**A Bacillus thuringiensis Crystal Protein with Selective Cytotoxic Action to Human Cells**

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Bacillus thuringiensis crystal proteins, well known to be toxic to certain insects but not pathogenic to mammals, are used as insecticidal proteins in agriculture and forest management. We here identified a crystal protein that is non-insecticidal and non-hemolytic but has strong cytotoxic activity against various human cells with a markedly divergent target specificity, e.g. highly cytotoxic to HepG2 and Jurkat and less cytotoxic to the normal hepatocyte (HC) and HeLa. In slices of liver and colon cancer tissues, the toxin protein preferentially killed the cancer cells, leaving other cells unaffected. The cytotoxic effect of the protein is non-apoptotic with swelling and fragmentation of the susceptible cells, although the apoptotic process does occur when the cell damage proceeded slowly. The amino acid sequence deduced from the nucleotide sequence of the cloned gene of the protein has little sequence homology with the insecticidal crystal proteins of B. thuringiensis. These observations raise the presence of a new group of the B. thuringiensis toxin and the possibility of new applications for the protein in the medical field.

Bacillus thuringiensis is a Gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. The crystal proteins studied so far are toxic to the larvae of certain insects of several orders, and even to nematodes, mites, and protozoa, but not pathogenic to mammals, birds, amphibians, or reptiles (1, 2). Thus, this bacterium is a valuable source of insecticidal proteins for use in conventional sprayable formulations and in transgenic crops and may even be the most promising alternative to synthetic chemical pesticides used in commercial agriculture and forest management as these proteins are beneficial and friendly to the environment relative to chemical pesticides (1, 2).

Genes of the crystal proteins of B. thuringiensis appear to reside on plasmids, often as parts of composite structures that include a variety of transportable elements (2, 3). This high degree of genetic plasticity results in the remarkable diversity of B. thuringiensis strains and crystal proteins, and a growing number of the strains and toxin proteins are being isolated and cloned (1, 4). Earlier studies have shown that non-insecticidal B. thuringiensis strains are ubiquitous in natural environments and are even more widely distributed than insecticidal strains (5, 6).

Among the non-insecticidal B. thuringiensis isolates, we found isolates with a novel property, non-hemolytic but highly cytotoxic to a wide range of mammalian cells, including human cancer cells (7–11). The cytotoxic proteins were heterogeneous in cytotoxicity spectra, and some were active on a wide range of human cells, whereas the others killed a few specific cells. These proteins are not allied to the class of Cyt proteins, which also exhibit cytotoxicity against mammalian cells but do have hemolytic and insecticidal activities (1).

The B. thuringiensis A1547 (designated previously as strain 94-F-45-14), belonging to serovar dabaota, is one of the strains with parasporal crystal proteins that are non-hemolytic but are cytotoxic against human leukemic T cells, MOLT-4 (7–9). In the present study, we obtained the gene for the purified toxin protein, examined the cytotoxic activity of the expressed recombinant toxin protein against the cultured human cells and the cancer tissues, and found that the toxin protein has strong cytotoxic activity against various human cells with markedly divergent target specificity and preferentially kills the liver and colon cancer cells, leaving the normal cells in the tumor tissue slice unaffected.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Culture Media**—The organisms used in this study was the soil isolate B. thuringiensis A1547, belonging to serovar dabaota (7–9). The bacteria were grown on nutrient medium, containing 0.1% meat extract, 0.1% polypeptone, and 0.2% NaCl, pH 7.6, at 27 °C for 8 days.

**Human Cells and Culture of the Cells**—Human cells used, MOLT-4, Jurkat, HL-60, Sawano, HepG2, HeLa, TCS, HC, A549, MRC-5, and Caco-2 cells, were obtained from Riken Cell Bank (Tsukuba, Japan). All cells were maintained under conditions recommended by the manufacturer. Normal T-cells were prepared from buffy coats obtained from the Fukuoka Red Cross Blood Center (Fukuoka, Japan) and were separated from lymphocytes and cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum and 30 μg/ml kanamycin at 37 °C.

**Isolation of Toxin Protein from Parasporal Crystals**—Sporulated cells of B. thuringiensis strain A1547 were harvested and washed three times and disrupted in distilled water. After purification of the parasporal crystals using a biphasic separation technique (12), the crystals were solubilized with 50 mM Na2CO3 (pH 10.0) at 37 °C for 1 h in the presence of 10 mM dithiothreitol and 1 mM EDTA and were treated with proteinase K (final concentration, 10 μg/ml) at 37 °C for 30 min followed by the addition of 1 mM phenylmethylsulfonyl fluoride to stop the proteolysis. The proteinase-treated proteins were applied to DE-52 ion-exchange column, and the toxin protein was eluted with 50 mM NaCl in 20 mM Tris-HCl buffer, pH 8.0. The active fraction was then subjected to gel filtration with Sephacryl S-300.

