Lethal effects of *Spodoptera exigua* nucleopolyhedrovirus isolated in Shiga Prefecture, Japan, on larvae of the beet armyworm, *Spodoptera exigua* (Lepidoptera: Noctuidae)

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Abstract

*Spodoptera exigua* nucleopolyhedrovirus (SpeiNPV) isolated in Shiga Prefecture, Japan, was bioassayed with all five stages of the beet armyworm to determine lethal doses and survival times at three rearing temperatures: 20°C, 25°C, and 30°C. Rearing temperature did not significantly affect the susceptibility of *S. exigua* larvae to SpeiNPV. The median lethal dose (LD$_{50}$) per insect for neonate, second-, third-, fourth-, and fifth-stage larvae was 2.5, 11.2, 5.5, 32.4, and 181.8 polyhedral occlusion bodies (POBs), respectively. These data demonstrate that this SpeiNPV isolate is extremely infectious, even to later stages of *S. exigua* larvae. The LD$_{50}$ values corrected per unit larval body weight tended to decrease with larval age, from about 60 POBs for neonates to just a few POBs for each of the last three stages. This pattern is atypical for NPV-lepidopteran host insect systems, emphasizing the high infectivity of this NPV isolate. Rearing temperature and larval stage at the time of viral treatment significantly affected survival times, while viral dosage did not. Survival time decreased with increased rearing temperature, and increased with larval stage at the time of treatment. Median survival time (ST$_{50}$) was between 3 to 14 d, depending on the rearing temperature and larval stage at the time of viral treatment.

Key words: Nucleopolyhedrovirus, lethal dose, survival time, *Spodoptera exigua*

INTRODUCTION

Nucleopolyhedroviruses (NPVs) are one of the most important entomopathogens being developed as microbial insecticides for pest control programs in forestry, agriculture, and horticulture (Hunter-Fujita et al., 1998); they are safe for non-target organisms, environmentally persistent, and highly virulent against target insect pests. In addition to their advantages as microbial insecticides, they can also be used in more ecologically based strategies, such as inoculative releases and environmental manipulation, given some ecological understanding of host-NPV interrelationships. Since it is clear that NPVs have a substantial impact on the natural population dynamics of insects (Fuxa and Tanada, 1987; Elkinton and Liebhold, 1990; Myers and Rothman, 1996), efforts are being made to increase the currently limited knowledge of NPV ecology (Cory et al., 1997).

Successful application of control agents requires information about biological activities, such as dose-mortality, time-response relations, sublethal effects, and changes in susceptibility as host development proceeds. This information should significantly affect host-pathogen interrelationships, because virulence and pathogenicity are components of virus transmission (Andreadis, 1987).

*Spodoptera exigua* is an extremely polyphagous insect pest that is widely distributed in tropical, subtropical, and temperate regions of the world. Its hosts include more than 200 plant species, including economically important crops, such as cabbage, radish, and tomato (Smits, 1987). Although chemical insecticides have been used to control *S. exigua*, this method has become difficult since the species has developed resistance to many of the chemical agents in use (Brewer and Trumble, 1989). In Japan, *S. exigua* has seriously damaged crops, particularly welsh onion, since the early 1980s (Horikiri, 1986). Effective control of *S. exigua* by *S. exigua* NPV (SpeiNPV) has been...
demonstrated in the field (Gelernter et al., 1986; Kolodny-Hirsch et al., 1993) and in greenhouses (Smits, 1987). A SpeiNPV isolate from Florida has already been formulated and marketed (Hunter-Fujita et al., 1998). This SpeiNPV-based formulation is one current alternative to using chemical insecticides in *S. exigua* control in Japan. However, the release of exotic agents could entail some risks, such as unexpected environmental impact and gene transfer to native organisms (Muñoz et al., 1997). In addition, it seems likely that NPVs can locally adapt to their host (Shapiro et al., 1984; Fuxa, 1987). Fuxa (1987) documented that *S. frugiperda* NPV from distant sources was less virulent than NPV isolated where the insects were collected. Therefore, it is of value to study the biological activity of indigenous NPVs to estimate the degree to which viral agents can be developed for local insect control.

In this paper, we determined the lethal doses and survival times of SpeiNPV isolated in Shiga Prefecture, Japan, for all five stages of *S. exigua* larvae at three rearing temperatures: 20°C, 25°C, and 30°C. This isolate showed surprisingly high infectivity against *S. exigua* larvae.

