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NOTE

Insecticidal Efficacy of a Recombinant Baculovirus Expressing JHE-KK, a Modified Juvenile Hormone Esterase

In the Lepidoptera, the titer of juvenile hormone is regulated in part by juvenile hormone esterase (JHE), and development depends upon both the timely expression of JHE and its rapid removal from the hemolymph by the pericardial cells. Mutation of two lysine residues in JHE resulted in decreased efficiency of lysosomal targeting in the pericardial cells (Bonning *et al.*, 1997). Lys29 and Lys524 were mutated to arginine and three modified forms of JHE were produced: JHE-29 and JHE-524 with the single mutations and JHE-KK with both mutations. The modified JHEs were expressed in insect cells by using the recombinant baculovirus *Autographa californica* multiple nucleocapsid nucleopolyhedrovirus (AcMNPV). The lysine mutations did not significantly affect the kinetic parameters of the enzymes, the *in vitro* or *in vivo* baculovirus expression levels, or the rate of removal of the enzymes from the hemolymph by the pericardial cells (Bonning *et al.*, 1997). Here we report on the insecticidal effects of the recombinant baculoviruses expressing modified JHEs on two major insect pest species, the cabbage looper *Trichoplusia ni* Hübner and the tobacco budworm *Heliothis virescens* F.

For comparison of the median survival time of larvae infected with wild-type AcMNPV clone C6 with those of larvae infected with the recombinant viruses AcJHE, AcJHE-29, AcJHE-524, or AcJHE-KK, neonate larvae of *T. ni* and *H. virescens* were infected with 1000 or 2000 pibs/μl, respectively, by droplet feeding (Hughes *et al.*, 1986). Following infection, larvae were transferred to cups of semi-synthetic diet (Hunter *et al.*, 1984; BioServ) at 26°C, 16L:8D, and mortality was monitored every 4, 6, or 8 h according to the mortality rate. Thirty larvae were infected per bioassay for each virus, and bioassays were replicated three times. Median survival time and 95% confidence intervals were obtained using the Kaplan Meier Estimator to give nonparametric estimates of survival functions for the insects under each treatment (Collett, 1994; Kalbfleisch and Prentice, 1980). The survival times were estimated as the midpoints of the intervals in which they died. Lethal doses (LD_{50}) were determined for each virus for both *T. ni* and *H. virescens* by infection of third instars with one of five viral doses, 30 larvae per dose. For these bioassays, larvae were infected with virus inoculated onto a plug of diet, as described previously (Bonning *et al.*, 1995). Bioassays were replicated three times and data analyzed using a probit analysis program (Russell *et al.*, 1977).

For quantification of feeding damage, neonate larvae of *H. virescens* were infected by droplet feeding at 2000 pibs/μl with wild type virus, AcJHE-KK or AcAaIT, a recombinant virus which expresses an insect-specific scorpion toxin (McCutchen *et al.*, 1991; Stewart *et al.*, 1991). Feeding damage caused to lettuce by virus-infected larvae was compared with that caused by uninfected larvae. Thirty larvae per treatment were transferred individually to petri dishes containing damp filter paper and a piece of iceburg lettuce (variety Salinas) which were sealed with parafilm and maintained at 26°C. Pieces of iceberg lettuce were changed every 48 h and the area of each piece was scanned before and after feeding by using a CI-202 area meter (CID Inc., Vancouver, Washington State). The bioassay was ended when the control virus-infected larvae died. Data were analyzed by one-way ANOVA followed by the Fisher PLSD means comparison test (Steel and Torrie, 1980). Feeding damage assays were also carried out using cotton leaves (variety Acala SJ-2). Because the amount of cotton leaf consumed by uninfected or infected larvae is only about 20% that of lettuce, feeding assays on cotton were done using third instars for accurate determination of feeding differences between treatments. Larvae reared to third instar were starved for 24 h prior to infection with 3000 pibs per diet plug (Bonning *et al.*, 1995). Larvae that had completely consumed the diet plug after 16 h were transferred to prescanned leaves, and the protocol was continued as described above.

