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Inhibitory Effects of Carcinogenic Mycotoxins on Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase and Ribonuclease H

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Fourteen mycotoxins were tested for inhibitory effects on ribonucleic acid polymerase of rat liver and *Escherichia coli* and nuclear ribonuclease H of rat liver and *Tetrahymena pyriformis*. These enzymes were strongly inhibited by (−)-luteoskyrin, (+)-rugulosin, patulin, and PR toxin.

Mycotoxic problems arising from naturally occurring pollutants in food and feed have been reported by many investigators, and their effects on human and animal health have been reviewed by Ciegler (4) and Enomoto and Saito (5). Although the toxic effects of mycotoxins on animals have been studied from pathological, toxicological, and biochemical standpoints, the basic action mechanisms of most mycotoxins are still poorly understood.

In a previous paper we reported that the cytotoxic trichothecenes from *Fusarium* spp. inhibit protein synthesis of rabbit reticulocytes (20) and that patulin from *Penicillium* and *Aspergillus* spp. inhibits the Na^+-dependent amino acid transport system of rabbit reticulocytes (24). Furthermore, carcinogenic mycotoxins, such as aflatoxins, sterigmatocystins from *Aspergillus* spp., and (−)-luteoskyrin and (+)-rugulosin from *Penicillium* spp. were proved to interfere with deoxyribonucleic acid (DNA) recombination in *Bacillus subtilis* (22). Mutagenicities of aflatoxins, sterigmatocystins, PR toxin, and crotocin were also demonstrated (23).

In an effort to understand the action mechanism of these mycotoxins in a more detailed manner, we investigated their effects on ribonucleic acid (RNA) polymerase and ribonuclease H. In this communication, we report the in vitro inhibitory effects of mycotoxins on RNA polymerase (EC 2.7.7.6) of rat liver nuclei and *Escherichia coli* and on nuclear ribonuclease H (EC 3.1.4.34) of rat liver and *Tetrahymena pyriformis*.

MATERIALS AND METHODS

Chemicals. The following mycotoxins were isolated from culture filtrates or fungal mats of the corresponding fungi according to the methods already reported: (−)-luteoskyrin and chloropeptide (Cl peptide) from *Penicillium islandicum* Sopp (9, 21); (+)-rugulosin from *Penicillium rugulosum* Thom (19); citrinin from *Penicillium citrinum*; patulin from *Penicillium patulum*; penicillic acid from *Penicillium olivino-viride*; fusarenone-X from *Fusarium nivale* (26, 28); zearalenone from *Fusarium roseum* (9). Sterigmatocystin, *O*-acetyl-sterigmatocystin, chaetoglobosin A, and PR toxin were generously supplied by U. Hatsuda (Tottori University), M. Yamazaki (Chiba University), S. Natori (National Institute of Hygiene), and R. Wei (National Yang-Ming Medical College, Taiwan), respectively. All these mycotoxins were homogeneously pure with respect to several analytical criteria such as thin-layer chromatography and infrared and nuclear magnetic resonance spectra. Aflatoxin B_1 and cytochalasin B were products of Markor Chemical Ltd. and Aldrich Co. Inc., respectively. α-Amanitin, actinomycin D, and cycloheximide were purchased from Boehringer Mannheim, and *N*-ethylmaleimide was from Sigma. Rifampin derivatives were kindly supplied by Gruppo Leptit (Milano). All the test compounds were dissolved into dimethyl sulfoxide (DMSO). [4-^{14}C]uridine 5′-triphosphate (UTP) (50 Ci/mol) was purchased from Radiochemical Centre (Amersham, England).

Isolation of rat liver nuclei. Nuclei were isolated essentially by the method of Higashinakagawa et al. (8). The livers of Wistar rats weighing 300 to 350 g were perfused with 0.25 M sucrose–10 mM $MgCl_2$ and homogenized in 10 volumes of 2.3 M sucrose–10 mM $MgCl_2$ with a Potter-Elvehjem homogenizer. The homogenate was filtered through four layers of gauze and centrifuged at 100,000 × *g* for 1 h. The nuclear pellet was suspended in 0.34 M sucrose–1 mM $MgCl_2$ and centrifuged at 800 × *g* for 15 min. The pellet was suspended in buffer [0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.9), 0.25 M sucrose, 5 mM $MgCl_2$, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 0.5 mM dithiothreitol (DTT)] and used for nuclear RNA synthesis.

