KOR. JOUR. MICROBIOL. March, 1991 p. 34-39 Vol. 29, No. 1

cis-Diamminedichloroplatinum (II) (CDDP) Inhibits Bluetongue Virus (BTV) Core Associated Transcriptase Activity

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CDDP를 처리한 Bluetongue Virus Core의 전사저해

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ABSTRACT: The BTV core associated transcriptase activity, assayed by acid precipitable counts, was reduced to an undetectable level after treat the core with 100 μM CDDP. When the RNA transcripts prepared from the CDDP treated BTV core were analysed on agarose-urea gel, it was observed that the band intensity of the large size RNA was reduced while the band intensity of the small size RNA was enhanced. Northern blot analysis showed that much of the small size RNAs appeared to be prematurely terminated transcripts. These results suggest that CDDP adduction to the template RNA blocks chain elongation process of the virion bound transcriptase that is ultimately responsible for the inactivation of BTV infectivity.

KEY WORDS□CDDP, Bluetongue Virus (BTV), Transcriptase, Northern blot

Bluetongue is a disease of domestic and wild ruminants caused by bluetongue virus (BTV), a member of family Reoviridae. The capsid of BTV is composed of four major and three minor polypeptides, which contains ten genomic segments of dsRNA size ranged from 0.5×10^6 to 2.8×10^6 daltons (Verwoerd *et al.*, 1972). The ten segments of RNA are roughly distributed into three groups namely, large (segment 1, 2, and 3), medium (segment 4, 5 and 6), and small (segment 7, 8, 9 and 10), based on their size (Verwoerd and Huismans, 1972; Van Dijk and Huismans, 1980). Treatment of the virion with chymotrypsin in the presence of $MgCl_2$ removes capsid proteins P_2 and P_5. The resultant core exhibits RNA dependent RNA polymerase (transcriptase) activity *in vitro* (Van Dijk and Huismans, 1980).

Although CDDP has been established as a potent antitumor chemotherapeutic agent, its molecular mode of action is not clearly elucidated yet (Roberts and Pera, 1983). It has been shown that CDDP interacts with biomacromolecules especially with DNA and to the lesser extent with protein (Lippard and Hoeschele, 1979). It has been proposed that CDDP adduction to DNA, most likely by intrastrand crosslinks and mono-adducts (Girault *et al.*, 1982 and Marcellis *et al.*, 1982) is responsible for the observed inhibition of DNA replication for the effectiveness as an antitumor activity (Pinto and Lippard 1985).

We have found that relatively impure preparations of BTV infectivity was reduced to an undetectable level after treat the virus with 100 μM CDDP and that CDDP preferentially binds to guanine residues of genomic dsRNA generating local denaturation (manuscripts in preparation). In addition, local perturbation of RNA conformation resulted in the formation of compact and kinked structure.

Transcriptase activity of CDDP-treated BTV

core was analyzed in order to study if transcription inhibition was a likely cause for BTV inactivation by CDDP. The transcriptase activity of BTV core was reduced to an undetectable level after treat the core with 100 μM of CDDP. It was suggested that CDDP adduction to the template RNA blocks chain elongation of the virion bound transcriptase that is ultimately responsible for the inactivation of BTV infectivity.

MATERIALS AND METHODS

Virus and cell

BHK 21 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (GIBCO). BTV grown in BHK 21 cells was harvested after 24 to 48 hr postinfection. The cells were pelleted, resuspended in 2 mM Tris-HCl buffer (pH 8.8), sonicated three times, and centrifuged at 11,000×g for 10 min. To the supernatant an equal volume of 2X lysis buffer (1% Triton X-100, 200 mM KCl, 20 mM $MgCl_2$, 7.2 mM $CaCl_2$, 2 mM Tris-HCl, pH 8.8) was added, incubated for 15 min at 37℃, and centrifuged at 11,000×g for 10 min. The supernatant was layered on top of a 40% sucrose cushion and centrifuged in a Beckman SW 27 rotor at 24,000 rpm for 2 hr. The pellet was resuspended in 2 mM Tris-HCl buffer (pH 8.8), loaded on top of preformed 10 to 40% sucrose gradient, and centrifuged in a Beckman SW 41 rotor at 24,000 rpm for 70 min. The light scattering band was collected, diluted in 2 mM Tris-HCl buffer (pH 8.8), pelleted in a Beckman SW 41 rotor at 24,000 rpm for 100 min, and resuspended with 2 mM Tris-HCl buffer (pH 8.8).

