Effects of Bacillus thuringiensis Cry1Ab Toxin on Mammalian Cells

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ABSTRACT. The Cry proteins produced by Bacillus thuringiensis are considered to be highly specific insecticidal proteins. Judged to be safe for humans and farm animals due to their insect-oriented selective toxicity, the proteins have been utilized as a biological pesticide and introduced into genetically modified plants. However, some critical fundamental characters of the Cry proteins remain unclear, and the direct effects of activated Cry proteins on mammalian cells have not yet been fully confirmed. Therefore, in this study we employed primary cultured bovine hepatocytes as a model system to determine if Cry1Ab, a Cry protein, affects mammalian cells. There were no significant changes in the secretion of albumin or the morphology of the Cry1Ab-treated cells. The LDH release showed a tendency to increase after the administration of Cry1Ab, but not significantly. Taking these results on bovine hepatocytes into consideration, Cry1Ab has little acute toxicity on mammalian cells.

KEY WORDS: albumin secretion, Bacillus thuringiensis Cry1Ab toxin, LDH release, primary cultured bovine hepatocyte.

Bacillus thuringiensis (Bt) is a Gram-positive endospore-forming bacterium characterized by the presence of a protein crystal within the cytoplasm during sporulation [13]. When this crystal is ingested by target insects, it develops an insecticidal activity due to partial digestion of the protoxin inside the midgut of the insect [7, 18]. The insecticidal proteins in the crystal form two different families, Cry and Cyt, which have been classified on the basis of amino acid identity into more than 30 Cry subgroups and two Cyt subgroups [3]. The Cyt proteins are well known to have cytolytic activity against a broad range of cells including mammalian erythrocytes [8, 19], while the toxicity of the Cry proteins is considered to be highly specific because in vivo experiments show that non-target organisms exposed to high levels of the Cry proteins remain virtually unaffected [2]. Due to this selectivity of the Cry proteins, Bt has been utilized for many years in agriculture as a pesticide that is considered to be safe for mammals, including humans and livestock [16]. Further, plants genetically modified to express the Cry proteins are being put into commercial use [2]. However some critical, fundamental characters of the Cry proteins have not yet been elucidated completely [1] (e.g. the mode of action, the mechanism of expression for their high specificity). Moreover, in field of entomology, experiments on tissue culture cells have clarified that Cry proteins have a fairly general capacity to form large nonspecific pores under certain conditions, including high Cry protein concentrations, long incubation times, and relatively low pHs [18]. However, to our knowledge, there have been few reports about the direct effects of activated Cry proteins on mammalian cells. Therefore, we investigated whether Cry1Ab, a Cry protein, affects mammalian cells in vitro. We used primary cultured bovine hepatocytes because they provide a simple, manageable model system for evaluating the effects of xenobiotics in a completely serum-free medium [20]. Furthermore, unlike other established cell lines, primary cultured bovine hepatocytes have various well-differentiated functions [9, 22]. Therefore, it is possible to evaluate not only cell viability but also albumin secretion, which reflects cellular protein synthesis.

MATERIALS AND METHODS

Purification of Cry1Ab protein: Cry1Ab protein was purified from a recombinant Escherichia coli (E. coli) strain JM109, kindly supplied by Dr. Y. Kanda of Saga University, as follows. First, the strain was grown in LB medium containing ampicillin (50 µg/ml) and isopropyl-β-D-thiogalactopyranoside (IPTG, 100 mM) to induce the expression of cloned genes in the vector plasmids [11]. Recombinant Cry1Ab protoxin in E. coli JM109 was purified as previously described [10]. The Cry1Ab protoxin was activated by trypsinization, and the activated Cry1Ab was further dialyzed against PBS(−) (pH 7.4). Then the Cry1Ab was further purified by a modification of the preferential precipitation method [14]. Briefly, the Cry1Ab was dialyzed (4°C, overnight) against 0.05 M Tris-HCl (pH 9.0), which preferentially precipitated Cry1Ab. The precipitate was resuspended in PBS(−) (pH 7.4). The purity of the Cry1Ab was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), using a 20–5% gradient gel, and the protein concentration was determined with a colorimetric kit (Advanced Protein Assay Reagent, Cytoskeleton, U.S.A.).

