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FURTHER DEVELOPMENT OF A RECOMBINANT BACULOVIRUS INSECTICIDE EXPRESSING THE ENZYME JUVENILE HORMONE ESTERASE FROM *HELIOTHIS VIRESCENS*

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Abstract—The speed of action of baculovirus insecticides may be enhanced by insertion of genes encoding enzymes, hormones or toxins into the viral genome. The coding sequence for juvenile hormone esterase (JHE) of *Heliothis virescens* was inserted into a baculovirus to produce the recombinant virus AcUW2-(B).JHE which produces polyhedra. These polyhedra are essential for viability of viral insecticides in the field and increase oral infectivity. This virus was purified by a new, rapid method. JHE expression by AcUW2 (B).JHE in larvae of *Trichoplusia ni* was twice the maximum naturally occurring JHE activity in the haemolymph of *H. virescens* seen during the final larval stadium. Significantly more JHE activity (130 nm substrate hydrolysed/min/ml) was expressed *in vitro* in spinner culture than by the polyhedrin negative virus expressing JHE. *In vitro* studies revealed that the viral promoter for the p10 protein gene used for expression of JHE in AcUW2 (B).JHE is active 4 h before the polyhedrin gene promoter used in the polyhedrin negative virus. These observations illustrate the use of JHE as a soluble, exported reporter enzyme for use in baculovirus studies. The recombinant virus AcUW2 (B).JHE was slightly faster acting than the recombinant control virus AcUW2 (B).lacZ, but did not improve LT50, LD50 values or reduce weight gain of infected larvae, compared to the wild type control virus. Implications for the involvement of JHE in physiological regulation of metamorphosis, and for use in recombinant baculoviruses as insecticides are considered.

Key Word Index: juvenile hormone esterase; baculovirus insecticide; *Heliothis virescens*; reporter enzyme.

INTRODUCTION

The restricted host range of baculoviruses to a limited number of insect species is advantageous for their use as insecticidal agents, avoiding harmful effects on non-target and beneficial species. Baculoviruses have been studied extensively for the control of insect pests and have proved successful in a number of control programmes such as that of *Neodiprion sertifer* (the pine sawfly), *Lymantria dispar* (the gypsy moth) and *Heliothis* sp. (the cotton bollworm) (Entwistle and Evans, 1985). A primary limitation of the use of baculoviruses as insecticides, however, is the delay in action on the target species. Considerable feeding damage may be done to crops after viral infection of the pest, during replication of the virus and prior to death of the caterpillar (Benz, 1986). Genetic engineering of baculoviruses to insert the sequences of specific toxins, enzymes or insect hormones which disrupt the development of pest insects when expressed at inappropriate times, may enhance the speed of action of these insecticidal agents.

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Regulation of JH titre in Lepidoptera

The titre of juvenile hormone (JH) in the haemolymph of Lepidoptera is intrinsically involved in development of the larvae (Sehnal, 1985). Absence of JH results in loss of larval commitment of the tissues (Riddiford, 1980; Sehnal, 1981), and is necessary for release of prothoracicotrophic hormone (PTTH) which leads to events culminating in pupation and metamorphosis. Specifically, release of ecdysone results in reprogramming of larval tissues to become pupal at the moult (Sehnal, 1989). The JH titre is finely controlled both by the rate of biosynthesis (deKort and Granger, 1981) and the highly efficient enzyme, juvenile hormone esterase (JHE) (Hanzlik and Hammock, 1988). Hydrolysis of JH by JHE is the major route of degradation in the prewandering Lepidoptera studied (Hammock, 1985). Hence, a drop in JH titre controlled in part by JHE, preceeds cessation of feeding prior to moulting, and also allows initiation of metamorphosis late in the last larval instar. In Lepidoptera, 2 peaks of JHE activity in the final instar contribute to a rapid decline in JH leading to the onset of pupation (Hammock, 1985).