Preparation of BTV core

BTV at a concentration of 210 μg/m*l* was treated with 100 mM Tris-HCl (pH 8.0), 600 mM $MgCl_2$, and 32 μg/m*l* of chymotrypsin. The mixture was incubated for 1 hr at 37℃, diluted 1:3 with 2 mM Tris-HCl (pH 8.8), and pelleted through 40% sucrose cushion in a Beckman 50.1 rotor at 40,000 rpm for 45 min. The core was resuspended in 2 mM Tris-HCl (pH 8.8) buffer at a concentration of 4 μg/μ*l* and stored in 4℃.

CDDP reaction

Purified BTV or BTV core was diluted 1:4 with 20 mM KH_2PO_4 (pH 7.2). An equal volume of CDDP of twice the final concentration prepared in 20 mM KH_2PO_4 (pH 7.2), was added. The reaction mixture was incubated in the dark for 1 hr at 37℃, diluted 1:3 with 2 mM Tris-HCl (pH 8.8), and pelleted through 40% sucrose cushion in a Beckman SW 50.1 rotor at 40 K rpm for 45 min. The supernant was carefully removed and the pellet was resuspended in 2 mM Tris-HCl (pH 8.8).

Transcriptase assay

The transcription reaction was done as described by Van Dijk and Huismans (1980) with a minor modification. Briefly, the reaction mixture contained 100 mM Tris-HCl, pH 8.0, 1.7 mM each of GTP, CTP, and ATP, 20 μCi of [α-^{32}P] UTP (specific activity 410 μCi/mmole, Amersham), 6 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mg/m*l* pyruvate kinase, 2 mM dithiothreitol (DTT), 0.25 mM S-adenosyl-L-Methionine (SAM), 7.5 mM phosphoenol pyruvate (PEP), 100 units of RNasin (Promega Biotech), and 20 μg of BTV core. After 5 hr of incubation at 28℃, 20 μ*l* aliquot of each sample was diluted with 5 m*l* of 5% TCA in 10 mM sodium pyrophosphate and held for 30 min in the ice water bath. The precipitated RNA was collected on Whatman GF/C filters washed 5 times with 5 m*l* of ice cold 5% TCA containing 10 mM sodium pyrophosphate once with 5 m*l* of 95% ethanol, dried, and counted in a Beckman liquid scintillation counter.

RNA purification

The unincorporated [α-^{32}P]-UTP was removed by spin dialysis (Maniatis *et al.*, 1982) and the soluble RNA transcripts were separated from viral cores by centrifuging the reaction mixture in a Beckman SW 50.1 rotor at 40,000 rpm for 45 min. The supernatant was phenol extracted, washed with chloroform, ethanol precipitated, pelleted, vacuum dried, and resuspended in 0.1 X TE (1 mM Tris, 0.1 mM EDTA).

Electrophoresis

Purified RNA transcripts released from the viral core were separated by electrophoresis in a slab gel containing 1.5% agarose, 7 M urea, 36 mM Tris-HCl (pH 7.8), 30 mM NaH_2PO_4, and 1 mM EDTA. Prior to electrophoresis, the gel was solidified by setting in the cold room overnight. The gel was run for 16 hr at a constant voltage of 50 V, rinsed with deionized water to remove urea, dried, and exposed to Kodak XR-5 film for autoradiography. BTV genomic RNA was run in 1% agarose gel prepared in TEA buffer (40 mM Tris-Acetate, 2 mM EDTA, pH 8.0) at 1.5 V/cm for 16 h.