Enzymes. Rat liver RNA polymerases I and II were prepared from the isolated nuclei by the method of Roeder and Rutter (14). *Escherichia coli* RNA polymerase was isolated by the method of Burgess (2).

The purification procedures of nuclear ribonuclease H of rat liver and *T. pyriformis* were described previously (17, 18).

Assay conditions of RNA synthesis. (i) Nuclear RNA synthesis. A 0.2-μmol amount of adenosine 5'-triphosphate (ATP), 0.05 μmol each of guanosine 5'-triphosphate (GTP) and cytidine 5'-triphosphate (CTP), 0.25 μmol of $MnCl_2$, 0.1 μCi of [^{14}C]UTP, 0.05 μmol of DTT, 12 μmol of $(NH_4)_2SO_4$, and 5 μmol of Tris-hydrochloride (pH 7.9) in a total volume of 50 μl were mixed with 50 μl of the nuclear suspension (containing 50 to 100 μg of DNA), 40 μl of distilled water, and 10 μl of a test compound solution. The mixture was incubated at 37°C for 15 min and chilled in an ice bath. A 100-μl portion was placed on a Whatman GF/C filter. The filters were washed five times with cold 5% trichloroacetic acid containing 1% $Na_4P_2O_7$ and twice with ethanol. The filters were dried under an infrared lamp and counted for radioactivity with a liquid scintillation spectrometer by using toluene scintillator.

(ii) RNA polymerase I. A 0.2-μmol portion of ATP, 0.05 μmol each of GTP and CTP, 0.25 μmol of $MnCl_2$, 5 μmol of Tris-hydrochloride (pH 7.9), 0.05 μmol of DTT, 20 μg of native calf thymus DNA, and 0.1 μCi of [^{14}C]UTP in a total volume of 50 μl were mixed with 50 μl of RNA polymerase I solution, 40 μl of distilled water, and 10 μl of a test compound solution. The mixture was incubated for 30 min at 37°C. The radioactivity of the acid-insoluble fraction was counted as described above.

(iii) RNA polymerase II. The assay for RNA polymerase II was the same as described for RNA polymerase I except that it contained 16.5 μmol of $(NH_4)_2SO_4$ and heat-denatured DNA.

(iv) *E. coli* RNA polymerase. The reaction mixture for *E. coli* RNA polymerase contained, in a final volume of 150 μl, 0.2 μmol of ATP, 0.05 μmol each of GTP and CTP, 0.01 μmol of DTT, 1.5 μmol of $MgCl_2$, 22.4 μmol of KCl, 0.015 μmol of EDTA, 0.1 μCi of [^{14}C]UTP, 6 μmol of Tris-hydrochloride (pH 7.9), 20 μg of native calf thymus DNA, and 50 μl of *E. coli* RNA polymerase solution. The reaction mixture was incubated for 15 min at 37°C, and the radioactivity was counted as described above.

All assays were performed in duplicate. In control assays 10 μl of DMSO was added instead of the test solution. Control experiments revealed that DMSO used as a solvent caused a slight inhibition (around 0 to 6%) of RNA synthesis at a final concentration of 6.7%. Background assays containing enzymes but terminated at time zero were subtracted from experimental values.

Assay conditions of ribonuclease H. [^{14}C]RNA-DNA hybrid was prepared from heat-denatured calf thymus DNA (100°C, 5 min) and rat liver RNA polymerase II (18). The hybrid (2,000 to 2,500 cpm) was incubated with the enzyme in 300 μl of assay mixture containing 15.2 μmol of Tris-hydrochloride (pH 7.9), 0.15 μmol of DTT, and 10 μl of a test solution. The assay mixture was made to contain 7.6 μmol of $(NH_4)_2SO_4$ and 5.2 μmol of $MgCl_2$ for rat liver ribonuclease H (18), and 3 μmol of $(NH_4)_2SO_4$ and 1.5 μmol of $MgCl_2$ for *Tetrahymena* ribonuclease H (17). After incubation for 30 min at 37°C, acid-insoluble material was precipitated by the addition of 200 μl of cold 15% trichloroacetic acid and 100 μl of yeast RNA (2 mg/ml) as co-precipitant. The mixture was centrifuged at 1,600 × *g* for 15 min. The radioactivity in the acid-soluble supernatant fraction was determined in a liquid scintillation spectrometer using Bray's scintillator (3). All assays were performed in duplicate. In control assays 10 μl of DMSO was added instead of the test solution. This much of DMSO (3.3%) inhibited ribonuclease H activity by about 17%.