**Assay of Cytotoxic Activity**—The cytotoxic activity was measured by the 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2H tetrazolium bromide (MTT) colorimetric assay.}
BT Toxin with Selective Cytocidal Action to Human Cells

Expression and Purification of Recombinant Toxin Proteins—Plasmids, pET-37k and pET-30k, were constructed from the genes Images 3'-oligolabeling module then used for selection of positive plaques. DNA sequencing of the positive clone was done using the Thermo sequenase per mixed cycle sequencing kit (Amersham Biosciences). The degenerate primer, designed from the N-terminal amino acid sequence of the 30-kDa toxin protein, was labeled using Gene Images 3'-terminus. A hexahistidine tag was introduced into the C termini of both proteins. Escherichia coli BL21 (DE3) cells transformed with each plasmid were cultured in LB medium containing 0.1 mM isopropyl β-D-thiogalactopyranoside for 24 h at 37°C. After harvesting the cells by centrifugation for 10 min at 1,000 × g, the cells were suspended in 10 mM Tris-HCl buffer, pH 7.5, containing 10 mM MgSO4, 50 mM KCl, and 10% glycerol, and then lysed by sonication followed by solubilization with 50 mM Na2CO3 (pH 10.0) at 37°C for 1 h in the presence of 10 mM dithiothreitol and 1 mM EDTA. The soluble extract was recovered by centrifugation for 20 min at 15,000 × g. The extract was loaded on a nickel-chelating Sepharose column (Amersham Biosciences; His-trap) and eluted with 500 mM imidazole. The purity was confirmed by SDS-PAGE.

Culture of Human Liver Tissue Slice—The cancer tissue was isolated immediately after surgical resection. The cancer tissue was cut into about 3-mm-diameter pieces. After washing with cold phosphate-buffered saline, the fragments were cultured in Dulbecco’s modified Eagle’s medium, supplemented with 10% fetal calf serum and an antibiotic mixture containing 100 ng/ml streptomycin, 100 units/ml penicillin, and 5 mg/ml fungizone, at 37°C. The tissue was added to the culture medium (final concentration, 0.3 μg/ml), and the tissue fragments were further incubated for 8 h. The tissues were then fixed with 20% formaldehyde and embedded in paraffin. After being stained with hematoxylin and eosin, the tissue was observed under a light microscope.

Assay of Caspase Activity—Cells were incubated with the toxin protein (0.1 or 10 μg/ml), staurosporine (10 μg/ml), Triton X-100 (0.1%), at 37°C for the indicated times. After lysis of the cells, the fluorogenic peptide substrate, z-DEVD-rhodamine 110, was added and incubated at the room temperature. The caspase activation (fold) was determined on the basis of the relative intensity of fluorescence to the mock-incubated cells. For inhibition experiments, cells were pretreated with or without caspase inhibitor, Z-VAD-fmk (100 μM), at 37°C for 1 h and then incubated with the toxin protein (0.1 or 10 μg/ml), staurosporine (10 μg/ml) at 37°C for 20 h. A half-portion of each sample was subjected to the caspase assay, and for another half, the cell proliferation was measured as described above.

RESULTS AND DISCUSSION

Cloning of a Cytocidal Protein of B. thuringiensis A1547 and Its Expression in E. coli—After treatment of the parasporal inclusions of B. thuringiensis A1547 strain with proteinase K, the toxin protein was purified through two column chromatographies, measuring their cytotoxic effects on MOLT-4 cells using the MTT assay system. The cytocidal activity was associated with a protein with a molecular mass of 30 kDa. Without protease digestion, the inclusion proteins, like the Cry toxins, exhibited no cytocidal activity (15, 16). The N-terminal amino
acid sequence of the proteinase K-activated 30-kDa protein was determined to be Asp-Val-Ile-Arg-Glu-Tyr-leu-Met-Phe-Asn. The gene for the cytocidal 30-kDa protein was cloned from a phage library prepared from a plasmid DNA of the bacteria, using as the probes degenerated oligonucleotides designed from the N-terminal amino acid acid sequence. The amino acid sequence deduced from the nucleotide sequence of the cloned gene is shown in Fig. 1. The gene was 1,014 bp long and encoded a polypeptide of 338 amino acid residues with a predicted molecular mass of 37,446. The N-terminal amino acid sequence of the proteinase K-activated protein appears in the deduced sequence. Proteinase K would cleave between threonine 51 and aspartic acid 52, and the molecular weight of the N-terminal truncated protein was calculated to be 31,703. The block sequences conserved in the Cry proteins (1, 3) were not detectable in the sequence of the 37-kDa precursor protein. The protein exhibits a weak sequence homology only with Cry 15Aa (17) among the established Cry and Cyt proteins (3).