**MATERIALS AND METHODS**

**Insect.** *S. exigua* was originally supplied as eggs by the Agro-Kanesho Co., Ltd. (Tokorozawa, Saitama) and reared continuously in our laboratory. The insects were originally collected at Kagoshima Prefecture. Larvae were reared until pupation on INSECTA artificial diet (Nihon Nosan-Kogyo Co., Ltd., Yokohama, Kanagawa) in plastic cases (15 cm diameter, 10 cm height) at 25°C with a 16-h photoperiod. Adults were transferred into a paper bag (8×15×20 cm) with a crude sugar solution for food. In order to preclude the possible effect of rearing density on baculovirus susceptibility (Kunimi and Yamada, 1990; Goulson and Cory, 1995; Wilson and Reeson, 1998), larvae used in the bioassay were reared in groups of 200, 100, 80, and 50 during the first, second, third, and fourth stages, respectively.

**Virus.** The virus used in this study was NPV isolated from *S. exigua* larvae collected at a welsh onion field in Shiga Prefecture (SpeiNPV #1, Kondo et al., 1994), kindly supplied by Dr. A. Kondo of the Shiga Prefecture Agricultural General Center. According to DNA endonuclease analysis, its genomic DNA is very similar to that of standard *S. exigua* NPV strains (Kondo et al., 1994). The virus was produced in fourth stage *S. exigua* larvae at 25°C. Viral polyhedral occlusion bodies (POBs) were purified by homogenization and density gradient centrifugation, and suspended in 0.05 M sodium phosphate buffer (pH 7.7). POBs in the suspension were counted with a Thoma hemocytometer and stored at 4°C until use.

**Bioassay.** Larvae were bioassayed using a modified droplet feeding method (Hughes and Wood, 1981; Hughes et al., 1986). Head capsule slippage was used to determine when larvae were beginning to molt. First- and second-stage molting larvae were each transferred into one well of a 96-well tissue culture plate, and third- and fourth-stage molting larvae to a 24-well tissue culture plate, all without food. The newly molted larvae were collected after 20–24 h for the bioassay. Neonate and newly molted larvae were allowed to feed on droplets of POB suspension containing 10% sucrose and 1% blue food coloring (Kyoritsu Food Co., Ltd., Tokyo). As a control, larvae at each stage were fed droplets of the same solution without POB. After ingesting the suspension, larvae were immediately transferred into 30-ml cups containing fresh artificial diet. The volume of suspension ingested by larvae at each stage was determined by the fluorescent dye method of Kunimi and Fuxa (1996) for first- to third-stage larvae, and by the gravimetric method for the fourth and fifth stages. The gravimetric method consisted of individually weighing 20 larvae before and after they drank the droplet of suspension; the difference in weight was divided by the density of the suspension (1.0445). Four doses plus the control were used for the first and third stages, five doses plus the control for the second and fourth stages, and six doses plus the control for the fifth stage. Experiments included 30 larvae per dose per rearing temperature, and were replicated three times for the first to third stages and two times for the fourth and fifth stages. Larvae were reared at 20°C, 25°C, or 30°C with a 16-h photoperiod. To measure mortality, they were observed daily for 10 d for the first stage, for 14 d for the second stage, and until pupation for the other stages. Tissue smears were prepared from dead larvae and examined for the presence of POBs under phase contrast microscopy.
**Data analysis.** The Kaplan-Meier product limit estimator was used to estimate median survival time (ST$_{50}$) per treatment. The significance of differences among survival times was tested using the survival time module, and pairwise comparisons made using the log rank test, in the STATISTICA computer program (Anonymous, 1995). Insects that survived the experiments were excluded from the survival time analyses. It is important to note that NPVs may cause persistent infections, during which the virus replicates, but hosts do not exhibit any symptoms of infection (Hughes et al., 1997). Survivors in this study likely would not have died from NPV infections even if the experiments were continued for a longer period. Thus, our analysis of survival times examined only the acute effects of overt infection during the larval stages. Percent mortality data were arcsin square-root transformed and analyzed using ANOVA. Mortality data were analyzed by probit analysis (Finney, 1978) using the microcomputer program POLO-PC (Russell et al., 1977).