RESULTS

Inhibitory effects of mycotoxins on DNA-dependent RNA polymerase. Effects of 11 mycotoxins on RNA synthesis were examined, and the results are summarized in Table 1. Among these mycotoxins, seven compounds marked by an asterisk (*) have already been reported to be carcinogenic or co-carcinogenic to animals and positive in the Rec assay (22). (+)-Rugulosin, citrinin, and PR toxin were inhibitory in all four RNA synthetic systems. (−)-Luteoskyrin and patulin inhibited RNA synthesis by isolated nuclei and RNA polymerase I and II.

To investigate the mechanism of the inhibition of transcription by PR toxin, further experiments were performed with the in vitro RNA synthetic system by purified rat liver RNA polymerases I and II in the presence of native or heat-denatured DNA as a template. Both enzymes were inhibited by PR toxin in a dose-dependent manner (Fig. 1). As for RNA polymerase I, which prefers native DNA as template, PR toxin inhibited the transcription directed by either native or denatured DNA to a similar extent. However, as for RNA polymerase II, which prefers denatured DNA as template, the transcription directed by denatured DNA was more markedly inhibited by this mycotoxin than that by native DNA.

To investigate the inhibitory effects of PR toxin on RNA chain initiation and chain elongation, the mycotoxin was added to the reaction mixture at 0 or 3 min. RNA synthesis by RNA polymerase I was inhibited whether the mycotoxin was added before or after initiation of the reaction, whereas RNA synthesis by RNA polymerase II was inhibited only when the mycotoxin was added before the initiation (Fig. 2).

In the case of citrinin, the inhibition of RNA synthesis was dose dependent. No marked difference was observed between the two types of template (Fig. 3).

Bisfuranoid mycotoxin (*O*-acetylsterigmatocystin), macrocycles (cytochalasin B and zearalenone), a cyclic peptide (C1 peptide), and a trichothecene (fusarenone-X) showed no remarkable effect on RNA synthesis by the RNA

TABLE 1. *Inhibition of DNA-dependent RNA polymerases[a] by mycotoxins*

Mycotoxin	Concn (μmol/ml)	Inhibition of RNA synthesis (%)			
		Rat liver			*E. coli*
		Nuclei	I	II	
*(−)-Luteoskyrin	0.146		77	77	0
	0.291	39	89	87	0
	0.583	40	98	96	5
	1.166	46			27
*(+)-Rugulosin	0.131				8
	0.262	0			21
	0.524	2			38
	1.049	21	96	36	58
*O-Acetylsterigmatocystin	0.237	0			0
	0.473	0			0
	0.946	5			0
	1.893	10			0
*Citrinin	0.335	12	0	27	45
	0.669	18	8	26	38
	1.338	30	13	63	50
	2.677	49	67	82	
*Patulin	0.544				14
	1.087				10
	2.174				13
	4.348	61	29	84	0
	8.697	70			
*Penicillic acid	0.984	19			5
	1.968	21			9
	3.936	20	0	31	15
	7.873	29			32
PR toxin	0.262	3	57	34	9
	0.523	5	54	48	33
	1.047	22	61	67	54
	2.094	25	73	77	66
Cytochalasin B	0.175				27
	0.350				38
	0.700				23
	1.400	0	15	17	25
*Cl-peptide	0.139		0	1	0
	0.277		0	0	0
	0.544	0			0
	1.109	4	0	0	10
Fusarenone-X	0.236		1	7	4
	0.472		0	0	6
	0.945		0	6	0
	1.889	12	0	0	3
	3.779	8			
Zearalenone	0.263		0	3	
	0.526		0	0	
	1.052		0	3	

[a] Under the control assay conditions, the incorporations of [^{14}C]UMP into the acid-insoluble fraction by rat liver nuclei, rat liver RNA polymerases I and II, and *E. coli* RNA polymerase were 1,533, 543, 1,143, and 5,374 cpm/assay, respectively. *, Reported to be carcinogenic or co-carcinogenic to animals and positive in the Rec assay (22).

polymerases of animal and bacterial origins as summarized in Table 1.

