Effect of nucleopolyhedrovirus infection on antibacterial activity and antibacterial peptide production in larvae of the silkworm, *Bombyx mori* (Lepidoptera: Bombycidae)

Seiichi Furukawa,1 Aki Sagisaka,1 Hiromitsu Tanaka1 and Minoru Yamakawa1,2,*

1 Innate Immunity Research Unit, National Institute of Agrobiological Sciences; Tsukuba 305–8634, Japan
2 Graduate School of Life and Environmental Sciences, University of Tsukuba; Tsukuba 305–8572, Japan

(Received 25 May 2006; Accepted 27 September 2006)

**Abstract**

Effects of nucleopolyhedrovirus (NPV) on antibacterial peptide gene expression and peptide production in *Bombyx mori* larvae remain unclear. Antibacterial activity was first examined with the hemolymph from *B. mori* larvae infected with *B. mori* NPV (BmNPV) and injected with bacterial lipopolysaccharide (LPS) 72 h postinfection (p.i.). No antibacterial activity was detected. Expression of four antibacterial peptide genes were next analyzed in *B. mori* larvae infected with BmNPV. Although antibacterial peptide genes were activated upon injection of LPS in the larvae infected with BmNPV, none were triggered by BmNPV alone. Production of the antibacterial peptide cecropin B was next examined by Western blotting. Cecropin B was not detected from 72 to 96 h p.i. The absence of cecropin B was coincident with an increase in BmNPV cysteine proteinase (CP) activity. Cecropin B production was confirmed at 96 h p.i. in the larvae infected with CP-deficient BmNPV, indicating that the antibacterial peptides are degraded by BmNPV-CP at a very late stage of viral infection. These results suggest that BmNPV neither activates nor suppresses gene expression of antibacterial peptides and that absence of antibacterial activity is due to BmNPV-CP in *B. mori* larvae.

**Key words:** *Bombyx mori*; NPV; gene expression; peptide production; antibacterial peptides

**INTRODUCTION**

Antibacterial responses in the insect immune system have been the most extensively studied (Hoffmann et al., 1999; Stöven et al., 2000; De Gregorio et al., 2002). On the other hand, insect immunity against viral pathogens still remains obscure (Washburn et al., 1996). Of many viruses, *Bombyx mori* nucleopolyhedrovirus (BmNPV) is the most important virus in the sericultural industry, because farmers often suffer severe economic losses caused by this virus. Nonetheless, studies on immune responses of the silkworm against BmNPV are still in their infancy.

The immune reactions against NPV can be divided into two phases depending on the infection cycle: primary defense against occlusion-derived virus (ODV) in the alimentary canal and secondary defense against the budded virus (BV) in other tissues such as the fat body, trachea and hemocytes. Recently, a lipase and a serine protease from the digestive juice of *B. mori* larvae have been demonstrated to show antiviral activity against BmNPV (Ponnuvel et al., 2003; Nakazawa et al., 2004). These two digestive enzymes are speculated to play an important role in the primary defense. Concerning the secondary defense stage, it has been shown that apoptosis is initiated during NPV replication in insect cells and specific viral gene products, P35 and P49, are responsible for blocking the apoptotic response (Clem et al., 1991; Zoog et al., 2002). Moreover, it was previously demonstrated in the giant silk moth *Hyalophora cecropia* that an *Autographa californica* baculovirus (AcNPV) neither activates NF-κB nor induces *Cecropin, Attacin* or *Lysozyme* gene expression (Sun and Faye, 1992). In addition, it has been reported that gene expression of hemolin, one hemolymph protein, is induced by...
NPV infection in the Chinese oak silk moth _Antheraea pernyi_ and that no _Attacin_ gene expression or antibacterial activity is observed (Hirai et al., 2004).