Polyacrylamide gel electrophoresis

BTV was disrupted by boiling for 3 min in electrophoresis sample buffer (60 mM Tris-HCl, pH 6.8, 4% SDS, 40% glycerol, 3% dithiothreitol, 0.005 % bromophenol blue). Samples were applied to 10% acrylamide gel (acrylamide/bisacrylamide weight ratio, 37.5:1) and subjected to electrophoresis as described by Laemmli (1970).

Northern blot analysis

BTV genomic RNA, separated in 1.0% agarose gel, was denatured in 0.4 N NaOH for 15 min, neutralized in 12 mM Tris (pH 7.5), 6 mM sodium acetate, and 0.3 mM EDTA for 30 min, and transferred to nylon membrane (New England Nuclear) by electroblotting. The membrane was prehybridized in 10 m*l* of 50% formamide, 1% SDS, 1 M sodium chloride, and 10% dextran sulfate overnight at 42℃. ^{32}P labeled RNA transcripts and denatured salmon sperm DNA (150 μg/m*l*) were added into the bag. The bag was resealed and incubated overnight at 42℃. The membrane was washed twice with 100 m*l* of 2X SSC (1X SSC: 150 mM NaCl-15 mM sodium citrate) at room temperature for 5 min, twice with 200 m*l* of 2X SSC and 1% SDS at 65℃ for 30 min, twice with 100 m*l* of 0.1 X SSC at room temperature for 30 min, dried, and exposed to Kodak XR-5 film for autoradiography.

RESULTS

Chymotrypsin digestion of BTV.

Purified BTV treated with CDDP was digested with chymotrypsin and run in 10% SDS polyacrylamide gel electrophoresis. CDDP treatment of BTV prior to digestion with chymotrypsin did not affect the removal of the two major polypeptides (P_2 and P_5) from the viral capsid (Fig. 1, lanes 3 and 4). Similar band patterns were also obtained with BTV core treated with 100 μM CDDP after chymotrypsin digestion (Fig. 1, lane 5). The absence of new protein bands with higher molecular weight confirms our previous observation that concentrations of up to 500 μM of CDDP did not generate protein-protein crosslink. The fact that BTV infectivity was completely lost after treat the virus with 100 μM CDDP strongly indicates that any CDDP adduction to protein most likely plays no role for the viral inactivation.

***In vitro* transcription of CDDP treated BTV core.**

BTV cores treated with different concentrations of CDDP were purified and the transcriptase activity associated with the core was assayed by acid precipitable counts (Table 1). A reduction of total TCA precipitable counts by approximately 50% was observed with the BTV core treated with 2.5 μM CDDP. The transcriptase activity was completely lost after treat the BTV core with 100 μM CDDP. These results are parallel to the inactivation experiments indicating that inhibition of the transcriptase activity is the major cause for the inactivation of BTV by CDDP. Psoralen inactivated reovirus core also showed reduced

