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Identification of RNA Species in the RNA–Toxin Complex and Structure of the Complex in *Clostridium botulinum* Type E

Masaru Kitamura[1]

Laboratory of Bacterial Toxins, Department of Bacteriology, National Institute of Infectious Diseases (formerly National Institute of Health), 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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***Clostridium botulinum* type E toxin was isolated in the form of a complex with RNA(s) from bacterial cells. Characterization of the complexed RNA remains to be elucidated. The RNA is identified here as ribosomal RNA (rRNA) having 23S and 16S components. The RNA–toxin complexes were found to be made up of three types with different molecular sizes. The three types of RNA–toxin complex are toxin bound to both the 23S and 16S rRNA, toxin bound to the 16S rRNA and a small amount of 23S rRNA, and toxin bound only to the 16S rRNA.** © 2002 Elsevier Science (USA)

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When *Clostridium botulinum* type E was cultured at pH 6.3, the toxin was retained in the bacterial cell (1). The toxin could be extracted with 0.2 M phosphate buffer (pH 6.0) from the bacterial cells (2, 3). The extracted toxin was observed to form complexes with ribonucleic acid (RNA) (2). The RNA–toxin complexes (15–19S) (4, 5) consisted of three molecule components: the neurotoxin (15-kDa, 7.3S), nontoxic protein (15-kDa, 7.3S) and ribonucleic acid (RNA) (4, 6, 7). The RNA was shown to bind only to the neurotoxin and the molecular size of RNA binding with the neurotoxin was estimated as 12–13S (4). The bound RNA was not related to toxic activity (2). The purification of *C. botulinum* type E toxin was performed from the RNA–toxin complexes after digestion by ribonuclease (EC 3.4.4.4) treatment (3). We successfully purified and characterized RNA-free *C. botulinum* type E toxin in 1968 (3). The purified toxin (35-kDa, 12S), consisting of neurotoxin and nontoxic protein (6, 7) was dissociated into two 7S subunits when pH was raised above pH 7.2 (4, 6). When type E neurotoxin was treated with trypsin, toxic potency was increased 200–400 times (2, 3, 8). While Type E neurotoxin is a single polypeptide chain and was nicked by trypsin at the same position as other toxin types (A–G), between the L chain (50-kDa) and H chain (100-kDa) (9). The RNA–toxin complexes are found as several types of differing molecular size (4, 5). It has been suggested that RNA–toxin complexes may not be an artifact of the process of toxin preparation (4). The RNA–toxin complexes of *C. botulinum* type E seemed to be unique because this type of RNA–toxin complex was not found in botulinum type A or in the tetanus toxins prepared by the same extraction procedures. Questions remaining to be answered are: what is the function of the RNA, if any, and what is the type of the RNA. The present work specifically answers the second question.

MATERIALS AND METHODS

Preparations of the RNA–toxin complexes. Clostridium botulinum type E 35396 strain was cultured in 2% peptone, 0.5% yeast extract, 1% glucose and 0.05%, sodium thioglycolate media (2000 ml) at pH 6.4 for 4 days at 30°C (3) according to the previous method (3). The RNA–toxin complexes were extracted from the cells (wet wt: 1.82 g) in 0.2 M phosphate buffer, pH 6.0, at 37°C for 90 min according to the previous method (3). The extracted RNA–toxin complexes were concentrated to 50% in volume using ammonium sulfate and were run through a CM-Sephadex column at pH 6.0 to remove a large amount of contaminating proteins (3) and then the percolated fraction containing the RNA–toxin complex containing 64 mg of protein (named RNA–toxin fraction) were used as the starting material in the following experiments.

Purification of the RNA–toxin. Preparation of the RNA–toxin fraction by sucrose-density gradient ultracentrifugation of the RNA–toxin fraction is described in the legend to Fig. 1. The isolated RNA–toxin complexes were then called RNA–toxin.

Toxicity of toxin. Determination of toxicity of the toxins after activation by trypsin treatment was performed by the mouse intravenous injection method as previously reported (10).

