PREFERENTIAL *IN VIVO* INHIBITION OF RIBOSOMAL RIBONUCLEIC ACID SYNTHESIS IN MOUSE LIVER BY THE EXOTOXIN OF *BACILLUS THÜRINGIENSIS*

V.V. MACKEDONSKI, A.A. HADJIOLOV
Biochemical Research Laboratory, Bulgarian Academy of Sciences, Sofia, Bulgaria
and
K. SEBESTA
Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Science, Prague, Czechoslovakia

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1. Introduction

The biosynthesis of rRNA in animal cells appears to be more sensitive to a variety of inhibitors as compared to non-ribosomal RNA species (see [1]). Perry et al. [2] proposed that the higher sensitivity of rRNA synthesis to actinomycin D may be due to the sequential transcription of clustered rRNA genes. The model would explain the preferential inhibition of rRNA synthesis by inhibitors acting on the DNA template. However, a preferential inhibition of rRNA synthesis was observed also with cordycepin [3], causing the termination of growing RNA chains, and even with the non-specific inhibitor, 2, 4-dinitrophenol [4]. Therefore, factors besides the structure of rRNA genes may be also important in conferring a higher sensitivity to rRNA synthesis.

The exotoxin of *Bac. thüringiensis* has been isolated and characterized chemically by Sebesta et al. [5, 6] as a structural analogue of ATP. In the present work it is shown that *in vivo* administration of low doses of *Bac. thüringiensis* exotoxin inhibits more strongly the synthesis of liver rRNA, while the synthesis of nuclear "DNA-like" RNA is less affected. Part of these results has been reported previously [7].

2. Methods

2.1. *Conditions of in vivo labelling*

Experiments were carried out with albino mice of 20–25 g body weight. The highly purified exotoxin was dissolved in saline and injected i.p. simultaneously with 200 μCi of carrier-free ^{32}P-phosphate or 15 μCi of 6-^{14}C-orotic acid (specific activity 15.1 mCi per mmole). In another set of experiments the exotoxin was given 30 min before the administration of 6-^{14}C-orotic acid.

2.2. *Isolation and fractionation of nuclear RNAs*

A 10% liver homogenate in 0.14 M NaCl was mixed immediately with an equal volume of cold phenol saturated with 0.14 M NaCl pH 6.0 containing 0.1% 8-hydroxyquinoline. The mixture was shaken for 15 min at 4° and centrifuged at 5000 rpm in the cold. The water layer, containing the "cytoplasmic RNA" fraction was aspirated and discarded. The nuclear RNAs, entrapped in the interphase layer, were extracted by hot phenol treatment at different temperatures according to Georgiev [8]. The fraction of nuclear "rRNA" was extracted at 45°, while the fraction of nuclear "DNA-like RNA" was extracted from the residual interphase layer at 85°.

2.3. *Purification of RNA preparations*

The nuclear RNA fractions were deproteinized 3–5 times with cold phenol and freed from ^{32}P-phosphate and other contaminants by passage through a column of Dowex-1, formate form. The nuclear "DNA-like RNA" was freed from DNA by treatment with deoxyribonuclease I (Worthington) at a concentration of 10 μg per ml for 30 min at 25°. After hydrolysis the mixture was deproteinized 3 times with cold phenol and RNA precipitated with 3 vol of 96%

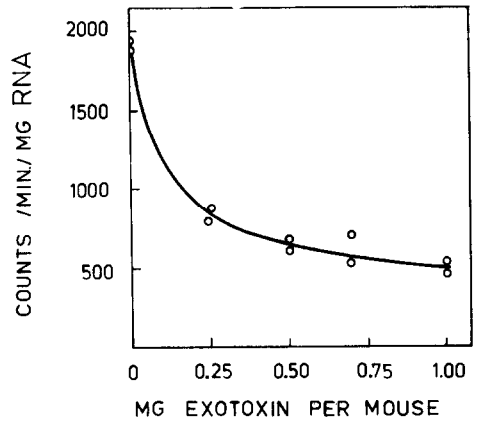

Fig. 1. Inhibition of the *in vivo* ^{32}P-phosphate incorporation into total high molecular weight RNA of mouse liver by the *Bac. thüringiensis* exotoxin. The animals were injected i. p. simultaneously with 200 μCi per mouse of carrier-free ^{32}P-phosphate and varying amounts of *Bac. thüringiensis* exotoxin for 90 min.

ethanol containing 1% potassium acetate.