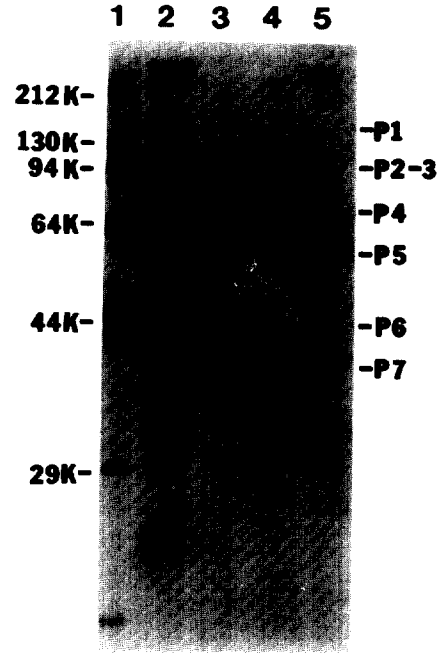

Fig. 1. *10% SDS polyacylamide gel electrophoresis of BTV core.*
BTV 10 (40 μg) was incubated with 8 μg of chymotrypsin in a reaction mixture containing 100 mM Tris-HCl (pH 8.0) and 600 mM $MgCl_2$ at 37℃. After 1 hr of digestion, the reaction mixture was diluted 1:3 with 2 mM Tris-HCl (pH 8.8) and pelleted through 40% sucrose cushion in a Beckman SW 50.1 rotor for 40 min at 40 K rpm. The pellet was dissolved in Laemmli sample buffer and run in 10% SDS PAGE. (1) molecular weight standard marker; (2) BTV 10; (3) BTV 10 digested with chymotrypsin; (4) BTV 10 treated with 100 μM CDDP and digested with chymotrypsin; (5) BTV 10 digested with chymotrypsin and treated with 100 μM CDDP.

Table 1. *BTV core associated RNA transcriptase activity assayed by acid precipitable counts.*

CDDP (μM)	Acid precipitable counts (cpm)	Transcriptase activity (%)
0	7638	100
2.5	3993	55
10	1829	23.9
25	633	8.3
100	39	0.5

transcriptase activity *in vitro* (Nakashima *et al.*, 1979).

Agarose-urea gel electrophoresis of the RNA transcripts

The RNA transcripts produced from CDDP-treated and untreated BTV cores were run in 1.5% agarose gel containing 7 M urea. No detectable transcripts were released from BTV cores treated with 25 μM and 100 μM CDDP (Fig. 2, lanes 4 and 5). Several transcripts were produced by the BTV cores treated with 2.5 μM and 10 μM CDDP (Fig. 2 lanes 2 and 3) but abundance was reduced compared to the untreated BTV core (Fig. 2, lane 1). Large size species of full length RNAs were disappeared while small size transcripts were still visible. The absence of large size species of full length transcripts indicated that CDDP treated core might produce prematurely terminated transcripts comparative to untreated core which produces full length transcripts as well as prematurely terminated transcripts.

Northern blot hybridization

To investigate if CDDP treated BTV core produces prematurely terminated transcripts, the purified RNA transcripts were hybridized back to the denatured BTV genomic RNA. As shown in Fig. 3 both RNA transcripts produced from cores either untreated (lane 1) or treated with 10 μM CDDP (lane 2) were hybridized back to all 10 segments although full length species of large size transcripts produced from CDDP-treated core were absent in agarose-urea gel (Fig. 2, lanes 2 and 3). This result also indicated that CDDP-treated BTV core produces prematurely terminated transcripts that could be hybridized back to the genomic RNA.

DISCUSSION

CDDP treatment of BTV prior to or after chymotrypsin digestion did not alter the

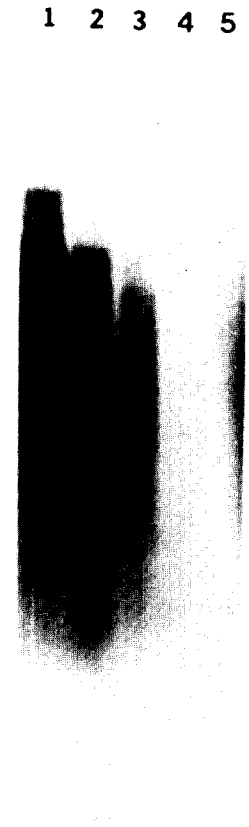

Fig. 2. *1.5% agarose-7 M urea gel electrophoresis of the RNA transcripts produced from BTV core.* The transcription reaction was done as described in Materials and Methods. After 5 hr of incubation at 28℃, RNAs were purified and run in slab gel containing 1.5% agarose, 7 M urea, 36 mM Tris-HCl (pH 7.8), 30 mM NaH_2PO_4, and 1 mM EDTA, at 40 constant voltage for 18 hr. The gel was washed with distilled water to remove urea, dried, and exposed to Kodak XR-5 film. (1) control; (2) 2.5 μM CDDP; (3) 10 μM CDDP; (4) 25 μM CDDP; (5) 100 μM CDDP.