Effects of inhibitors and mycotoxins on ribonuclease H of rat liver and protozoa. Control experiments with antibiotics and metabolic inhibitors revealed that, as summarized in Table 2, the rifampin derivative AF/013 inhibited the nuclear ribonuclease H of rat liver in

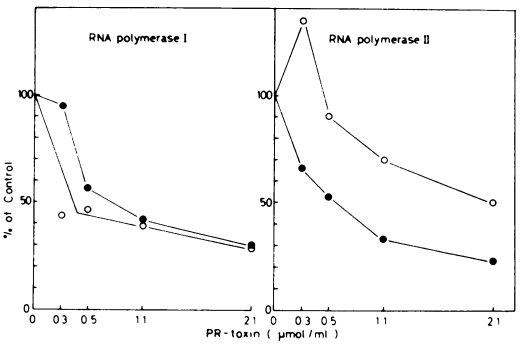

FIG. 1. *Dose response curve of the inhibition of rat liver RNA polymerases I and II by PR toxin. Both enzymes were assayed with native and heat-denatured calf thymus DNA as template. ○, Native; ●, heat-denatured DNA.*

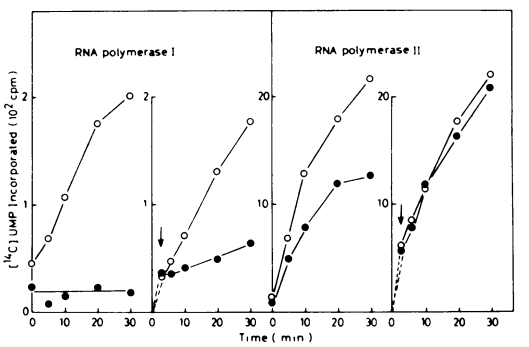

FIG. 2. *Time course of RNA synthesis by RNA polymerases I and II in the presence of PR toxin added before and after the initiation of the reaction. At the times indicated by the arrows (3 min), control assays received 10 μl of DMSO and test assays received 2.1 μmol of PR toxin dissolved in DMSO per ml. ○, Control; ●, addition of PR toxin (2.1 μmol/ml).*

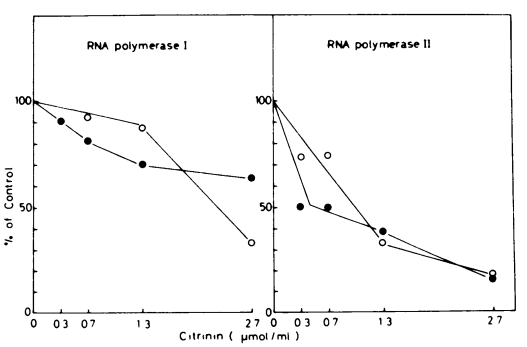

FIG. 3. *Dose response curves of the inhibition of rat liver RNA polymerases I and II by citrinin. Both enzymes were assayed with native and heat-denatured calf thymus DNA as template. ○, Native DNA; ●, heat-denatured DNA.*

TABLE 2. *Inhibitory effects of inhibitors and mycotoxins on ribonuclease H*[a]

