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An Entomopathogenic Bacterium, *Xenorhabdus nematophila*, Causes Hemocyte Apoptosis of Beet Armyworm, *Spodoptera exigua*

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Abstract Entomopathogenic nematode, *Steinernema carpocapsae*, carries the symbiotic bacterium, *Xenorhabdus nematophila*, and enter host insect hemocoel. In the infected host, nematodes release the bacteria, which in turn cause lethal hemolymph septicemia. This study was designed to test a hypothesis that hemolymph septicemia of beet armyworm, *Spodoptera exigua*, is triggered by *X. nematophila* through a programmed cell death (= apoptosis) of the hemocytes. Morphological changes of the hemocytes after the bacterial infection were similar to cell changes undergoing a typical apoptosis. At 4-8 h post-infection, the cell membrane blebbing and apoptotic vesicles were observed and the nuclear membrane was broken apart. At 12 h post-infection, the overall cell shape was lost externally and nuclear condensation was evident internally, where genomic DNA was fragmented into small pieces. Proportion of apoptotic hemocytes increased with post-infection time and reached a maximum level when the hemocytes were in complete septicemia. Increase of FAD-glucose dehydrogenase (EC 1.1.99.10) activity preceded initial hemocyte apoptosis after the bacterial infection. The infected plasma or the culture broth of *X. nematophila* contained apoptosis-inducing factors. The apoptosis factors were extracted into organic fraction and turned out to be relatively high hydrophobic chemical nature after further fractionation assay. These results indicate that *X. nematophila* induces hemocyte apoptosis by its secretory material(s), which lead to hemolymph septicemia and immunodepression of the target insect hosts.

Key words apoptosis, apoptotic vesicle, DNA fragmentation, membrane blebbing, septicemia, *Spodoptera exigua*, *Xenorhabdus nematophila*

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Introduction

Insects can express immune responses, which are divided in humoral and cellular defenses against nonself materials. Humoral defense responses include production of antimicrobial peptides (Meister *et al.*, 2000; Lowenberger, 2001), reactive intermediates of oxygen or nitrogen (Vass and Nappi, 2001), and the complex enzymatic cascades that regulate coagulation or melanization of hemolymph (Gillespie *et al.*, 1997). In contrast, cellular defense responses refer to hemocyte responses of nodulation, phagocytosis, and encapsulation depending on pathogen nature (Schmidt *et al.*, 2001; Lavine and Strand, 2002).

Many microorganisms, including virus and bacteria, may induce apoptosis of host cells during their pathogenesis (Chen and Zychlinsky, 1994; Zychlinsky and Sansonetti, 1997). Apoptosis or programmed cell death is a dynamic process by which unwanted or diseased cells are disassembled in a rapid but systematic manner (Manji and Friesen, 2001) and apoptotic cells undergo a series of characteristic alterations in morphology that include chromatin condensation, DNA fragmentation, cell shrinkage, and membrane blebbing (Schwartz *et al.*, 1993; Robertson *et al.*, 2000; Manji and Friensen, 2001).

Xenorhabdus nematophila is a symbiotic bacterium of entomopathogenic nematode, *Steinernema carpocapsae*, and highly pathogenic to target insects (Akhurst, 1980; Park *et al.*, 1999). *X. nematophila* causes hemolymph septicemia, which leads to death of the target hosts including, *Spodoptera exigua* (Park and Kim, 2000). Park and Kim (2000) also reported that *X. nematophila* depresses cellular immune response such as nodule formation by blocking eicosanoid biosynthesis pathway because the eicosanoids play a significant role in immune mediation in response to bacterial infection (Stanley, 2000). But the

ultimate hemolymph septicemia is caused by the cytotoxic effect of *X. nematophila* against the hemocytes of the target insects (Ribeiro *et al.*, 1999; Park and Kim, 2000). This study was designed to test a hypothesis that *X. nematophila* induces apoptosis, which gives its cytotoxic action on the hemocytes of *S. exigua.* We also isolated several apoptosis-inducing factors from the bacterial culture broth.