Development of baculovirus insecticide expressing JHE

As precocious development of most crop pests will dramatically reduce feeding damage, anti-juvenile

hormone activity has been the target of extensive effort in the agricultural chemical industry (Staal, 1986). Several widely different structures show anti-juvenile hormone activity. These compounds generally reduce feeding damage even at doses below that needed to induce complete precocious pupation (David Cerf, Sandoz, pers. commun.). Since we have demonstrated that injection of JHE into larvae of *Manduca sexta* clearly shows anti-JH effects (Philpott and Hammock, 1990), we hoped that precocious expression of the enzyme in a baculovirus system might reduce feeding damage in virus infected insects.

Thus, the coding sequence of JHE from *Heliothis virescens* (Hanzlik *et al.*, 1989) was inserted into the baculovirus *Autographa californica* nuclear polyhedrosis virus (AcNPV) behind the promoter for the polyhedrin gene to produce the virus AcRP23.JHE (Hammock *et al.*, 1990a). The expressed enzyme had anti-JH activity, and infection of insects with the recombinant virus resulted in cessation of feeding and subsequent mortality. As the JHE gene sequence was inserted in place of the polyhedrin gene necessary for production of polyhedra, the virus was non-occluded, and therefore less viable under field conditions. Occlusion is necessary for persistence of the virus in the environment and efficient oral infection of noctuid larvae, and consequently is essential for practical use of viral insecticides in the field. The aim of this research was to further the development of a baculovirus insecticide expressing JHE by insertion of the same JHE coding sequence behind a repeated p10 protein promoter in a stable polyhedrin-positive AcNPV. Such an occluded recombinant virus has potential for use as an insecticide in field trials, and provides a simple method for field monitoring by detection of virally expressed JHE using either a rapid and sensitive radiochemical assay for JHE activity (Hammock and Roe, 1985), or a spectrophotometric substrate (McCutchen *et al.*, in preparation). In addition, this construct allowed us to compare the effects of the polyhedrin positive and polyhedrin negative viruses *in vitro*, and *in vivo* on lepidopteran larvae.

MATERIALS AND METHODS

Virus construction

The 1.73 kb coding sequence of JHE from *H. virescens* was isolated from pJHE16B (Hammock *et al.*, 1990b) using standard procedures (Maniatis *et al.*, 1990). This fragment was ligated into the unique Bgl II cloning site of the plasmid vector pAcUW2 (B) (Weyer *et al.*, 1990). Insertion of the JHE sequence downstream of a duplicated p10 protein promoter produced the recombinant plasmid pAcUW2(B).JHE with both the polyhedrin and p10 genes. The correct insert orientation was verified by restriction analysis. Digestions, ligation, transformation and plasmid purification were carried out using standard techniques (Maniatis *et al.*, 1990; Possee, 1986).

Viruses were propagated in *Spodoptera frugiperda* (Sf) cells IPLB-Sf-21 (Vaughn *et al.*, 1977) in TC100 medium containing 5% foetal calf serum (FCS) and 1% Penicillin-Streptomycin (PS) at 28°C, and titrated as described by Brown and Faulkner, (1977). Cells were co-transfected by calcium precipitation with the plasmid pAcUW2 (B).JHE and DNA purified from AcRP8 (Matsuura *et al.*, 1987; Possee, 1986).

Virus purification

The recombinant virus was plaque purified by screening for polyhedrin-positive plaques in Sf 21 cells (Summers and Smith, 1987) and for JHE activity (Hammock *et al.*, 1990a). Cores were taken from the agar with a Pasteur pipette and vortexed in 0.2 ml TC100 with 5% FCS. Only plaques with enzyme activity over 0.1 nm substrate hydrolysed/min/ml were purified by further rounds of plaque assay in Sf 21 cells.

A second, novel method of purification of the recombinant virus was also used; a single plaque selected from a low titre plaque assay dish from the first round of plaque purification was used to infect Sf cells. The isolated plaque was suspended in 0.5 ml TC100 with 5% and PS. Sf cells in 35 mm dishes were infected with 100 μl of the inoculum from the plaque which contained polyhedrin negative as well as polyhedrin positive virus. These cells were harvested 5 days post infection and treated with sodium dodecyl sulphate (final concentration 1%) to remove any polyhedrin negative, non-recombinant virus in the media. The pelleted polyhedra were then washed with water to remove levels of detergent potentially harmful to lepidopteran larvae.