We have concentrated our studies on antibacterial peptides from _B. mori_ (Yamakawa and Tanaka, 1999). Some antibacterial peptides are known to not only have antibacterial properties but also antifungal and antiparasitical properties (Kimbrell, 1991). However, whether antibacterial peptides are involved in defense reactions against viral infection has not been extensively analyzed. In this study, we focused on the effects of BmNPV on gene expression of _B. mori_ antibacterial peptides to determine if BmNPV infection causes up or down regulation of these peptides genes. We also investigated whether BmNPV induces or shuts down the production of cecropin B by using antibodies against the peptide.

**MATERIALS AND METHODS**

_Insects._ _B. mori_ larvae (Tokai×Asahi strain) were reared on artificial diet (Nihonnosanko) at 25°C under a controlled environment (11 h light and 13 h dark). Fifth instar larvae (1 d) were used in the experiments.

_Infection of _B. mori_ larvae with BmNPV-BV, injection of lipopolysaccharide (LPS) and collection of hemolymph._ Larvae were first infected with BmNPV-BV (8×10⁵ TCID₅₀/larva). Naive larvae were also used as controls. Lipopolysaccharide (Sigma, 20 μg/larva) was injected into the larval 72 h postinfection (p.i.). As a control saline instead of LPS was injected. Larvae were kept at 25°C for 24 h. Hemolymph was collected by cutting off a leg into ice-cooled tubes containing phenylthiourea (Sigma, 50 μg/ml). For kinetic experiments, hemolymph was collected 0, 4, 8, 12 and 24 h after LPS injection from larvae infected with BmNPV (72 h p.i.). Hemolymph was heat-treated at 95°C for 15 min and centrifuged at 10,000×g for 5 min. One microliter of the supernatant was loaded onto the gel with size markers. As a positive control, 1 μg of chemically synthesized cecropin B (Kadono-Okuda et al., 1995) was loaded onto the gel. Preparation of polyclonal antibodies against cecropin B from _B. mori_ was as reported previously (Nakazawa et al., 2003). Western blotting was conducted as described by Sambrook and Russel (2001).

_Infection of _B. mori_ larvae with BmNPV-BV, injection of lipopolysaccharide (LPS) and collection of hemolymph._ Larvae were first infected with BmNPV-BV (8×10⁵ TCID₅₀/larva). Naive larvae were also used as controls. Lipopolysaccharide (Sigma, 20 μg/larva) was injected into the larval 72 h postinfection (p.i.). As a control saline instead of LPS was injected. Larvae were kept at 25°C for 24 h. Hemolymph was collected by cutting off a leg into ice-cooled tubes containing phenylthiourea (Sigma, 50 μg/ml). For kinetic experiments, hemolymph was collected 0, 4, 8, 12 and 24 h after LPS injection from larvae infected with BmNPV (72 h p.i.). Hemolymph was heat-treated at 95°C for 15 min and centrifuged at 10,000×g for 5 min. One microliter of the supernatant was loaded onto the gel with size markers. As a positive control, 1 μg of chemically synthesized cecropin B (Kadono-Okuda et al., 1995) was loaded onto the gel. Preparation of polyclonal antibodies against cecropin B from _B. mori_ was as reported previously (Nakazawa et al., 2003). Western blotting was conducted as described by Sambrook and Russel (2001).

**Measurement of CP activity._** _B. mori_ larvae were infected with BmNPV-BV and hemolymph collected at different time intervals (0, 24, 48, 72 and 96 h p.i.). Infection conditions were the same centrifuged, washed once in 10 mm sodium phosphate buffer (pH 7.4) and suspended in the same buffer. Melted LB broth (10 ml) containing agar and 2–6×10⁵ mid-exponential-phase cultured bacteria was poured into sterile Petri dishes (9 cm diameter). Samples (3 μl each) were applied to wells (2 mm diameter) in the LB agar plates. The plates were incubated at 37°C overnight. Antibacterial activity was detected as clear inhibition zones after incubation.