The reduced efficiency of targeting of JHE-KK to the pericardial cell lysosomes was associated with an insecticidal effect. Larvae of *H. virescens* and *T. ni* infected with the recombinant baculovirus AcJHE-KK were killed significantly faster (by approximately 25%) than larvae infected with the wild type virus (Table 1A). Of the larvae infected with AcJHE-KK (n = 186), 17% exhibited symptoms of apparent contractive paralysis and all died early. The paralysis was unusual and different from that caused by the scorpion toxin AaIT in that while the body was contracted, the prolegs were protruded. This observation suggests that the nervous control of body musculature may have been affected by

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TABLE 1A

Survival Times of Larvae of *T. ni* and *H. virescens* Infected *per os* with Recombinant Baculoviruses Expressing Modified JHE

Host and virus	Median ST[a] (h)	95% CL Lower	95% CL Upper
T. ni			
AcMNPV	111	101	111
AcJHE	113	103	117
AcJHE-29	114[b]	114	123
AcJHE-524	119[b]	116	133
AcJHE-KK	83[c]	83	83
H. virescens			
AcMNPV	117	107	121
AcJHE	116	106	120
AcJHE-29	131	121	154
AcJHE-524	107	107	131
AcJHE-KK	90[c]	82	98

[a] ST, survival time of infected larvae. First instar larvae ($n = 30$) were infected at 1000 or 2000 pibs/μl for *T. ni* and *H. virescens*, respectively. Bioassays were replicated three times. Data presented are for one representative replicate. Median ST and 95% CL (confidence limits) were determined by the Kaplan Meier Estimator.

[b] Significantly higher than median ST for AcMNPV C6 at the 95% confidence level.

[c] Significantly lower than median ST for AcMNPV C6 at the 95% confidence level.

TABLE 1B

Dose–Mortality Response of *H. virescens* and *T. ni* Infected *per os* with Recombinant Baculoviruses Expressing Modified JHE

Host and virus	LD_{50}[a] (PIBS)	95% Fiducial limit Lower	95% Fiducial limit Upper	Slope[b]	Heterogeneity[c]
T. ni					
AcMNPV	22	8	45	1.42 ± 0.27	0.24
AcJHE	110[d]	53	162	0.98 ± 0.20	1.20
AcJHE-29	215[d]	67	689	0.89 ± 0.25	0.40
AcJHE-524	31	20	50	1.64 ± 0.22	1.06
AcJHE-KK	108[d]	54	218	0.91 ± 0.21	1.32
H. virescens					
AcMNPV	106	73	156	1.20 ± 0.25	0.28
AcJHE	53	25	82	1.29 ± 0.59	0.22
AcJHE-29	108	41	293	1.10 ± 0.26	0.35
AcJHE-524	131	65	220	1.74 ± 0.30	1.19
AcJHE-KK	97	14	233	0.97 ± 0.20	1.55

[a] Lethal doses were determined for third instar larvae. Groups of 30 larvae were infected at five different doses. Bioassays were replicated three times and log-dose probit parameters calculated with the POLO probit analysis program (Russell *et al.*, 1977).

[b] Slope ± standard error.

[c] Heterogeneity factor equals the χ^2 divided by the degrees of freedom (POLO-PC manual, LeOra software, 1994).

[d] Significantly higher than the LD_{50} of AcMNPV at the 95% confidence level.

infection with the virus AcJHE-KK. Median survival times of *H. virescens* infected with the viruses AcJHE, AcJHE-29, and AcJHE-524 were not significantly different from those for the wild-type, nonengineered virus. The median survival times of *T. ni* following infection with AcJHE-29 and AcJHE-524, however, were significantly higher than those for the wild-type virus (Table 1A; $P < 0.05$). The lethal doses for the recombinant viruses were not significantly different from the wild-type virus in *H. virescens* ($P > 0.05$; Table 1B), but those for AcJHE, AcJHE-29, and AcJHE-KK in *T. ni* were significantly higher than for the wild-type virus ($P < 0.05$; Table 1B). The reason for these apparent increases in median survival time and lethal doses for recombinant viruses in *T. ni* are unclear.