Larval infection was used for amplification of the purified virus. In order to grow the recombinant virus to high titres in larvae, the polyhedra derived from cells infected by a single plaque were used to inoculate plugs of diet dispensed into the wells of a multi-well sterile plate. Thirty, early third instar larvae of the cabbage looper *Trichoplusia ni* were placed individually in these wells with the virus coated diet plugs. After 24 h, the infected larvae were given fresh diet. Plaque assay of haemolymph from these larvae was used to confirm the absence of polyhedrin negative virus in the preparation. Larvae were frozen at fourth instar when pale from virus infection, but before the onset of melanization.

To purify virus from the infected larvae, the cadavers were homogenized in 0.1% SDS in water. This concentration of detergent would destroy any non-occluded virus present. Virus was separated from debris by differential centrifugation at 4°C. The pellet was washed twice in double distilled water and repelleted before resuspension in a small volume of water. Virus was stored at −70 C, or 4 C for short periods.

Baculovirus expression of JHE in vitro

Propagation of AcNPV and recombinant virus in Sf 21 cells was as described previously (Possee, 1986; Possee & Howard, 1987). Spinner flasks of Sf 21 cells were infected at 5×10^5 cells/ml with 10 pfu per cell of AcNPV C6 (wild type control virus), AcUW2 (B).JHE or AcRP23.JHE. Aliquots (1 ml) of cells and medium were removed from the spinner flasks at various times post infection. Cells were separated from medium by centrifugation and lysed by three cycles of freeze–thawing. All samples (lysed cells and media) from spinner flasks were then assayed for JHE activity (Hammock and Roe, 1985) to monitor viral production of JHE and export from the cells.

Bioassay

T. ni were maintained in the laboratory on a semi-synthetic diet (Hunter *et al.*, 1984). Five doses of AcUW2(B).JHE, the recombinant control virus containing the coding sequence for lacZ [AcUW2(B).lacZ] (Weyer *et al.*, 1990) or wild type AcNPV C6, between 12 and 1000 polyhedrin inclusion bodies (pibs) in TE (10 mM Tris–HCl pH 8.0.1 mM EDTA) were given on plugs of diet to mid-second instar larvae, 50 larvae being infected per dose. Each larva was isolated in a microtitre plate well with a plug of diet previously inoculated with virus. Larvae that had completely fed on the diet 48 h later, were transferred to individual tubs containing artificial diet and maintained at 24°C. Mortality was scored and LD50 values were determined using probit analysis (Finney, 1971). Bioassays were replicated at least three times.

To determine effects of infection with wild type virus, AcUW2(B). JHE or the recombinant control virus on feeding

inhibition and hence weight gain, mid-second instar larvae were starved for 6 h before infection by droplet feeding on 2×10^6 pibs/ml (Hughes *et al.*, 1986). Approximately 100 larvae were set up for each virus. Half of each of the treatments were weighed 72 h after feeding, and the other half of each treatment weighed 96 h after feeding.

To determine the LT50 for the viruses, neonates were infected using the droplet feeding method at 100 pibs/μl. Mortality was monitored daily at 4, 6 or 8 h intervals according to the mortality rate. LT50 values were determined with the Vistat Program (Boyce Thompson Institute, Ithaca, New York, 1990).

Baculovirus expression of JHE in the haemolymph

JHE activity in the haemolymph of *T. ni* larvae was monitored during the course of infection with wild type AcNPV C6, recombinant control virus or AcUW2(B).JHE. Mid-second instar larvae were starved overnight before virus infection by droplet feeding (Hughes *et al.*, 1986). Three larvae per treatment were bled at each time point post infection, each larva being bled only once. Haemolymph was collected in calibrated micropipettes from an incision made in the proleg. Micropipettes were treated with 1% phenylthiourea (PTU) to prevent the action of tyrosinases in the larval haemolymph. Samples were stored at −20°C before being assayed for JHE activity.