2.4. *Agar gel electrophoresis of RNA*

This was carried out according to the technique of Tsanev and Staynov [9] under conditions specified in a previous paper [4]. In some experiments the dried agar gel film was sectioned at 2 mm and each section counted in a Packard "Tri-Carb" scintillation spectrometer with a toluene–PPO–POPOP phosphor.

2.5. *Nucleotide composition of RNA*

The nucleotide composition of total and ^{32}P-labelled RNA was determined by Katz and Comb [10] as specified previously [4].

3. Results

3.1. *Inhibition of in vivo nuclear RNA synthesis*

In a first series of experiments the effect of increasing doses of exotoxin on the labelling of total mouse liver RNA was followed. The results (fig. 1) show, in agreement with previous studies [11] that the exotoxin of *Bac. thüringiensis* inhibits the *in vivo* synthesis of liver RNA. The inhibitory effect is dose dependent. About 70% inhibition of ^{32}P-phosphate incorporation is attained by 0.5 mg exotoxin per mouse. However, the inhibition reaches a saturation level at doses of about 1 mg per mouse, thus indicating the existence in nuclei of some exotoxin resistant RNA synthesis.

3.2. *Inhibition of nuclear "ribosomal" and "DNA-like" RNA synthesis*

The rate of labelling of nuclear "DNA-like" RNA in control mice is 5–10-fold higher than that of nuclear rRNA. Following exotoxin treatment the labelling of nuclear rRNA is inhibited by 90–95%. On the other hand, with the same doses of exotoxin, a rather high labelling of nuclear "DNA-like" RNA is

Table 1
Inhibition of the incorporation of 6-^{14}C-orotic acid into nuclear "ribosomal" and "DNA-like" RNA fractions of mouse liver by the exotoxin of *Bac. thüringiensis.*

RNA fraction	Labelling time (min)	Exotoxin* (mg/mouse)	Specific activity of RNA (counts/min per A_{260} unit of RNA)		Residual incorporation (%)
			Control	Exotoxin treated	
"Ribosomal"	60	0.5	1,490	110	7.4
	120	0.15	4,753	578	12.0
"DNA-like"	60	0.5	17,592	4,354	24.8
	120	0.15	25,427	13,376	52.6

* The exotoxin was given intraperitoneally 30 min before the labelled precursor.