On lettuce, a 50% reduction in feeding damage was seen for larvae infected with AcJHE-KK compared with larvae infected with the wild-type virus (PLSD = 0.588, $P < 0.05$; Table 2). The amount of feeding damage caused by AcJHE-KK-infected larvae on lettuce was not significantly different from that caused by larvae infected with AcAaIT (Table 2B; PLSD = 0.588, $P > 0.05$). A 54% reduction in feeding damage was seen on cotton for insects infected with AcJHE-KK relative to damage caused by insects infected with the wild-type virus (Table 2A). Under these conditions, AcAaIT gave a 35% reduction in feeding damage compared with the wild-type virus. There was wide variation in the amount of feeding damage caused by individual larvae on cotton in these tests (Bi *et al.*, 1997; Duffey *et al.*, 1995), and as a result of this, statistical analysis indicated that none of the treatments on cotton were significantly different from the control treatments (Table 2B).

Kunimi *et al.* (1996) have shown that the reduction in survival time of third to fifth instar *T. ni* infected with AcJHE-KK is 5–8% relative to the wild-type virus, compared to a 25% reduction reported for neonates in the current study. These results suggest that earlier

TABLE 2A

Feeding Damage to Iceburg Lettuce and to Cotton Leaves Caused by Larvae of *H. virescens* when Infected with Recombinant Baculoviruses Compared with Wild-Type Virus and Uninfected Controls

Treatment	Mean area consumed (cm^2)[a] Lettuce[b]	SE	Cotton[c]	SE
Uninfected[d]	2.88	0.34	6.93	2.03
AcMNPV[e]	1.46	0.28	4.52	1.02
AcJHE-KK	0.73	0.17	2.08	0.37
AcAaIT[f]	0.39	0.11	2.92	0.36

[a] $n = 20$–30 larvae per treatment.

[b] Feeding damage caused by neonate larvae.

[c] Feeding damage caused by third instar larvae. Data in this column are not significantly different from each other (see Table 2B).

[d] Damage caused by uninfected larvae was monitored only until all AcMNPV C6-infected larvae had died.

[e] Control non-engineered, parent virus, AcMNPV clone C6.

[f] Baculovirus expressing an insect-specific scorpion toxin (McCutchen *et al.* 1991).

TABLE 2B
Statistical Analysis of Feeding Damage Data[a]

Treatment comparison	Fisher PLSD	
	Lettuce	Cotton
AcMNPV vs Uninfected	0.621*	7.763
AcMNPV vs AcJHE-KK	0.588*	7.763
AcMNPV vs AcAaIT	0.582*	7.763
AcAaIT vs AcJHE-KK	0.588	7.763

[a] Groups compared by one-way ANOVA followed by Fisher PLSD means comparison test.
* Significantly different compared by one-way ANOVA ($P < 0.05$).

instars of this species are more susceptible to the insecticidal effects of JHE-KK, although the physiological basis for this observation is currently unclear. AcJHE-KK did not reduce the survival time of the soybean looper *Pseudoplusia includens* compared to the wild-type virus (Kunimi *et al.,* 1997), suggesting that AcJHE-KK may not be more effective than the wild-type virus against all insect species. Other studies on AcJHE-KK have addressed the sensitivity of this virus to ultraviolet light (Ignoffo *et al.,* 1997), effects on nontarget organisms (McCutchen *et al.,* 1996), synergism with chemical insecticides (McCutchen *et al.,* 1997), and altered ecology relative to the wild-type virus (Fuxa *et al.,* 1998).

Overexpression of insect hormones or enzymes using a baculovirus vector for insect pest control has thus far met with limited success (Black *et al.,* 1997; Bonning and Hammock, 1996). This may be explained in part by the regulatory and feedback mechanisms that insects have for each hormone and enzyme involved in development, and the complexities of hormone interaction. For JHE-KK, we have modified the insect protein juvenile hormone esterase by site-directed mutagenesis to disrupt lysosomal targeting. In so doing, we have bypassed the mechanisms which regulate the titer of JHE and generated an insecticidal agent from an insect protein which does not normally have an insecticidal effect. The mechanism of insecticidal activity of JHE-KK is under investigation.

Key Words: Autographa californica; nucleopolyhedrovirus; insecticidal efficacy; juvenile hormone esterase.

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