RESULTS

Construction of the recombinant virus AcUW2(B).-JHE

Construction of the transfer vector pAcUW2(B).-JHE encoding the JHE gene behind a duplicated p10 protein promoter was the first step in engineering the recombinant baculovirus. This transfer vector has the sequence for both the polyhedrin and p10 genes, with the JHE coding sequence in the unique Bgl II cloning site under control of a duplicated p10 promoter (Fig. 1). Restriction profiles of the transfer vector confirmed that the JHE coding sequence had been inserted into the plasmid in the correct orientation. Sf21 cells were cotransfected with pAcUW2(B).JHE and AcRP8 DNA. JHE activity was detected in the cotransfection mixture. The virus AcUW2(B).JHE was purified by five rounds of plaque purification by screening for polyhedrin positive plaques with JHE activity over 0.1 nm substrate hydrolysed/min/ml.

Purification by SDS treatment of cells infected with virus from a single plaque isolate, and subsequent feeding to *T. ni* larvae was quicker than the five rounds

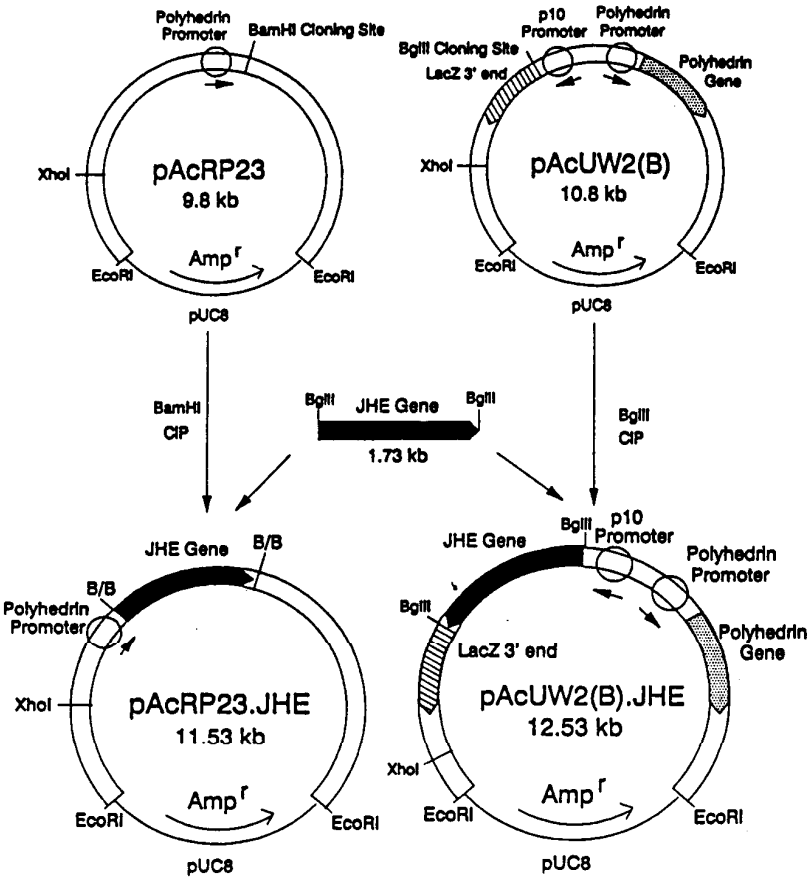

Fig. 1. Construction of the transfer vectors pAcUW2(B).JHE and pAcRP23.JHE. The 1.73 kb coding sequence of JHE from *H. virescens* was isolated from pJHE16B and ligated into the cut and dephosphorylated vectors to produce pAcRP23.JHE and pAcUW2(B).JHE. The viruses AcRP23.JHE and AcUW2(B).JHE were derived from these plasmids by transfection of Sf21 cells. B/B: Bam H1/Bgl-II fusion; CIP: calf intestinal phosphatase treatment.