Inhibitor or mycotoxin	Concn (μmol/ml)	Inhibition of ribonuclease H (%)			
		Rat liver	*T. pyriformis*		
			H-1	H-2	H-3
Rifampin	0.002		29	0	24
	0.024		26	0	25
	0.030	0			
	0.059	0			
	0.237		22	0	27
AF/013	0.002		7	0	1
	0.012	90			
	0.024	99	88	85	93
	0.235		100	100	100
AF/ABDPcis	0.002	0	18	1	10
	0.021		17	1	10
	0.210		100	98	100
Actinomycin D	0.024	9			
α-Amanitin	0.002	5			
N-Ethylmaleimide	0.005	84			
	0.010	85			
	0.020	90			
Cycloheximide	0.107	0			
(−)-Luteoskyrin	0.174	94			
	0.348		100	98	96
(+)-Rugulosin	0.157	83			
	0.313		100	99	100
Aflatoxin B_1	0.321	9			
	0.641		0	2	29
Sterigmatocystin	0.309	4			
	0.617		57	1	14
Cytochalasin B	0.209	0			
Chaetoglobosin A	0.419		98	98	93
Citrinin	0.400	17			
Patulin	0.324	62			
	0.535		7	27	43
	1.071	47	47	29	54
	2.141		55	37	70
Penicillic acid	0.588	30			
PR toxin	1.175		0	0	0
Fusarenone-X	0.564		0	0	16

[a] Under the control assay conditions, the degradations of [^{14}C]RNA-DNA hybrid into the acid-soluble fraction by rat liver ribonuclease H, *T. pyriformis* ribonuclease H-1, H-2, and H-3 were 1,154, 410, 224 and 436 cpm/assay, respectively.

agreement with the previous report (17). Actinomycin D, α-amanitin, and cycloheximide showed no inhibition. *N*-Ethylmaleimide inhibited the rat enzyme.

Among the 8 mycotoxins tested, patulin, PR toxin, penicillic acid, (+)-rugulosin, and (−)-luteoskyrin inhibited the rat enzyme activity (Table 2).

Ribonuclease H-1, H-2, and H-3 of *T. pyriformis* were also inhibited by (−)-luteoskyrin, (+)-rugulosin, and patulin. In addition, the cytochalasin derivative, chaetoglobosin A, inhibited three enzyme activities. The sensitivities of the three types of protozoan ribonuclease H to the mycotoxins were not so much different from each other.

To clarify the mechanism of inhibition of ribonuclease H by patulin, a further experiment was made by using purified ribonuclease H-3. In this experiment, the enzyme preparation gave rise to 1,155 cpm of acid-soluble ^{14}C-labeled nucleotides from [^{14}C]RNA-DNA hybrid. Concentrations of 1.07 mM and 2.14 mM patulin inhibited the enzyme activity by 75 and 85%, respectively (Fig. 4). These inhibitions were not observed in the presence of DTT. On the other hand, the inhibitory effect of AF/013 on ribonuclease H was not abolished by the addition of DTT.

DISCUSSION

(+)-Rugulosin, an anthraquinoid hepato-carcinogen from *P. rugulosum* Thom (unpublished data), and citrinin, a renal co-carcinogen from *P. citrinum* (16), behaved like actinomycin D, which binds with DNA and inhibits RNA synthesis (6). Luteoskyrin, an anthraquinoid hepato-carcinogen from *P. islandicum* Sopp (30), inhibited selectively the RNA synthesis by isolated rat liver nuclei and by RNA polymerases of rat liver in agreement with our previous finding that this mycotoxin interferes with nuclear RNA synthesis in Ehrlich ascites tumor cells in vivo (27). Our previous experiments have demonstrated that (−)-luteoskyrin binds in vitro with DNA molecules in the presence of magnesium ions, forming a complex (DNA-Mg^{2+}-luteoskyrin) (25). However, the present experiment did not exert any inhibitory effect by this mycotoxin on the RNA polymerase activity of *E. coli*. We would like to conclude that luteoskyrin possesses selective affinity to the polym-

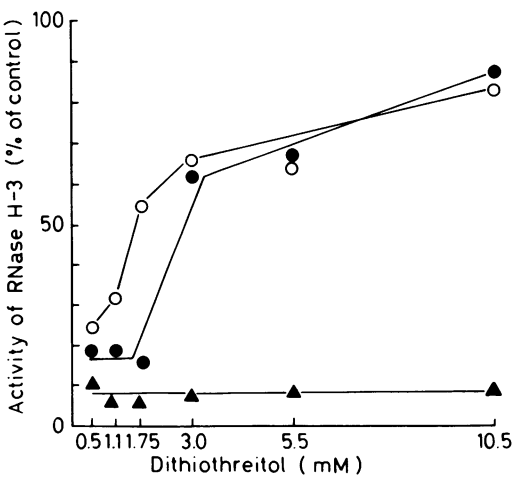

FIG. 4. *Effects of DTT on the inhibition of Tetrahymena ribonuclease H-3 by patulin and AF/013.* ○, *1.07 mM patulin;* ●, *2.14 mM patulin;* ▲, *0.44 mM AF/013.*

erase of animal origin in contrast to the result of Ruet et al. (15).