**Northern blot analysis._** Total RNA was extracted from the fat body of larvae at different time intervals (0, 4, 8, 12 and 24 h) after LPS injection. Northern blot analysis (Sambrook and Russel, 2001) was conducted with 3.5 μg of the total RNA and specific probes labeled with DIG-conjugated dUTP (Roche Diagnostics) using cDNAs for cecropin B (Kato et al., 1993), attacin (Sugiyama et al., 1995), lebocin (Chowdhury et al., 1995) or moricin (Furukawa et al., 1997). Polyhedrin gene expression of BmNPV was also analyzed with a specific probe prepared by PCR using BmNPV genomic DNA as a template and forward and reverse primers (5’-AAGCGCAAGAAGCACCTAGT-3’ for forward primer and 5’-TGTACTCGCTGTGGAT-GTTC-3’ for reverse primer taken from the genomic sequence data base, GenBank accession no. L33180).

**Western blot analysis._** The larvae were first infected with BmNPV-BV or cysteine protease (CP)-deficient BmNPV-BV 72 h before LPS injection. As controls, naive larvae were injected with LPS. Hemolymph was collected at the indicated time intervals (0, 4, 8 and 24 h) after LPS injection. Conditions for BmNPV-BV infection and LPS injection were the same as described above. The hemolymph was heat-treated at 95°C for 15 min and centrifuged at 10,000×g for 5 min. One microliter of the supernatant was loaded onto the gel with size markers. As a positive control, 1 μg of chemically synthesized cecropin B (Kadono-Okuda et al., 1995) was loaded onto the gel. Preparation of polyclonal antibodies against cecropin B from _B. mori_ was as reported previously (Nakazawa et al., 2003). Western blotting was conducted as described by Sambrook and Russel (2001).
as described above. Hemolymph was collected into a tube with phenylthiourea to prevent melanization. Activity of CP was measured with 10 μl of the hemolymph using Azocoll (Sigma) as a substrate according to the methods of Kobayashi et al. (1985).

Degradation of cecropin B in the hemolymph from larvae infected with BmNPV-BV. B. mori larvae were infected with BmNPV-BV or CP-deficient BmNPV-BV and hemolymph was collected at different time intervals (24, 48, 72 and 96 h). The hemolymph samples (6 μl each) were mixed with hemolymph (1 μl) containing endogenous cecropin B from larvae 12 h after injection with LPS (20 μg/larva). The mixtures were incubated at room temperature for 1, 4 and 16 h, then electrophoresed and Western blotting conducted.

RESULTS

Effect of BmNPV infection on the induction of antibacterial activity

Antibacterial activity against E. coli of the hemolymph from B. mori larvae infected with BmNPV-BV and then injected with saline instead of LPS was analyzed first by the inhibition zone assay. Antibacterial activity was not detected in the larvae infected with BmNPV (Fig. 1A), showing BmNPV does not induce activity. Antibacterial activity was next examined using hemolymph from larvae infected with BmNPV-BV and then injected with saline instead of LPS 72 h p.i. as a control, larvae were injected with saline instead of BmNPV. BV(+) and BV(−) denote larvae infected with BmNPV or injected with saline, respectively. Panel B: Time course of induction of antibacterial activity in larvae infected with BmNPV (BV(+)). Naive larvae (BV(−)) were used as controls. Hemolymph was collected 0, 4, 8, 12 and 24 h (72, 76, 80, 84 and 96 h p.i., respectively) after LPS injection.