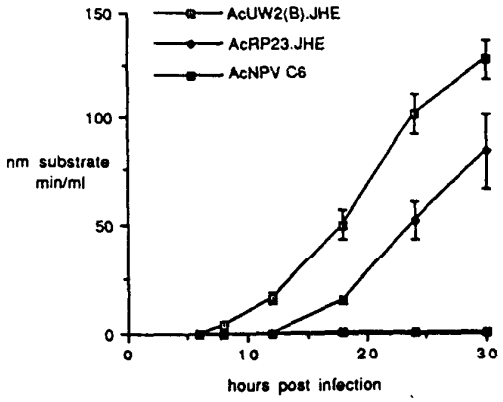

Fig. 2. JHE activity with standard deviations in the media of spinner cultures of Sf21 cells at various times post infection with wild type AcNPV C6, AcUW2(B).JHE or AcRP23.JHE. Samples were assayed in triplicate. Maximum expression of 130 nm substrate hydrolysed/min/ml was attained for AcUW2(B).JHE and 105 nm substrate hydrolysed/min/ml for AcRP23.JHE at 48 h post infection. Earlier and significantly higher expression was observed for AcUW2(B).JHE when similar experiments were carried out in Petri dishes and large flat cultures.

of plaque assays required to purify the recombinant virus, taking 2 as opposed to 4 weeks.

Viral titres obtained from larval haemolymph are significantly higher than those attainable from cell culture, emphasizing use of larvae as viral amplification tools. Thirty virus-infected *T. ni* larvae yielded 10^{10} pibs NPV of recombinant virus derived from a single clone.

Baculovirus expression of JHE in vitro

Only 2–4% of JHE activity was present in cells from spinner flasks infected with AcUW2(B).JHE or AcRP23.JHE, the rest being detected in the cell media. *In vitro* expression of JHE in spinner culture was detected at 8 h post infection in AcUW2(B).JHE infected cultures, and at 12 h post infection in AcRP23.JHE infected cultures (Fig. 2). The difference in expression levels from 8 to 30 h post infection is significant at the 5% confidence level. JHE activity in the media of spinner flask cultures of Sf 21 cells infected with AcUW2(B).JHE reached a maximum at 48 h post infection of *ca* 130 nm of substrate hydrolysed/min/ml. This was significantly higher than yields attained from AcRP23.JHE infected cell culture (105 nm substrate hydrolysed/min/ml; data not shown). No activity was detected in AcNPV C6 infected cultures at any time.

Baculovirus expression of JHE in vivo

The LD50 for second instar larvae using the engineered baculovirus AcUW2(B).JHE was lower than the recombinant control virus but not significantly so. The wildtype AcNPV gave significantly more effective kill than either AcUW2(B).JHE or AcUW2(B).lacZ (Table 1).

LT50 values for neonate larvae infected with AcUW2(B).JHE and the recombinant control viruses were 118 ± 1 and 139 ± 2 h respectively. The LT50 of the wild type virus was 116 ± 0.5 h (Table 1).

Table 1. LD50 (pibs) and LT50 (h) values with 95% confidence limits for *T. ni* larvae infected with AcUW2(B).JHE, wild type virus (AcNPV) or AcUW2(B).lacZ. For LD50 data larvae were infected mid-second instar. Neonates were infected for LT50 experiments

	LD50 (pibs)	95% CL	LT50 (h)	95% CL
AcNPV	16	10–27	116	115–116
AcUW2(B).JHE	74	53–102	118	117–119
AcUW2(B).lacZ	123	75–201	139	137–140

Table 2. Median weights (mg) of *T. ni* larvae 72 and 96 h post infection at second instar with wild type (AcNPV), engineered control virus (AcUW2(B).lacZ) or AcUW2(B).JHE. The experiment was repeated 3 times. Fifty larvae were used/virus/time point

	Median weight (mg) (minimum–maximum)	
	72 h	96 h
AcNPV	3.4 (1–16.2)	4.2 (0.6–21.3)
AcUW2(B).JHE	3.8 (1.3–13.2)	4.6 (0.9–17.2)
AcUW2(B).lacZ	4.5 (1.3–19.8)	4.4 (1.2–19.9)

Larvae infected with AcUW2(B).JHE and weighed 72 and 96 h post infection weighed slightly less than larvae infected with wild type or engineered control virus (Table 2). Statistical analysis by 2 way ANOVA showed this difference to be insignificant.