Recombinant toxin proteins of the full-length and N-terminal truncated proteins, tagged with a hexa-histidine at the C terminus, were expressed in Escherichia coli strain BL21 and purified on a nickel-chelating column. The full-length protein had no apparent cytocidal activity, and the proteolytic processing was essential for activation of the toxic protein. On the

### Table I
Cytocidal activity of B. thuringiensis parasporal toxin protein to various cultured human cells

| Cell          | Characteristics             | LD_{50}  |
|---------------|-----------------------------|----------|
| MOLT-4        | Leukemic T cell             | 0.044    |
| Jurkat        | Leukemic T cell             | 0.015    |
| HL-60         | Leukemic T cell             | 0.066    |
| T cell        | Normal T cell               | 0.148    |
| HC            | Normal hepatocyte           | >10      |
| HepG2         | Hepatocyte cancer           | 0.023    |
| HeLa          | Uterus (cervix) cancer      | >10      |
| Sawano        | Uterus cancer               | 0.041    |
| TCS           | Uterus (cervix) cancer      | >10      |
| UMSMC         | Normal uterus               | 9.28     |
| MRC-5         | Normal embryonic lung fibroblast | 7.15   |
| A549          | Lung cancer                 | >10      |
| CACO-2        | Colon cancer                | 4.86     |

LD_{50} values were calculated from the data shown in Fig. 2.

**Fig. 3. Cytotoxic effect of parasporal toxin protein on hepatocellular carcinoma cells.** The cancer tissue of moderately differentiated hepatocellular carcinoma, Edmondson type II, from a 65-year-old male was isolated immediately after surgical resection. The patient gave written informed consent. The tissue pieces were treated with the toxin protein (final concentration, 0.3 μg/ml) for 8 h at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. A, the cancer tissue exposed to the toxin protein. B, the cancer tissue without this exposure. C and D, the non-cancer area of the cancer tissue exposed to the toxin protein. The original magnification is ×200.

**Fig. 4. Cytotoxic effect of parasporal toxin protein on colon cancer cells.** The cancer tissue was isolated from a front portion of advanced carcinoma in the serosa of the colon immediately after surgical resection. The tissue pieces were treated with the toxin protein (final concentration, 1.5 μg/ml) for 8 h at 37 °C in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum. A and B, the cancer tissue exposed to the toxin protein. C, the cancer tissue without toxin exposure. D, the non-cancer area of the cancer tissue exposed the toxin protein. In A and C, the original magnification is of ×200, and in B and D, the original magnification is of ×400. In A, the region containing the dead cells is marked with arrows. Arrows in B and D indicate pyknotic nuclei in the cancer cells. Arrowheads in D indicate blood vessels and endothelial cells.
other hand, the N-terminal truncated protein had a molecular mass of 31 kDa, as seen by SDS-PAGE, and had substantially the same cytotoxicity to MOLT-4 cells as that of the activated 30-kDa protein from the parasporal inclusion of B. thuringiensis A1547 strain.

Cytocidal Activity of the Recombinant Toxin Protein against Human Cells—Dose responses of the recombinant N-terminal-truncated toxin protein against various cultured human cells were monitored using the MTT assay and the LD50 values 24 h after the administration. The cytotoxity largely varied from cells to cells. Among the cells examined, the toxin protein was highly cytotoxic to MOLT-4, Jurkat, Sawano, and HepG2 cells, and the LD50 values against those cells were as low as 10–40 ng/ml. On the other hand, some cells, including HeLa, TCS, HC, A549, and MRC-5, were resistant to the toxin protein. Although we found no general rule for specificity of the cells or no common characteristics for sensitive or resistant cells, some cells derived from the tumor tissues seem more sensitive to the toxin protein than did those from the normal tissues, i.e. HepG2 versus HC cells.