**RESULTS**

**Volumes ingested**

The volume ingested by larvae of each stage increased with host stage at the time of treatment. Mean volumes ingested by neonate to fifth stage larvae were 0.01±0.001 (mean±SE), 0.12±0.01, 0.48±0.04, 3.87±0.22, and 13.87±1.04 µl, respectively. Coefficients of variation of ingested volumes were 34.94, 35.67, 38.45, 25.30, and 35.34% for first to fifth stage larvae, respectively.

**Infectivity**

Figure 1 summarizes mortality in *Spodoptera exigua* larvae that ingested solutions of varying POB concentrations, at three rearing temperatures. The infectivity of SpeiNPV was significantly affected by viral concentration (first stage larvae: $F=245.642$, df=3, 24, $p<0.01$, second stage larvae: $F=199.740$, df=4, 30, $p<0.01$, third stage larvae: $F=172.880$, df=3, 24, $p<0.01$, fourth stage larvae: $F=107.762$, df=4, 15, $p<0.01$, fifth stage larvae: $F=101.104$, df=5, 18, $p<0.01$), but not rearing temperature (first stage larvae: $F=1.664$, df=2, 24, $p>0.05$, second stage larvae: $F=0.820$, df=2, 30, $p>0.05$, third stage larvae: $F=1.485$, df=2, 24,

![Fig. 1. Mortality in *Spodoptera exigua* larvae that ingested solutions of varying SpeiNPV concentrations, at three rearing temperatures. Neonate or newly molted larvae were allowed to feed on droplets of polyhedral suspensions containing 10% sucrose, and then were incubated at 20°C, 25°C, or 30°C.](image-url)
Survival times

Median survival times (ST50) for *S. exigua* larvae infected with SpeiNPV are summarized in Table 3. Survival time was strongly influenced by rearing temperature, regardless of host stage at the time of viral treatment (Tables 3, 4), but not by viral concentration (Table 3, first stage larvae: $\chi^2=1.087$, df=3, $p>0.05$, second stage larvae: $\chi^2=1.666$, df=4, $p>0.05$, third stage larvae: $\chi^2=2.622$, df=3, $p>0.05$, fourth stage larvae: $\chi^2=2.861$, df=4, $p>0.05$, fifth stage larvae: $\chi^2=5.909$, df=5, $p>0.05$). Survival time decreased significantly with increasing rearing temperature: survival times were significantly shorter at 30°C than at 25°C, and shorter at 25°C than at 20°C (Tables 3, 4). Additionally, survival times significantly increased with host stage at the time of viral treatment (Table 3, $\chi^2=411.637$, df=4, $p<0.00001$).

**DISCUSSION**

The recent development of droplet feeding, a novel oral bioassay technique, allows us to quantify the biological activities of entomopathogens more precisely than ever (e.g., Hughes et al., 1983; Sait et al., 1994; Kunimi et al., 1997), because it allows for synchronized ingestion of relatively consistent amounts of pathogen. Previous studies with lepidopteran insects showed coefficients of variation of...
ingested volumes ranging from 30 to 60% (Hughes and Wood, 1986; Smits, 1987; Ridout and Fenlon, 1991; Kunimi and Fuxa, 1996). The coefficients of variation in this study were well within this range. Accordingly, our bioassay likely yielded precise data with which to examine the response of test insects.

The SpeiNPV used in our study exhibited extremely high infectivity. The LD<sub>50</sub> value for neonate insects was a few POBs, close to the theoretical minimum (Huber and Hughes, 1984). Smits (1987) reported that SpeiNPV isolated from California was highly infective to <i>S. exigua</i> larvae, with an LD<sub>50</sub> of 3 POBs per neonate insect. Low LD<sub>50</sub> values have been reported for several other viruses: <i>Heliothis zea</i> SNPV (Allen and Ignoffo, 1969), <i>H. armigera</i> SNPV and MNPV and <i>H. virescens</i> SNPV in their homologous hosts (Hughes et al., 1983), <i>Mamestra brassicae</i> MNPV in <i>Plusia gamma</i> (Allaway and Payne, 1984), and two recombinant and one wild type <i>A. californica</i> MNPV in <i>Pseudoplusia includens</i> (Kunimi et al., 1997).