JHE activity monitored in haemolymph samples during the course of viral infection remained low for uninfected and wild type AcNPV infected *T. ni* larvae, fluctuating around 5 nm substrate hydrolysed/min/ml (Fig. 3). In larvae infected with AcUW2(B).JHE, JHE activity increased rapidly after 48 h post infection to over 100 nm substrate hydrolysed/min/ml.

DISCUSSION

Rapid purification of the AcUW2(B).JHE was achieved by feeding *T. ni* larvae with 1% SDS treated cells that had been infected with the virus from a

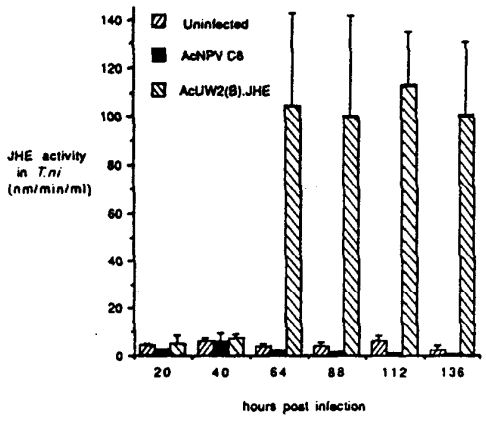

Fig. 3. JHE activity with standard deviations in haemolymph samples taken from uninfected *T. ni* larvae, or larvae infected at second instar with wild type AcNPV C6 or AcUW2(B).JHE. Three larvae were bled/time point/treatment. Assays of diluted haemolymph samples for JHE activity were carried out in triplicate.

single cotransfection plaque isolate. Selection of a well isolated plaque on a low titre plaque assay dish is important to minimise chances of isolating polyhedra containing polyhedrin negative virus. The likelihood of such co-occlusion is unknown, but subsequent plaque assay can be used to confirm the absence of contaminating polyhedrin negative virus. SDS treatment destroyed any non-recombinant, polyhedrin negative virus present in the media.

This method was considerably quicker than plaque purification of the recombinant polyhedrin positive virus, which routinely takes four or five rounds of plaque purification. Large amounts of virus were produced on infection of larvae, illustrating the effective use of larvae as a tool for viral amplification at an early stage in the virus purification process.

Assay of JHE activity *in vitro* in cells and media showed that the majority of JHE (96–98%) was exported from the cells as predicted from the presence of the 19 amino acid leader sequence (data not shown). As JHE is exported and JHE activity is easily monitored, it is a useful reporter enzyme for optimization of NPV culture conditions and analysis of promoter activities. Expression yields were significantly higher for AcUW2(B).JHE than for AcRP23.JHE in cell culture (130 and 105 nm substrate hydrolysed/min/ml respectively).

In vitro data indicate that JHE expression by AcUW2(B).JHE begins about 8 h post infection, and later than 12 h post infection by AcRP23.JHE. JHE expression controlled by the p10 promoter in this viral construct is initiated at least 4 h before JHE expression under the control of the polyhedrin promoter, as illustrated by expression of JHE under control of the two promoters in the two viruses *in vitro*. This difference in timing of expression initiated by the p10 protein and polyhedrin promoters effective in the very late phase of gene expression in virus infection has not previously been reported, and illustrates the use of JHE as a reporter enzyme.

JHE activity in the haemolymph of *T. ni* larvae infected at second instar with AcUW2(B).JHE remained high from 56 to 144 hpi at about 105 nm/min/ml. This is about twice the maximum JHE activity seen in blood from *H. virescens* at final instar, and about 10 times the levels of activity detected in AcRP23.JHE infected larvae (bled at third instar) which were infected as neonates (Hammock *et al.*, 1990a). The low titres detected in AcRP23.JHE infected larvae may be attributed to the stage of initial infection (first instar) and the lower oral infectivity of the polyhedrin negative virus.