Electrophoresis of the RNA. Electrophoresis of the RNA–toxin complex was performed in a 3% agarose gel (No. A-9539, Sigma Chemical Co., St. Louis, MO) in Tris-phosphate buffer, pH 8.0, at

[1] Address correspondence and reprint requests to author. Fax: 81-03-5285-1159. E-mail: kmasaru@nih.go.jp.

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4°C. After electrophoresis, the RNA position in the agarose gel was detected by visualizing ethylene blue dye under UV light.

Preparation of anti-toxin IgG. Anti-serum against type E neurotoxin purified by the method described previously (3) was produced in rabbits. The anti-toxin IgG in the rabbit's serum was purified by protein G affinity column (HiTrap affinity columns, Pharmacia Biotech, Uppsala, Sweden). The anti-toxin IgG formed a single immunoprecipitation line against the crude RNA-complexed toxins of type E in an agar-gel-diffusion test (between well 2 and well 4 in Fig. 3). The purified anti-toxin IgG was used in immuno-blot tests for detection of toxin on electrophoresis gels and for isolation of RNA–toxin complexes by immunocomplex precipitation. Anti-type E toxin prepared in horse with the cell extract of *C. botulinum* type E was purchased from Chiba Serum Institute.

Immuno-blot test. Immuno-blot detection tests of the RNA–toxin complexes were performed using the KPL Western blot kit as directed in the guidelines for the kit (KPL, MD).

Identification of RNA. The RNA in the RNA–toxin complexes was identified by hybridization methods with riboprobes containing 23S and 16S mixed rRNAs or the transfer RNA (tRNA) of *Escherichia coli* (Boehringer-Mannheim, Mannheim, Germany) used as an RNA standard probe. The RNA from RNA–toxin complexes was isolated by phenol extraction followed by precipitation in methanol–chloroform. Phosphorylation of the isolated RNA of RNA–toxin with radioactive phosphate [^{32}P], after dephosphorylation, was performed using the MEGALABEL kit (Takara, Ohotu, Japan). The preparation of DNA of *C. botulinum* type E was performed using the method of Franciosa *et al.* (11). The DNA of *C. botulinum* type E was digested by the restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, or *Pst*I (Takara) and then separated by 0.8% agarose gel electrophoresis. After electrophoresis, the digested DNAs were transferred to nylon membrane for subsequent hybridization with the RNA isolate. Autoradiographs were made after the DNA–RNA hybridization (Northern blot).

RESULTS AND DISCUSSION

The RNA–toxin fraction was centrifuged in a sucrose density gradient at pH 6.0 (Fig. 1). Toxicity was detected in broad fractions (fraction Nos. 10~35) with at least three peaks (Fig. 1A). When each collected fraction was run in an agar gel electrophoresis, RNA was detected essentially in the same fractions which exhibited higher toxicity (Fig. 1B1). The immuno-blot test also identified the toxin to be in the same fractions (Nos. 10~35) where the RNA was found (Fig. 1B2). When the RNA–toxin fraction was centrifuged at pH 8 in a sucrose density gradient, the RNA–toxin complex showed a smaller molecular weight, compared with that at pH 6 (data not shown). These results indicate that the RNA–toxin complex dissociates to subunits at pH 8.0, as shown in a previous report (4). The pooled toxic fractions (Nos. 18~31) (see Fig. 1A) were recentrifuged in sucrose density gradient at pH 6 to purify RNA–toxin. The partially purified RNA–toxins (fraction Nos. 18~31 in Fig. 1A were pooled) were used to characterize the RNA(s).

For the identification of RNA in RNA–toxin (fraction Nos. 18~31 in Fig. 1A), RNA was hybridized to *C. botulinum* DNA digested with restriction enzymes (as shown under Materials and Methods). The DNA of botulinum type E were digested by *Eco*RI, *Eco*RV, *Hin*dIII, or *Pst*I and then were run in 0.8% agarose gel electrophoresis and transferred to nylon-paper sheets. The nylon-paper sheet (with transferred DNA) was dipped in the [^{32}P]RNA from RNA–toxin, as well as the [^{32}P]tRNA and the [^{32}P]rRNA of *E. coli* for hybridization. [^{32}P]RNA from RNA–toxin appeared as a single band hybridizing to the DNA of *C. botulinum* type E digested by *Eco*RI or *Hin*dIII (Fig. 2A1) but only a trace appeared on DNA digested by *Pst*I. The labeled tRNA produced a single band hybridizing to the DNA of botulinum type E digested by *Eco*RI and two bands on the