Effect of BmNPV infection on expression of antibacterial peptide genes

Whether gene expression and production of antibacterial peptides were inhibited or these peptides were degraded were next examined. Gene expression of four B. mori antibacterial peptides that show antibacterial activity against E. coli was first analyzed by Northern blotting in larvae infected with BmNPV. Total RNA was extracted from the fat body, which is the main tissue for expression of antibacterial peptide genes (Kato et al., 1993; Chowdhury et al., 1995; Sugiyama et al., 1995; Taniai et al., 1996; Furukawa et al., 1997, 1999). Although no activation of antibacterial peptide genes encoding cecropin B (Kato et al., 1993; Taniai et al., 1995), attacin (Sugiyama et al., 1995), lebocin (Chowdhury et al., 1995; Furukawa et al., 1997) and moricin (Furukawa et al., 1999) by BmNPV was observed, these peptide genes were induced normally with time (4, 8, 12 and 24 h) by LPS in the larvae that were infected with BmNPV 72 h before LPS injection (Fig. 2). Under these conditions polyhedrin gene was expressed nor-
mally and BmNPV itself showed no ability to activate antibacterial peptide genes. The induction of these four antibacterial peptide genes was also observed in the uninfected control larvae (Fig. 2). These results indicated that expression of the four antibacterial peptide genes are not affected by BmNPV in the larvae 76–96 h p.i., suggesting that down-regulation of antibacterial peptide genes by BmNPV infection does not occur in the larvae at the late stage of infection.

Effect of BmNPV infection on the production of cecropin B

Of four types of antibacterial peptides from B. mori, cecropin B is the only peptide for which an antibody is available (Nakazawa et al., 2003). Therefore, we focused on cecropin B to clarify the effects of BmNPV on the production of antibacterial peptides. Western blot analysis was conducted using polyclonal antibodies against cecropin B and hemolymph protein samples from larvae infected with BV. The experimental conditions were the same as the Northern blot analysis. No cecropin B was detected at 0, 4, 8, 12 and 24 h post LPS-injection in the larvae infected with BV, whereas hemolymph samples from saline-injected control larvae showed clear signals for cecropin B showing a typical induction pattern of the peptide synthesis, namely a gradual increase up to 12 h post LPS-injection and a decrease thereafter (Fig. 3). These results suggest either that the protein synthesis shutdown occurs in the larvae 76–96 h p.i. or that synthesized cecropin B is completely degraded in larvae at this infection stage.

Degradation of cecropin B by CP of BmNPV

Cysteine proteinases of BmNPV and AcNPV are hypothesized from deletion experiments of the viral CP gene to be involved in the destruction of insect tissues during the later stages of pathogenesis (Ohkawa et al., 1994; Slack et al., 1995). This led us to examine the effect of CP-deficient BmNPV on the synthesis of cecropin B. Cysteine proteinase activity in the hemolymph of larvae infected with BmNPV-BV was first measured to
clarify the relationship between the increment of CP activity and the degradation of antibacterial peptides. Hemolymph was collected from larvae 0, 24, 48, 72 and 96 h p.i. CP activity sharply increased during 72 to 96 h p.i. (Fig. 4). On the contrary, hemolymph from larvae injected with saline or infected with CP-deficient BmNPV (Ohkawa et al., 1994) did not show CP activity (Fig. 4), which is consistent with previously reported results (Suzuki et al., 1997). Hemolymph containing endogenous cecropin B was then incubated for 1, 4 or 16 h in vitro with hemolymph collected from larvae infected with BmNPV or CP-deficient BmNPV at different time intervals (24, 48, 72 and 96 h p.i.). Degradation was confirmed in the hemolymph collected from larvae 96 h p.i. with wild type BmNPV but not with CP-deficient BmNPV (Fig. 5), indicating coincidence of the timing of cecropin B degradation with increased CP activity (Fig. 4). These results suggest that BmNPV-CP possesses the ability to degrade secreted cecropin B in B. mori larvae.

**Production of cecropin B at the late stage of infection with CP-deficient BmNPV**

Western blot analysis was conducted to examine the production of cecropin B in B. mori larvae at the late stages of the infection (96 h p.i.) with CP-deficient BmNPV-BV. The result showed clear signals, although control hemolymph samples infected with wild type BmNPV-BV did not give the signal (Fig. 6). The results suggest that BmNPV-CP degrades the antibacterial peptides directly in the late stages of infection.