polypeptide band pattern in SDS-PAGE. This result supports our previous observation that CDDP did not generate protein-protein crosslinks. The CDDP-treated BTV core showed remarkable reduction in transcriptase activity. Approximately 50% of transcriptase activity was reduced after treat the core with 2.5 μM CDDP. This result is comparative to our earlier study of BTV inactivation by CDDP. After treat the virus with 2.5 μM

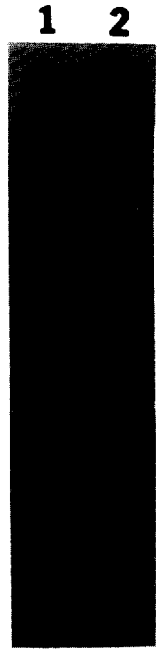

Fig. 3. *Northen blot analysis of the RNA transcripts produced from BTV core.*
Genomic dsRNA separated in 1% agarose gel, was denatured and hybridized with ^{32}P labeled RNA transcripts produced from BTV core (lane 1) and the RNA transcripts produced from BTV core treated with 10 μM CDDP (lane 2).

of CDDP, several orders of BTV infectivity was lost.

Partially inactivated reovirus core by psoralen also showed reduced transcriptase activity (Nakashima *et al.*, 1979). Full length species of all three kinds (large, medium, and small) of mRNA were synthesized from reovirus cores that retained only 5% of the transcriptase activity. This observed all or none phenomena suggests that psoralen adduction to the template RNA may have an effect on initiation step instead of elongation step.

BTV transcriptase and template RNA complex may not as stable as that of reovirus during elongation process which result in the production of prematurely terminated transcripts. The conformational change on template RNA by CDDP adduction may either physically block the process of transcriptase or unstablizes the transcriptase and template RNA complex so that the transcriptase fall off from the template leaving nascent RNA transcripts behind.

The early disappearance of full length species of large size RNA band could be explained by the larger target size RNAs compared to the medium and small size RNAs. Because of the larger target size, the probability of producing prematurely terminated RNA transcripts are greater than that of medium and small size RNA.

Reduction of BTV transcriptase activity by CDDP is probably derived from template RNA modification and not from protein modification because protein-protein crosslink was not formed after treat the virus with CDDP at high concentration (500 μM). This is confirmed by the finding that *E. coli* RNA polymerase exposed to CDDP for 48 hours was completely active in RNA synthesis *in vitro* while the enzyme activity is inhibited by the binding of CDDP to template DNA (Srivaster *et al.*, 1978). It appears that modified RNA template by CDDP blocks chain elongation of the core associated transcriptase that is ultimately responsible for the inactivation of BTV. However, it is still possible, although unlikely, that CDDP directly abolishes the catalytic activities of the transcriptase.

적 요

cis-Diamminedichloroplatinum(II)(CDDP) 처리한 Bluetongue virus core의 전사활성도를 TCA 침전법으로 정량 분석하였다. BTV core를 100 μM CDDP로 처리하였을 때 전사활성도는 검출되지 않을 정도로 낮아졌으며 합성된 RNA 전사물들을 agarose-urea gel로 전기영동한 결과 크기가 큰 RNA의 전사저해는 작은 RNA보다 더 낮은 CDDP 농도에서 일어남을 알았다. CDDP를 처리한 BTV core에서 합성된 RNA를 탐침으로 사용한 Northern blot 실험결과는 CDDP에 의해 전사의 조기종료가 일어남을 제시한다.

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(Received March 11, 1991)
(Accepted March 18, 1991)