The lipopolysaccharide (LPS) concentration in the Cry1Ab was measured by an automated turbidimetric-kinetic assay (ATK) kit (Limulus ES-II Test Wako, Wako, Japan) as described previously [17].

The toxicity of the Cry1Ab was confirmed by a bioassay on susceptible silkworm larvae.

Primary culture of bovine hepatocytes: Bovine primary hepatocytes were obtained from three one-week-old male
Holstein calves in accordance with the guidelines for animal use of the National Institute of Animal Health and cultured as previously described [9, 20]. Briefly, a modified serum-free culture medium (William’s medium) containing 10⁻⁹ M insulin, 10⁻⁹ M dexamethasone, 5 kIU aprogin, trace elements (0.1 µM CuSO₄·5H₂O, 3 mM H₂SeO₃, 50 pM ZnSO₄·7H₂O) and antibiotics (100 U/ml penicillin, 100 mg/ml streptomycin, 250 µU/ml amphotericin B) was used. One-hundred µl of a cell suspension (3 × 10⁵ cell/ml) in the modified serum-free medium was delivered to each well of a 96-well plate (Sumitomo Bakelite Co., Ltd, Japan) and pre-incubated at 37°C for 20 hr in a humidified atmosphere of 5% CO₂ in air. Then the medium was replaced with a fresh one containing various concentrations of Cry1Ab, as shown in the figures. After 24 hr and 48 hr incubation, we observed the bovine hepatocytes with a phase contrast microscope, and the supernatants were harvested for assays of albumin and LDH.

**ELISA assay of albumin:** The albumin concentration of the culture medium was measured using ELISA by the method of Yamanaka et al. [20]. Briefly, aliquots of bovine serum albumin (Seikagaku Co., Japan) and diluted culture medium were coated onto a flat-bottom 96-well plate (Nalge Nunc Co., U.S.A.). After blocking, they were reacted with purified rabbit anti-bovine albumin IgG antibody as the first antibody, and then alkaline phosphatase-conjugated anti-rabbit IgG goat IgG antibody (Sigma Co., U.S.A.) as the second antibody for color development. Finally, the albumin concentration in the medium was measured in an ELISA reader (Wako, Japan) at a 405 nm wavelength. Four wells were used for each dilution, and the assay was repeated three times.

**Lactate dehydrogenase release assay (LDH):** The LDH activity in the culture medium was measured in an ELISA reader (Wako, Japan) at a 490 nm wavelength, using a colorimetric kit (CytoTox 96 Non-radioactive Cytotoxicity Assay Kit, Promega, U.S.A.). The percentage of release was calculated using the formula: % LDH release = 100 × experimental LDH release/maximum LDH release. Maximum LDH release was obtained by complete solubilization of bovine hepatocytes with 0.1% Triton X-100. Prior to the experiment, it was confirmed that Cry1Ab itself does not induce any non-specific colorimetric reaction. At least four wells were used for each dilution, and the assay was repeated three times.

**Statistics:** The significance of the difference between mean values was evaluated by analysis of one-factor ANOVA and Dunnett’s post-hoc procedure. A level of p<0.05 was regarded as significant.

**RESULTS**

**Cry1Ab purification:** Figure 1 shows the purity, analyzed by SDS-PAGE, of the Cry1Ab used in this study. As shown in Lane 2, the Cry1Ab after purification was a pure mono-band as judged by silver stain. The mono-band was identified as Cry1Ab by immunoblot analysis using anti-Cry1Ab rabbit serum (Lane 3).

The LPS level of the Cry1Ab was 2.5 ng/ml. The Cry1Ab was diluted 1:100 in the medium when it was added to the 96-well plate. The LPS contamination was considered to be negligible because we detected no changes in the secretion of albumin from the bovine hepatocytes even at the LPS concentration of 1 µg/ml (data not shown). The bioassay showed that 2.6 µg/head of orally administered Cry1Ab toxin killed all the silkworm larvae the day after administration.

**Phase contrast microscopic observation:** Figure 2 illustrates the morphology of hepatocytes incubated with Cry1Ab. The bovine hepatocytes had formed a full monolayer in all wells with or without Cry1Ab at 48 hr after seeding. Even at a concentration of 2,000 ng/ml of Cry1Ab, no morphological changes were detected in the cells.