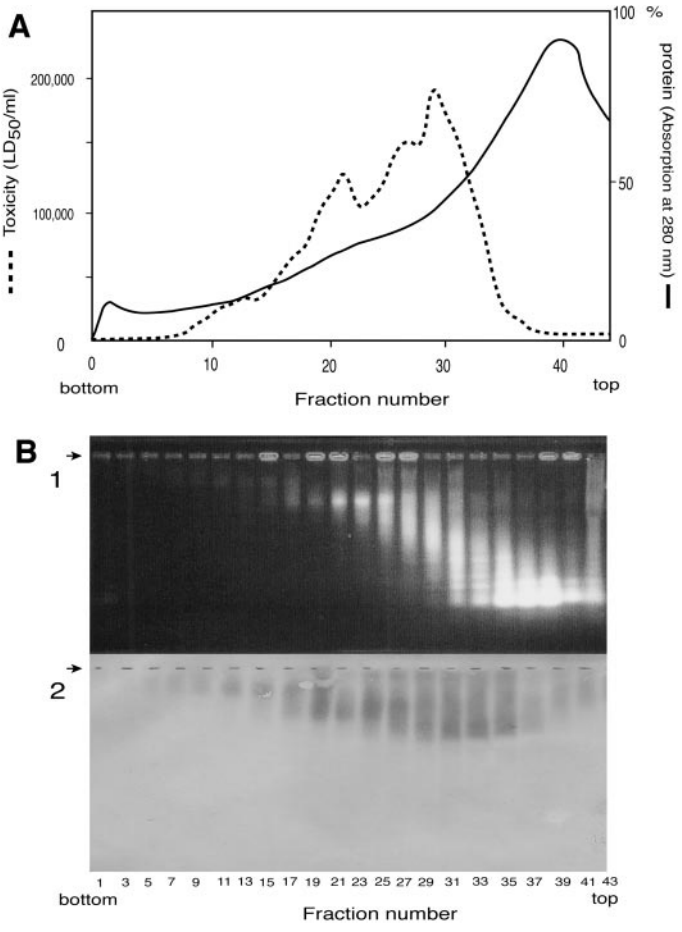

FIG. 1. Sucrose density gradient centrifugation of RNA–toxin complexes of *C. botulinum* type E, and analysis of toxins and RNA. (A) Pattern of protein quantity and toxicity in fractions collected after centrifugation. (B1) Pattern of RNA–toxin in each fraction on electrophoresis. (B2) Pattern of the immuno-blot test for RNA–toxin by anti-neurotoxin IgG. Linear density gradient of 5–20% (w/w) of RNase free sucrose (Nakarai Chem., Kyoto, Japan) was prepared at 0°C by mixing 4.9 ml each of 5% sucrose solution in a 0.05 M phosphate buffer, pH 6.0. Portions (0.5 ml) of RNA–toxin fraction layered on top of the sucrose gradient were spun in an RPS 40T in an Hitachi SCP 85H ultracentrifuge at 39,000 rev/min for 270 min at 5°C. After centrifugation, toxin content was determined by trypsin treatment and iv injection method (10). Electrophoresis of RNA–toxin separated by the centrifugation was performed in 3% of agarose. Immuno-blot test for RNA–toxin was performed by using anti-neurotoxin IgG. Arrows in B show the sample starting wells.