The LD<sub>50</sub> value for second stage insects was similar to those previously reported for other SpeiNPV isolates (Smits, 1987; Caballero et al., 1992; Hara et al., 1995). According to Smits (1987), the per-insect LD<sub>50</sub> values for third, fourth and fifth stage larvae infected with California-isolated SpeiNPV at 25°C were 39, 102, and 11,637 POBs, respectively. The LD<sub>50</sub> values in our study at 25°C for the

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<sup>a</sup> ST<sub>50</sub> values represent the median of replicates using data calculated by Kaplan-Meier product limit estimator. The values in parentheses indicate days to first death and last death.

<sup>b</sup> Viral polyhedral occlusion bodies/ml.

The SpeiNPV used in our study exhibited extremely high infectivity. The LD<sub>50</sub> value for neonate insects was a few POBs, close to the theoretical minimum (Huber and Hughes, 1984). Smits (1987) reported that SpeiNPV isolated from California was highly infective to <i>S. exigua</i> larvae, with an LD<sub>50</sub> of 3 POBs per neonate insect. Low LD<sub>50</sub> values have been reported for several other viruses: <i>Heliothis zea</i> SNPV (Allen and Ignoffo, 1969), <i>H. armigera</i> SNPV and MNPV and <i>H. virescens</i> SNPV in their homologous hosts (Hughes et al., 1983), <i>Mamestra brassicae</i> MNPV in <i>Plusia gamma</i> (Allaway and Payne, 1984), and two recombinant and one wild type <i>A. californica</i> MNPV in <i>Pseudoplusia includens</i> (Kunimi et al., 1997). The LD<sub>50</sub> value for second stage insects was similar to those previously reported for other SpeiNPV isolates (Smits, 1987; Caballero et al., 1992; Hara et al., 1995). According to Smits (1987), the per-insect LD<sub>50</sub> values for third, fourth and fifth stage larvae infected with California-isolated SpeiNPV at 25°C were 39, 102, and 11,637 POBs, respectively. The LD<sub>50</sub> values in our study at 25°C for the
corresponding stages were lower: 5.5, 32.4, and 181.8 POBs, respectively (Table 1). Thus, the SpeiNPV isolate from Shiga Prefecture was surprisingly active, even against later stages of S. exigua larvae. These differences between Smits’ (1987) work and ours could be due to either differences in test insects or NPV isolates, but it is difficult to tell which. Conversion of these per-insect LD50 values to LD50 values per mg larval body weight emphasized the high infectivity of the Shiga SpeiNPV isolate. The isolate also showed a pattern atypical for NPVs-lepidopteran host insect systems: LD50 values per unit larval body weight tended to decrease with larval stage at the time of viral treatment, from about 60 POBs for neonates to a few POBs for each of the last three stages (Table 2). Generally, the susceptibility of lepidopteran insects to NPVs decreases with stage, as explained by the “dilution effect”: the decrease in susceptibility is related to an increase in larval weight as development proceeds (Briese, 1986). In baculovirus-host systems in which the “dilution effect” can explain changes in susceptibility per stage, absolute LD50 values per insect increase as the host develops, whereas LD50 values per unit larval weight remain constant regardless of the stage at the time of viral exposure (Hochberg, 1991). Our result, however, cannot be explained by the “dilution effect.” The mechanism and ecological cause of this phenomenon need to be addressed in future research.

The slopes of the log dose-probit lines tended to
decrease as host stage at the time of viral treatments increased, indicating that variation in the response of test insects to the bioassay increased with age. This trend is consistent with that found by Smits (1987) for *S. exigua* infected with SpeiNPV from California. The increase in response variability could be due to increased variability either in ingested volume as host stage increased or in host susceptibility. Since the coefficient of variation for the ingested volume of each stage did not increase, the increased variability in response was most likely driven by increasing variability in host susceptibility. Decreasing slopes of log dose-probit lines with increasing host age has also been reported for *M. configurata* NPV (Bucher and Turnock, 1983) and *M. brassicae* NPV (Evans, 1983) in their homologous hosts. However, slopes of log dose-probit lines were independent of host age for *Hyphantria cunea* NPV (Boucias and Nordin, 1977), *H. armigera* NPV (Teakle et al., 1985), and *H. punctigera* NPV (Teakle et al., 1986) in their homologous hosts. The relationship between slopes of log dose-probit lines and host age depends on the combination of insect and NPV species.