Bioassay data indicate that AcUW2(B).JHE is no more effective at killing *T. ni* larvae than the recombinant control virus. The LT50 for neonates infected with AcUW2(B).JHE is slightly higher than for the wild type virus. Natural larval development is precisely controlled with JHE expression peaking on day 2 of the fifth larval instar, and again immediately before larval/pupal ecdysis (Hanzlik and Hammock, 1988). Feedback regulatory systems may dampen the effects of virally produced JHE, by enhancing expression of JH to restore homeostasis or increasing proteolytic breakdown of the enzyme. However, it is also likely that the JHE is being removed rapidly from the haemolymph. Otherwise, the JHE activity would continue to rise *in vivo*, rather than reaching a plateau (Fig. 3). Hence, the lack of significant insecticidal effect may be due to rapid proteolytic degradation of the expressed enzyme. Philpott and Hammock (1990) have shown that the affinity purified JHE from *Manduca sexta* is lost very rapidly from the haemolymph of early instars injected with the enzyme. More recent data indicate that the JHE expressed in the baculovirus system is extraordinarily stable chemically, but is rapidly eliminated *in vivo* (with a half life of only 1.2 h) by a saturable process (Ichinose *et al.*, 1992), and is likely to be removed from the haemolymph by the pericardial cells (Ichinose *et al.*, submitted for publication; Booth *et al.*, 1992).

Larvae infected with AcUW2(B).JHE at second instar showed no significant difference in growth compared to control larvae. Significant inhibition of feeding, growth and development was noted on infection of first instar *T. ni* larvae with the polyhedrin negative virus AcRP23.JHE (Hammock *et al.*, 1990a). The sensitivity of *T. ni* to virally expressed JHE may decrease during development as regulatory feedback mechanisms develop. However, during earlier larval instars, higher titres of JH must be overcome by the virally expressed JHE for a deleterious biological effect. The later stadia of *M. sexta* are more sensitive to the effects of JHE, possibly as the enzyme is more stable, or the biosynthesis of JH, or sensitivity of tissues to JH is lower (Philpott & Hammock, 1990).

The reduced insecticidal activity of the JHE and lacZ constructs compared to the wild type virus is not surprising based on analagous situations in the fields of plant biotechnology and insecticide resistance. Whether a new genetic make up is generated using molecular biology in the former case or selection pressure in the latter case, the resulting organism is usually genetically inferior to the wild type organism in the absence of strong selection pressure. Genetically engineered plants are subjected to careful plant breeding before plants are adapted for tests in a field crop situation. Similarly with insecticide resistance, even highly resistant populations quickly revert to susceptibility if pressure is removed shortly after resistance develops. However, if selection pressure is continued for many generations, the resulting resistant insect populations are far more stable.

Thus, viruses engineered with genes to improve one trait may be less competitive than the wild type virus in other ways. One may be able to anticipate such differences in engineered viruses and improve competitiveness by recombinant methods or the characteristics of promising viruses could be improved by classical selection methods analagous to those outlined above.

Expression of JHE by the engineered viruses may be at insufficient levels, or too late post infection to enhance the speed of kill by the baculoviruses. Given the finely controlled regulation of the JHE titre in larvae, the timing of viral expression of JHE may be critical. New virus constructs expressing JHE at higher levels and earlier in the viral cycle may disrupt larval development sufficiently for their use as viable alternative insecticides. However, in light of recent information concerning uptake of JHE from the haemolymph by pericardial cells (Ichinose *et al.*, submitted for publication; Booth *et al.*, 1992), work is

in progress to develop a mutant JHE gene coding for an enzyme which is more stable *in vivo*. Such a stabilized enzyme may be sufficiently active alone, and may synergize certain neuropeptides such as allatostatin on coexpression by baculovirus vectors.

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