Materials and Methods

Insect and bacterial cultures

S. exigua larvae were reared by the method of Park *et al.* (1999) and the fifth instar stage was used in this experiment. *X. nematophila* was isolated from the hemolymph of the fifth instar larvae of *S. exigua* infected with entomopathogenic nematode, *S. carpocapsae* (Park *et al.*, 1999). The bacteria were cultured in tryptic soy broth (TSB) (Difco, USA) media for overnight at 37℃ with shaking at 150 rpm rate.

In vivo cytotoxic analysis of *X. nematophila* on hemocytes

Bacterial injection (10^8 cfu/ml) followed the method of Park and Kim (2000). The fifth instar larvae were kept at 28℃ for different post-injection times (0, 4, 8, and 12 h) after hemocoelic injection with 2 µl of bacterial suspension by microsyringe (Hamilton, Nevada, USA). At each post-injection time, the treated larvae were bled by cutting off the abdominal prolegs with a pair of sterile scissors. Hemolymph was collected in a 1.5 ml cold (ca. 5℃) tube, mixed with 0.04 % trypan blue dye (1:1, v/v), and incubated for 5 min at 25℃. The hemocytes, which did not absorb the dye, were regarded as living cells ('dye-exclusion method') (Ribeiro *et al.*, 1999) and counted with a hemocytometer (Superior, Germany) under a phase contrast microscope (Olympus, Japan).

Morphology of hemocyte in *S. exigua* infected with *X. nematophila*

For external morphology, the hemocytes isolated from the larvae infected with *X. nematophila* at the different times were observed under the DIC mode of confocal razor microscope (Olympus, Japan). The hemocyte samples (ca. 50 µl) to be observed were collected in 1.5 ml cold (ca. 4℃) tube from the larvae and then mixed with 0.04 % methylen blue dye (1:1, v/v).

For ultrastructural study, the hemocytes were isolated from the larvae infected with *X. nematophila* at each post-injection time (0, 4, 8, and 12 h). Isolated hemocytes were fixed initially for 2 h at 25℃ in 0.2 M sodium phosphate buffer containing 3 % glutaraldehyde, post-fixed for 2 h in 2 % OsO_4 in the same buffer, and exposed overnight to 0.1 % aqueous uranyl acetate. Dehydration was performed with 30-100 % ethyl alcohol in six steps for each 30 min. The dehydrated hemocytes were embedded in spurr resin (EMS, WA, USA) and incubated at 70℃ for 18 h. Ultra-thin sectioning of 80 nm was performed by ultramicrotome with a glass knife. The hemocyte samples were double-stained with 2% uranyl acetate and 0.5% lead citrate for 15 min and 7 min on grids, respectively. The stained grids were examined on transmission electron microscope (EM 109, Zeiss, Germany) for ultrastructure of hemocytes.

For analysis of apoptosis determined by cell contour morphology, the hemocytes were observed under a phase contrast microscope to see the membrane blebbings or the apoptotic vesicles.

Glucose dehydrogenases (GLD) activity

GLD activity was measured by the method of Lovallo *et al.* (2002). Briefly, an extracted hemolymph sample (10 µl) was mixed with 990 µl of 2, 6-dichloroindophephenol (DCIP) reagent (50 µM Tris-HCl pH 7.0, 1 M D-glucose, 1 µM DCIP) and incubated for 45 min at 28℃. GLD activity was determined by change in absorbance per 5 minute at 600 nm on double-beam spectrophotometer (Kontron, Italy).

Genomic DNA (gDNA) analysis of the hemocytes

Hemolymph was extracted from the larvae injected with 2 µl of bacterial suspension (10^8 cfu/ml) or sterilized saline. Hemocytes were collected from the hemolymph by centrifugation at 8,000 rpm for 5 min at 4℃ and lysed by the extraction buffer containing 50 mM Tris (pH 8.0), 0.5 % sarkosyl, 0.5 mg/ml proteinase K, and 1 mM EDTA at 50℃ for 3 h. RNase A (0.5 µg/ml) were added and incubated at 50℃ for overnight. The gDNAs were extracted by phenol-chloroform-isoamyl alcohol. gDNAs were run in 0.9 % agarose gel at 70 volts. Both gDNAs of the infected or non-immunized larvae were visualized and compared under UV illumination after staining with ethidium bromide.

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