Survival time of SpeiNPV-infected larvae was a function of either rearing temperature or larval stage at the time of viral treatment. There seems to be a relationship between multiplication of the virus and survival time. NPVs kill their hosts quickly after multiplication plateaus (Shapiro et al., 1981; Vail and Collier, 1982; Shihe, 1989), and SpeiNPV is no exception (Takatsuka and Kunimi, unpublished data). Higher rearing temperature was related to shorter survival time with SpeiNPV, as in previous studies with other combinations of lepidopteran hosts and NPVs (Ignoffo, 1966; Tvermyr, 1969; Stairs, 1978; Boucias et al., 1980; Johnson et al., 1982). It seems likely that viral multiplication is related to developmental rates of host larvae, driving the relationship between survival time and host developmental rates (Hoover et al., 1998). The shorter survival times at higher temperatures seen in our study may be due in part to the fact that higher temperatures hasten developmental rates of host larvae, and, therefore, hasten multiplication of the virus. Gelernter and Federici (1986) reported a 2.5-d lethal time (LT$_{50}$) at 30°C for *S. exigua* neonate larvae infected with SpeiNPV isolated from California; Smits (1987) found an LT$_{50}$ value of 3.2 d for neonates at 25°C. Thus, at least for *S. exigua* neonates infected with SpeiNPV, there is some evidence that higher temperatures produce shorter survival times. Our results are consistent with this evidence, and suggest that it is true for all five stages. Our data also demonstrated that insects at later stages tend to have longer survival times, as is generally accepted. Smits (1987) also reported this trend for *S. exigua* infected with SpeiNPV from California. It is reasonable to infer that viruses that infect larger, later stage larvae have more time to multiply and plateau within their hosts. In fact, SpeiNPV that infected second stage *S. exigua* larvae reached a plateau 4 d post-infection, while SpeiNPV that infected fourth stage *S. exigua* larvae needed 5 d to reach a plateau (Takatsuka and Kunimi, unpublished data).

Viral doses had no effect on survival times of *S. exigua* larvae infected with SpeiNPV from Shiga Prefecture, regardless of stage at the time of viral treatment. Smits (1987), however, reported that LT$_{50}$ values for *S. exigua* infected with SpeiNPV isolated from California tended to decrease with increasing viral dose. The pattern Smits (1987) found has been reported for other NPV-lepidopteran insect systems, such as *Autographa californica* MNPV in *Trichoplusia ni*, *T. ni* MNPV in *T. ni*, and *H. zea* NPV in *H. zea* (van Beek et al., 1988). Huber and Hughes (1984) hypothesized that, at lower concentrations, most nuclear polyhedrosis in individual test insects begins with infection by a single or few virions, which require more time to multiply and kill the insect. In many cases, however, the effect of viral concentration on survival time is weak, with survival reduced by 1 d or less. Since we analyzed the data using nonparametric methods, and collected it only once a day, we may not have been able to detect such small differences in survival time among viral concentrations. For *Agrotis segetum* MNPV in *A. segetum*, however, lethal time was independent of viral concentration (Allaway and Payne, 1984). The time-mortality response should be further studied using appropriate techniques, observation intervals, and statistics (Farrar and Ridgway, 1998).

SpeiNPV from Shiga Prefecture had higher infectivity against *S. exigua* larvae, and almost the same killing time, compared to those in previous studies of SpeiNPV biological activity in which *S. exigua* was effectively controlled in the field (Gel-
erter et al., 1986) and greenhouse (Smits, 1987). Based on our data, the SpeiNPV from Shiga is a promising candidate for a control agent of *S. exigua* in Japan. In Smits’ study (1987), feeding by larvae of the first two stages explained about only 0.7% of the total consumption of chrysanthemum foliage, whereas 4%, 20%, and 75% occurred during the third, fourth, and fifth stages, respectively. A similar result was obtained for *S. exigua* larvae feeding on cabbage (Takatsuka and Kunimi, unpublished data). When these per-stage food consumption rate data are combined with the lethal dose data from our study, the last three stages appear to have a greater risk of infection per application rate. However, NPVs are usually applied to young larvae, in order to minimize damage to crops that are of high economic value. In addition, *S. exigua* larvae seem to invade the leaf blade of welsh onions immediately after hatching (Kawai et al., 1993). Therefore, particularly for welsh onion, precise timing of application, e.g., coincidental with larval hatch, is required. Nonetheless, the surprisingly high infectivity of SpeiNPV from Shiga Prefecture against later insect stages could prove useful for suppressing *S. exigua* populations, because larvae of later stages that have survived an initial NPV application would still be susceptible to infection.

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