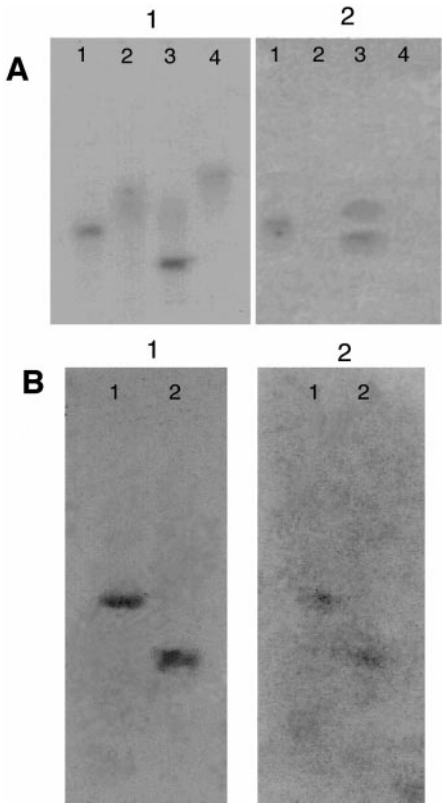

FIG. 2. DNA–RNA hybridization with probes of *C. botulinum* RNA–toxin, tRNA and rRNA against *C. botulinum* DNA digested with restriction enzymes. (A1) Hybridization with *C. botulinum* RNA probe (fraction No. 18~31 in Fig. 1A). (A2) Hybridization with tRNA probe. Lane 1, *Eco*RI; lane 2, *Eco*RV; lane 3, *Hin*dIII; lane 4, *Pst*I. (B1) Hybridization with botulinum RNA probe. Lane 1, *Eco*RI; lane 2, *Hin*dIII. (B2) Hybridization with rRNA probe. Lane 1, *Eco*RI; lane 2, *Hin*dIII.

*Hin*dIII-digested DNA (Fig. 2A2). The labeled rRNA formed a single band on the DNA digested by *Eco*RI or *Hin*dIII at essentially the same position as RNA from RNA–toxin (data not shown). Then we further hybridized either RNA from RNA–toxin or rRNA to the DNA digested by *Eco*RI or *Hin*dIII (Fig. 2B). The result showed that both RNA from RNA–toxin and rRNA produced single bands of hybridization at the same positions on the nylon-paper sheets of DNA digested by either *Eco*RI (Fig. 2, lane 1 in B1 and B2) or *Hin*dIII (Fig. 2, lane 2 in B1 and B2). These results demonstrate that RNA species in RNA–toxin is rRNA.

Although rRNAs are the most abundant RNA species in cells, it is not plausible that type E toxin preparation are simply contaminated with rRNAs because other types of toxin were not found as RNA–toxin complex in same preparation process (3). However, the purification of RNA–toxin was not sufficiently complete when using the sucrose density gradient centrifugation method as described above. Therefore, we tried isolation of the RNA–toxin using the anti-toxin IgG prepared by the purified neurotoxin (3). The anti-toxin IgG formed a single immunoprecipitation line against the extracted crude RNA–toxin of type E in an agar-gel diffusion test (between well 2 and well 4 in Fig. 3). The single precipitation line was fused (Fig. 3) with the purified neurotoxin which formed the single precipitation line (between well 2 and well 5 in Fig. 3) against either anti-toxin horse serum prepared by crude toxin extracted from cells or anti-neurotoxin IgG prepared with the purified neurotoxin. It means that anti-neurotoxin IgG was reacted with the toxin specifically. The RNA was detected by UV light after staining of ethylene blue dye in the single immunoprecipitation line, but not in the single line of the purified neurotoxin. These results show that RNA and toxin were collocated, i.e., that the RNA is bound to the toxin. The RNA–toxin in the three-toxic-peak fraction prepared at pH 6 in sucrose density gradient (Fig. 1A) was reacted with the anti-toxin IgG. The RNA–toxin-immunoprecipitate formed after incubation was isolated by centrifugation. The RNA–toxin-immunoprecipitate were washed to eliminate other substances by centrifugation for 20 min at 15,000*g* in 0.2 M phosphate buffer pH 6.0 by six times. After digestion of the isolated RNA–toxin-immunoprecipitate by proteinase K, RNA was extracted by the phenol method and run on agarose gel electrophoresis. The RNA–toxin sedimented at the higher density position in sucrose density gradient centrifugation (see Fig. 1) showed two bands corresponding to the position with either 23S or 16S rRNA on agarose gel electrophoresis (Fig. 4, lane 3). The RNA–toxin at the middle position in the gradient showed also two bands corresponding to either 16S rRNA or 23S rRNA in a small amount (Fig. 4, lane 4). On the other hand, the RNA–toxin at the lightest position in the gradient showed single band corresponding to 16S rRNA (Fig. 4, lane 5). These results clearly indicate that three different species of RNA–toxin complex exist. The three types of RNA–toxin are toxins binding with both the 23 S and 16 S rRNA, with the 16S rRNA