Cytotoxicity to the cancer cells was shown when we applied the toxin protein to cancer tissues isolated from moderately and well differentiated hepatocellular carcinoma (Edmondson type II and type I, respectively). The tissue pieces were treated with various concentrations of the toxin protein for 8 h at 37 °C, fixed, stained, and observed under a light microscope (Fig. 3). In the moderately differentiated cancer tissue, which has eosinophilic and abundant cytoplasm with enlarged atypical nuclei and forms glandular structures, treatment with the toxin protein (0.3 µg/ml) caused markedly degenerative appearance of the cells, and the nuclei and cell border of cytoplasm of the cells disappeared (Fig. 3A), as compared with the untreated cancer cells (Fig. 3B). Injury to non-neoplastic liver cells (Fig. 3C) and chronic inflammatory cells, blood vessels, and bile ducts in fibrous Gleeson’s sheath in non-neoplastic areas affected by chronic active hepatitis (Fig. 3D) was negligible, even after treatment with the toxin protein. Essentially the same observations were obtained with colon cancer tissues (Fig. 4). In the
cancer tissue exposed to the toxin protein (Fig. 4, A and B), there are markedly degenerative and desquamative appearances of the tall columnar cancer cells, and the number of dead cells was larger than the number of cells without toxin exposure (Fig. 4C). Some cancer cells had small, pyknotic, and irregular-shaped nuclei in a higher magnification (Fig. 4B). Little morphological change was observed in endothelial cells of the blood vessels, fibroblastic cells, and infiltrating inflammatory cells in the non-cancer areas of the same tissue shown in Fig. 4A, even after treatment with the toxin protein (Fig. 4D).

These results indicate that the toxin protein kills preferentially liver and colon cancer cells, leaving other cells in those tissues unaffected.

**Mechanism of Cytotoxic Action of the Toxic Protein**—We have examined the mechanism of cytotoxic actions of the toxin protein on the human cells (Fig. 5). Fig. 5A shows the time course of effects of the toxin protein on HepG2 cells, one of the most sensitive cells, measured using the MTT assay. With 10 μg/ml of the toxin protein, the cells rapidly lost respiratory activity, and none of the cells were viable within 8 h. Yet with a low dose (0.1 μg/ml), the cells gradually died, and half the number died at around 15 h. This time course is similar to that of staurosporine, an apoptosis inducer, used at the concentration of 10 μg/ml. The toxin protein led the cells to large morphological changes, and consequently, they died. Fig. 5B shows changes in the morphology of HepG2 cells to the toxin protein (0.1 μg/ml). The cells ballooned and started to detach from the dish around 4 h after exposure to the toxin administration, and then burst open and fragmented to death within 20 h. Such morphological changes were more clearly observed with HL60 cells, in which the cells ballooned at around 2 h and burst within 4 h (Fig. 5C).

No morphological change was observed in the resistant cells (data not shown). Morphological observations suggest that the cytotoxic effect of the toxin protein is non-apoptotic. To examine this in more detail, we studied DNA fragmentation and caspase activation. The faint ladder pattern was observed at this in more detail, we studied DNA fragmentation and cytotoxic effect of the toxin protein is non-apoptotic. To examine the mechanism of actions of the toxin protein (Fig. 4). These results indicate that the toxin protein kills preferentially liver and colon cancer cells, leaving the normal cells in the tumor tissue slice unaffected. This, taken together with the mammalian cell-recognition parasporels proteins reported previously (7–11), means the presence of a new distinct family of B. thuringiensis δ-endotoxins that are highly cytotoxic to a wide range of mammalian cells but are non-hemolytic and non-insecticidal, in addition to the established insecticidal Cry and Cyt protein families (9). Each toxin protein has its specific and distinct target spectrum against mammalian cells, and some proteins are strictly specific to certain human cancer cells, as the toxin protein reported in this report. This raises the possibility that we could screen the strains (and toxin proteins) cytotoxic to the specific cancer cell and use those proteins in the medical and biological fields.

Strict specificity on target cells suggests the presence of receptor-like protein and/or lipid at the surface of the sensitive cells, as in the case of the insecticidal Cry toxins. Non-apoptotic cytotoxic action with swelling and fragmentation of the susceptible cells suggest that the protein changes ion permeability of the cells, also as the Cry toxins (1, 18). Identification of the cell receptor is expected to provide some insight into the mechanism of target specificity and cytotoxicity of the new type of B. thuringiensis toxin protein.

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