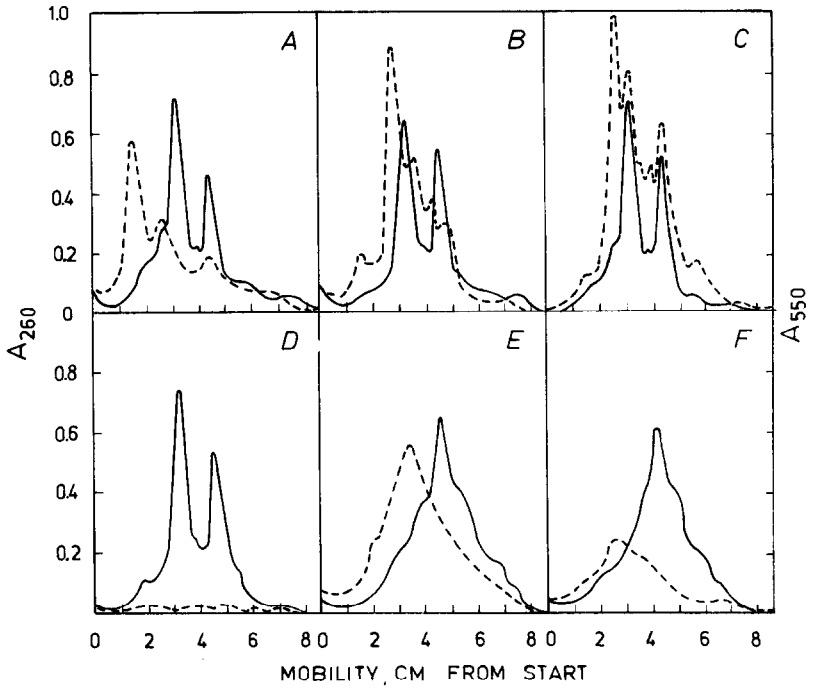

Fig. 2. Agar gel electrophoresis of nuclear "ribosomal" (A, B, C and D) and "DNA-like" (E and F) RNA of mouse liver after 6-^{14}C-orotic acid labelling *in vivo* for 20 min (A), 60 min (B) and 120 min (C, D, E and F). The agar gel electrophoresis profiles of RNAs from control (A, B, C and E) and pretreated for 30 min with the *Bac. thuringiensis* exotoxin (D and F) mice are presented. (———): Absorbancy recorded at 260 nm. (--------): Radioactivity recorded at 550 nm.

found, which amounts to about 25–50% of the labelling in control mice (table 1).

3.3. *Nucleotide composition of nuclear RNA fractions*

The GC/AU ratio of 1.60 obtained for the nuclear "rRNA" fraction is identical to that of cytoplasmic rRNA [1]. On the other hand, the GC/AU ratio of 0.88 recorded for the nuclear "DNA-like" RNA fraction reveals that when extracted under strictly specified conditions this fraction is clearly non-ribosomal by nucleotide composition. The composition of both labelled nuclear RNA fractions was identical to that of the total RNA in each fraction. Inhibition of RNA synthesis by the *Bac. thüringiensis* exotoxin does not cause any significant change in the nucleotide composition of either the total or the rapidly labelled components of the 2 nuclear RNA fractions studied.

3.4. *Agar gel electrophoresis of nuclear RNA fractions*

Information on the distribution of the label among different RNA species in the 2 nuclear fractions studied was obtained by agar gel electrophoresis (fig. 2). The nuclear "rRNA" fraction yields an electrophoretic profile with clearly delineated 28 S and 18 S RNA peaks [12]. The radioactivity profile of nuclear "rRNA" from control mice shows that the label is located mainly in fractions moving more slowly than 28 S RNA. At 20 min labelling, the radioactivity is mainly in the 45 S rRNA precursor. At longer times of labelling it is shifted to the 32 S and 18 S rRNA fractions, as well as to some minor fractions in the region between 28 S and 18 S RNA. Exotoxin pretreatment of mice abolished almost completely the labelling of the rRNA precursors, as well as the RNA fractions in the zone of the 2 rRNA peaks.

The UV-profile of the nuclear "DNA-like" RNA fraction shows a heterogeneous distribution with a clearly delimited peak in the zone of 18 S RNA [12]. Exotoxin administration does not change the recorded UV-profile. The distribution of the rapidly labelled

components is heterogeneous, the labelled species moving more slowly than the bulk of the UV-absorbing material. The labelled RNA species in exotoxin treated mice show an identical distribution, which indicates that the exotoxin does not inhibit any particular species of nuclear "DNA-like" RNA. Similar results were obtained in experiments where the exotoxin was administered simultaneously with the labelled precursor, 6-^{14}C-orotate or ^{32}P-phosphate.

Discussion

The presented results clearly show that *in vivo* the exotoxin of *Bac. thüringiensis* inhibits preferentially the synthesis of rRNA. On the other hand, *in vitro* experiments revealed that low doses of exotoxin inhibit much more strongly liver nucleoplasmic RNA polymerase and that it affects the enzyme molecule rather than the DNA template [13]. In this respect the exotoxin resembles α-amanitin, a highly selective inhibitor of liver nucleoplasmic RNA polymerase (see [14]). Yet, when given *in vivo* both α-amanitin [14, 15] and the *Bac. thüringiensis* exotoxin inhibit preferentially the synthesis of ribosomal RNA. The reason for the observed differences in the *in vivo* and *in vitro* action of these RNA polymerase inhibitors is not clear, but it is likely that both compounds might be useful in elucidating the higher sensitivity of rRNA synthesis in animal cells.

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