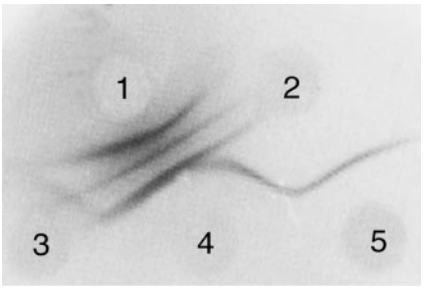

FIG. 3. Agar gel immuno-diffusion test on purified anti-neurotoxin IgG and toxin of *C. botulinum* type E. Well 1, anti-toxin house serum prepared by extracting toxin from cells. Well 2, anti-neurotoxin IgG prepared using the purified neurotoxin. Wells 3 and 5, purified neurotoxin. Well 4, RNA–toxin complex fraction. One band (line of reaction) can be observed between wells 2 and 4, and the single band was fused with the band of purified neurotoxin between wells 3 or 5 and wells 1 and 2.

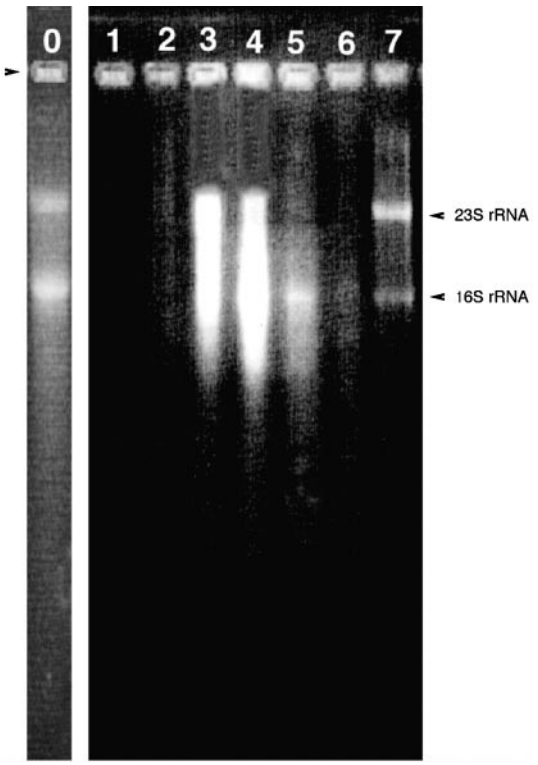

FIG. 4. Electrophoresis of RNA from the RNA–toxin immunoprecipitated by anti-neurotoxin-IgG of *C. botulinum* type E. The RNA–toxin in the three-toxic-peak fraction prepared at pH 6 in a sucrose density gradient (see Fig. 1A) was reacted with the anti-toxin IgG. The RNA–toxin immunoprecipitate formed after incubation was isolated by centrifugation. After digestion of the isolated RNA–toxin immunoprecipitate by proteinase K (Takara, Ohotu, Japan), RNA extracted by the phenol method was electrophoresed on a 3% of agarose gel. Lane 0, RNA–toxin fraction. Lane 1, fraction Nos. 1–9 in Fig. 1A. Lane 2, fraction Nos. 10–16 in Fig. 1A. Lane 3, fraction Nos. 17–22 in Fig. 1A. Lane 4, fraction Nos. 23–27 in Fig. 1A. Lane 5, fraction Nos. 28–32 in Fig. 1A. Lane 6, fraction Nos. 33–38 in Fig. 1A. Lane 7, 23S and 16S rRNA.

and a small amount of 23S rRNA, and with only the 16S rRNA. These systematical RNA binding to the toxin indicate that the binding is not artifact or nonspecific.

It is interesting to ask what the role of the RNA in RNA–toxin is. Whether the RNA–toxin complexes may be involved in the process of the biosynthesis of toxin protein or in the process of the secretion (release) of the toxin from bacterial cell, described in a previous report (5), is not clear at this time moment.

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