pulp industry, despite the volume of studies on biological activity found in other natural products.

Anti-HIV activity has been detected in a water-soluble lignin obtained from an

extract of the culture medium of *Lentinus edodes* mycelia (Suzuki et al., 1989), in a lignin sulfonate (Asanaka et al., 1989), and in pine cone extracts (Lai et al., 1990). We also found that the substances obtained from the cones of two other Japanese pine trees (*Pinus densiflora* Sieb. et Zucc., *Pinus thunbergii* Parl.), three Brazilian pine trees (*Pinus elliottii* var. Elliottii, *Pinus taeda* L. and *Pinus caribaea* var. Hondurenses), one Finnish pine tree (*Pinus sylvestris* L.), and from the seed shells of *Pinus parviflora* Sieb. et Zucc., completely inhibit plaque formation by influenza virus at 50 μg/ml (data not shown). These data suggest that the naturally occurring lignin family might have been overlooked as a promising candidate for the treatment of viral diseases.

Many molecular species of lignin are available, depending on the natural sources and methods of extraction. Lignin is, in general, a heterogeneous structure composed of phenylpropane units, and is usually a complex of a lignin skeleton with carbohydrates, uronic acid, or, sometimes, phenolic acids (Kirk and Obst, 1988). No success has been reported for the complete structural determination of high-molecular-weight lignins, due to their heterogeneity as a result of the random free radical coupling of phenylpropenoid precursors (Kirk and Obst, 1988). Further investigation is definitely needed to isolate and identify the principles responsible for their biological activity, including that shown by the Fr. VI and the lignins reported here.

Acknowledgements

We thank Drs. S. Tanuma and M. Nonoyama for valuable advice and Dr. A. Simpson for editing the manuscript. This study was supported in part by a Grant-in-Aid from the Ministry of Education, Science and Culture of Japan, the SHORAI Foundation for Science and Technology, a U.S. Public Health Services Grant U01 AI27280 from the National Institutes of Health, and a Grant for the Biodesign Research Program from RIKEN (K.N.).

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