**Albumin secretion:** Since albumin is an essential parameter of proteosynthesis in hepatocytes, we measured the albumin levels in the culture medium to evaluate whether Cry1Ab affects albumin secretion of the cultured hepatocytes. Figure 3 shows the albumin secretion of hepatocytes with or without Cry1Ab. In both control and experimental wells, the amount of albumin secretion was about 5 µg/ml at 24 hr and about 10 µg/ml at 48 hr after adding Cry1Ab.
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There were no statistically significant differences between control and experimental wells at each harvesting time, indicating that the Cry1Ab treatment did not affect the albumin secretion of hepatocytes.

**LDH release**: Figure 4 shows the LDH release, a parameter for evaluating the membrane integrity of the hepatocytes cultured with or without Cry1Ab [4]. In both control and experimental wells, the LDH release percentages were about 6% at 24 hr and about 11% at 48 hr after adding Cry1Ab. There were no statistically significant differences between control and experimental wells at each harvesting time. However, we found an increased tendency of cellular LDH release at 48 hr after adding Cry1Ab, but it was not significant.

**DISCUSSION**

Although Cry proteins are widely used as a highly selective biological insecticide to control the economically significant insect-borne pests in agriculture, there have been few reports evaluating quantitatively the direct effects of active Cry proteins on mammalian cells. Therefore, we investigated whether Cry1Ab affects mammalian cells, using primary cultured bovine hepatocytes.

In this study, we found no morphological changes in the hepatocytes incubated with Cry1Ab at various concentrations. This result is similar to previous morphological observations of Cry protein-treated mammalian cells [19]. We also confirmed that Cry1Ab induces no significant changes in the secretion of albumin and the release of LDH, even at a concentration of 2,000 ng/ml, which is high enough to kill some agronomically important insects [15, 21]. Therefore, like previous reports, we think that Cry proteins have little acute toxicity on mammalian cells. However, there is one noteworthy result of this study: the tendency of increased cellular LDH release. The LDH release from the primary cultured bovine hepatocytes increased with the administration of Cry1Ab, even though the release levels were not significantly different. Previously, Knowles et al. reported that in CF cells, a clonal culture derived from minced spruce budworm neonates, small molecules leaked out of the CF1 cells before large ones. They reasoned that the initial toxic event is the formation of a small hole in the plasma membrane, impermeable to intracellular macromolecules. As ions and water enter the cell, a further breakdown of the stretched plasma membrane will occur, allowing progressively larger molecules to leak out of the cell [5–7, 12]. In other words, experiments on tissue culture cells in the field of entomology clarified that Cry proteins have a fairly general capacity to form large nonspecific pores under certain conditions, including high Cry protein concentrations, long incubation times and relatively low pHs [18]. Therefore, our results in the LDH release assay might suggest that, even on mammalian cells, a similar phe-

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**Fig. 2.** Phase contrast microscopic observations. Times after the administration of Cry1Ab are indicated. Both control and Cry1Ab-treated (2,000 ng/ml) hepatocytes formed a full monolayer. No morphological changes were detected in any Cry1Ab-treated cells.
Fig. 3. Albumin secretion of bovine hepatocytes into the culture medium. The culture medium was harvested at 24 hr and 48 hr after adding Cry1Ab. The albumin was measured by ELISA. The data represent means ± standard error of three independent experiments.

Fig. 4. LDH release of bovine hepatocytes in response to Cry1Ab. Bovine hepatocytes (3 × 10^5 cells per well) were treated with Cry1Ab at various concentrations for 24 hr and 48 hr. Then the LDH activities of the supernatant were measured. The LDH release was expressed as % release as described in the Material and Methods section. The data represent means ± standard error of three independent experiments.
nomenon occurs due to a similar mode of action under certain conditions.

We conclude that there is little acute toxicity of Cry proteins on mammalian cells, judging from previous reports and our findings of no damage to the morphology, albumin synthesis and integrity of the cell membrane that was ascertained in the LDH release assay. However, it is premature to conclude the absolute harmlessness of Cry proteins on mammalian cells because, in this study, the LDH release showed a tendency of increase after the administration of Cry1Ab. Further elucidative study using other membrane integrity parameters such as ions is needed to resolve the influence of Cry proteins on mammalian cells.

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