PR toxin, a mycotoxin synthesized by *Penicillium roqueforti* (31), inhibited the RNA syntheses by both animal and bacterial RNA polymerases (Table 1). An interesting finding is that the initiation, as well as the elongation reaction, of RNA polymerase I of rat liver was impaired by PR toxin, but with RNA polymerase II PR toxin affected preferentially the initiation reaction (Fig. 2). Moulé et al. (12) reported that the initiation and elongation reactions of the RNA polymerases of rat liver and *E. coli* were affected by PR toxin, and this type of inhibition was abolished by the addition of ammonium sulfate. From kinetic analyses, they proposed that PR toxin acts in vitro at the enzyme level and an ammonium-PR toxin complex loses its inhibitory activity. Our previous experiments (13), however, demonstrated that PR toxin reacts with SH compounds such as glutathione and DTT in vitro. Since RNA polymerases are highly sensitive to the SH-blocking agent, PR toxin probably inactivates RNA polymerase through masking SH groups of the active center. The high sensitivity of RNA polymerase II to PR toxin (Fig. 1) may be related to the fact that the enzyme protein forms an initiation complex (DNA-RNA polymerase) at a low ionic strength in the absence of substrate and that the affinity of denatured DNA to the enzyme protein is higher than that of native DNA.

One of synthetic bisfuranoid mycotoxins, *O*-acetylsterigmatocystin, exhibited no significant inhibitory effects on RNA synthesis in vitro. The result was very similar to those of aflatoxin B_1 and sterigmatocystin (data not shown; 1). It is well known that the metabolic activation of the terminal bisfuran structure to an epoxide is required for the binding of bisfuranoids with DNA. Therefore, it may be possible that the in vitro inhibition of RNA synthesis by *O*-acetylsterigmatocystin is observed in the presence of a mixed-function oxygenase system.

Chloropeptide is a potent hepatotoxin of *P. islandicum* and induces tumorous changes in the liver of mice after long-term feeding (30). Although this mycotoxin possesses a cyclic structure similar to that of α-amanitin which specifically inhibits RNA polymerase II at low concentration (32), no inhibitions were observed in our RNA synthetic systems in the presence of the peptide.

Ribonuclease H specifically degrades the RNA moiety of the RNA-DNA hybrid. Interest has been increasing in this enzyme because it might play an important role in DNA replication and transcription (7, 11, 18). Therefore, we investigated the inhibitory effects of 11 mycotoxins on this enzyme activity (Table 2). Rat liver ribonuclease H was inhibited by the anthraquinones [(−)-luteoskyrin and (+)-rugulosin] and the lactones (patulin, penicillic acid, and PR toxin). In this respect, the inhibitors of ribonuclease H and those of RNA polymerase overlapped each other. Chaetoglobosin A, which is a cytochalasin derivative from *Chaetomium globosum* and induces polynucleic changes in cultured mammalian cells (29), exceptionally impaired the ribonuclease H derived from *Tetrahymena*.

As for the mechanism of the inhibition of ribonuclease H by patulin, penicillic acid, and PR toxin, we proposed that they act as an SH blocker, similar to the case of RNA polymerase, since these mycotoxins are highly reactive with SH compounds (13) and the enzyme itself is an SH enzyme. Recovery from the inhibition of patulin by the addition of DTT supports this assumption (Fig. 4). The mechanism of the inhibitory effects of two anthraquinones is not clear at the present time. From the evidence that the enzyme recognizes the DNA moiety of RNA-DNA hybrid in the initial phase of the enzymatic reaction (18), it is highly possible that this recognition step may be interrupted by the anthraquinones.

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