**DISCUSSION**

Invertebrate immune peptides having antibacterial activity are often reported to have antiviral activity. Tachyplesin I from the horseshoe crab was shown to have antiviral activity against vesicular stomatitis virus (Murakami et al., 1991) and human immunodeficiency virus (HIV) (Morimoto et al., 1991). Melittin and cecropin from insects have been shown to suppress replication of HIV by inhibiting viral gene expression (Wachinger et al., 1998) and alloferon from the blow fly *Calliphora vicinia* showed antiviral activity against influenza viruses A and B (Chernysh et al., 2002). Although the horseshoe crab and insects are not hosts for these viruses, these results suggest that some invertebrate antibacterial peptides have antiviral activity. However, it remains unclear whether these viruses can induce antibacterial peptide gene expression and peptide production in the horseshoe crab and insects.
Our results indicate that BmNPV neither induces antibacterial activity nor activates antibacterial peptide genes in B. mori larvae and that activation of antibacterial peptide genes by LPS in BmNPV-infected larvae are normal. However, degradation of the antibacterial peptide by BmNPV-CP occurs at the very late stage of infection (72–96 h p.i.) in B. mori larvae. To our knowledge, this is the first report of the effects of NPV infection on gene expression and production of antibacterial peptides in B. mori larvae. These results are consistent with data that NPV does not activate Attacin genes, nor induce antibacterial activity in A. pernyi (Hirai et al., 2004). Taken together with the results on H. cecropia (Sun and Faye, 1992) and A. pernyi (Hirai et al., 2004), our results suggest that baculoviruses do not provoke the NF-κB pathway which plays an important role in antibacterial peptide gene expression in lepidopteran insects. On the other hand, Hirai et al. (2004) speculated that Hemolin gene expression is induced upon virus infection by a pathway that does not involve NF-κB. It is important to examine antiviral activity of B. mori antibacterial peptides against BmNPV to determine whether antibacterial peptides are involved in immune reactions against BmNPV infection.

In B. mori, comparative expressed-sequence-tag (EST) analysis of differential gene expression profiles was conducted in BmNPV-infected BmN cells (Okano et al., 2001). The detailed analysis of host derived ESTs showed that expression of most of the major host genes is reduced with BmNPV infection (Okano et al., 2001). However, the expression of the mitochondria-encoded genes, cytochrome b oxidase, cytochrome c oxidase 1 and 3, is not affected by viral infection (Okano et al., 2001). Consistent with this, a previous report showed that expression of the Spodoptera frugiperda cytochrome oxidase 3 gene was stable until at least 24 h.p.i. during infection with AcNPV (Ooi and Miller, 1988). On the other hand, the ATP/ADP translocase and mitochondrial phosphate carrier protein genes of B. mori, that are encoded by the host nuclear genome and play important roles in mitochondrial metabolism, showed decreasing expression during infection (Okano et al., 2001). These observations suggest that BmNPV infection does not affect mitochondria-specific transcription during BmNPV infection. So far, the effects of host gene expression and protein synthesis by NPV were examined in most cases using culture cells. It still remains unclear whether down-regulation of global protein gene expression and shutdown of global protein synthesis by NPV observed in culture cells (Mazzacano et al., 1999; Okano et al., 2001) also occur in vivo. Analyses of proteins other than antibacterial peptides in NPV-infected larvae are necessary to compare the results from NPV-infected cultured cells.

ACKNOWLEDGEMENTS

We thank Dr. Shogo Matsumoto at RIKEN (The Institute of Physical and Chemical Research) for providing a cysteine proteinase-deficient BmNPV mutant (orf 104). This work was supported in part by a Grant-in-Aid (Insect Technology Program) from the Ministry of Agriculture, Forestry and Fisheries of Japan.

REFERENCES


Cecropin B in NPV-Infected B. mori Larvae


