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Competition between Wild-Type and Recombinant Nucleopolyhedroviruses in a Greenhouse Microcosm

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Wild-type *Autographa californica* nucleopolyhedrovirus (AcNPV or AcNPV.WT), AcNPV expressing a scorpion toxin (AcNPV.AaIT), and AcNPV expressing a mutated juvenile hormone esterase (AcJHE.SG) were compared in their capability to produce epizootics in larvae of *Trichoplusia ni* infesting collards in a greenhouse microcosm. Larvae treated in four different ways were released into 1.8-m^2 microplots in week 1. The four treatments included (1) uninfected larvae (control), (2) 100% AcNPV.WT-infected larvae (WT), (3) 100% AcNPV.AaIT-infected larvae (AaIT), and (4) 1:1 ratio of AcNPV.WT-infected and AcNPV.AaIT-infected larvae (WT+AaIT). On a weekly basis, larvae were sampled and new, uninfected larvae were added to all plots. Sampled larvae were reared until death and then subjected individually to DNA–DNA dot-blot hybridization assay to determine the proportion of insects infected with each virus in each plot. The entire experiment was repeated with AcJHE.SG in the place of AcNPV.AaIT. Epizootics of AcNPV.WT lasted 8 weeks after a single viral release in the replicated greenhouse microplots. AcJHE.SG epizootics also lasted 8 weeks after viral release, but this virus and AcNPV.AaIT were both out-competed by AcNPV.WT. AcNPV.AaIT was no longer detected in the *T. ni* population by the fourth week after release. AcNPV.WT also increased to greater numbers in soil than AcNPV.AaIT or AcJHE.SG after 8 weeks. Thus, it was possible to induce 8-week epizootics of AcNPV.WT in replicated microplots under artificial greenhouse conditions, and the wild-type virus out-competed the recombinant virus for a niche in this greenhouse microcosm, which reduces the probability that the recombinant virus will persist in an agroecosystem.

Key Words: **nucleopolyhedrovirus; recombinant baculovirus; viral persistence; viral competition; risk assessment.**

INTRODUCTION

Nucleopolyhedroviruses (NPVs) have major advantages for insect pest management. The primary reason for interest in the NPVs is their environmental safety, which, in turn, is due to their extreme host specificity. They are safe to humans, wildlife, livestock, plants, and beneficial insects (Fuxa, 1989; Laird *et al.*, 1990; Heinz *et al.*, 1995; McCutchen *et al.*, 1996). In addition, the NPVs are virulent and cause disease epizootics in their insect hosts, primarily the larvae of sawflies and moths (Fuxa, 1991).

The NPVs, though virulent in terms of their case-fatality rates, generally require approximately 1 week or longer to kill the host insect under field conditions. This slow speed is very deleterious when little damage from the pest can be tolerated. It also is disadvantageous when a user expects quick action in a "knock-down" or "firefighting" approach to insect control (Fuxa, 1991). Thus, the wild-type baculoviruses are excellent agents for classical biological control and for certain augmentation strategies. However, slow speed of kill often limits their utility for short-term control.

There have been several approaches to increasing the speed of kill of recombinant baculoviruses. For example, NPV was engineered to express a highly specific insect toxin (AaIT) from the North African scorpion *Androctonous australis* Hector. This toxin has been expressed in *Autographa californica* (Speyer) multiple embedded NPV (AcNPV) (Maeda *et al.*, 1991; McCutchen *et al.*, 1991; Stewart *et al.*, 1991). The recombinant virus, AcNPV.AaIT, reduces time of kill by 30% compared with wild-type AcNPV (AcNPV.WT) and stops insect feeding approximately 8–10 h before death (McCutchen *et al.*, 1991; Kunimi *et al.*, 1996). Another approach to increasing the speed of kill of baculoviruses involves the insertion of a mutated juvenile hormone esterase (JHE) gene. The resulting recombinant baculovirus, AcJHE.SG, expresses juvenile hormone esterase with the catalytic serine (Ser_{201}) mu-

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tated to Gly (Bonning *et al.*, 1995). This virus reduces feeding damage by 66%, and its lethal times are 20 to 30% lower than those for wild-type virus (Bonning *et al.*, 1995).

Despite the excellent safety record of natural or wild-type strains of NPVs, recombinant NPVs so far have not been registered for insect control. Much of the concern about the release of recombinant-DNA microorganisms is due to uncertainty about their potential for environmental persistence and subsequent transport and dispersal (Fuxa, 1990). Even an agent harmful to some environmental component might have low risk if it cannot persist or spread. The pathology of AcNPV.AaIT, in particular, has certain features—a high proportion of infected insects falling off plants onto soil, production of relatively low numbers of polyhedra, and cadavers that remain intact and therefore do not liberate virus on host plant leaves (McCutchen and Hammock, 1994; Hoover *et al.*, 1995)—that might decrease its ability to compete with wild-type virus for a niche in its habitat. Microcosms, or contained systems ranging from test tubes to greenhouses in which a portion of an ecosystem is duplicated, can provide data concerning how a recombinant organism performs or affects a portion of the environment (Omenn, 1986). The purpose of this research was to determine whether the recombinant viruses AcNPV.AaIT and AcJHE.SG can compete with AcNPV.WT for a niche in a plant/insect/virus microcosm.

MATERIALS AND METHODS

Greenhouse setup. Collards were grown in a greenhouse at a spacing of 30 cm between plants. There were 16 plots on four greenhouse benches (one block of 4 plots per bench). Each plot consisted of 16 evenly spaced plants grown in one 1.35 × 1.35 m flat container. Each plot was surrounded by a wooden, plastic-covered wall 20 cm high, with buffers of 45 cm between plots. The plants were watered with overhead sprinklers at a rate of 5 mm applied within 2 min once every 2 days to simulate rainfall on the foliage.

Viruses. The viruses (AcNPV.WT, AcNPV.AaIT, and AcJHE.SG) were grown in laboratory-reared cabbage loopers, *Trichoplusia ni* (Hübner). AcNPV.WT was provided by biosys, Inc. (Hanover, Maryland). AcJHE.SG and AcNPV.AaIT were obtained from Dr. B. D. Hammock (Department of Entomology, University of California, Davis) (McCutchen *et al.*, 1997; Bonning *et al.*, 1995). Viruses were purified by standard techniques of homogenization of dead insects followed by filtration and centrifugation. NPV preparations were quantified on an improved Neubauer hemacytometer. The *T. ni* were reared on artificial diet with standard techniques.

AcNPV.WT vs AcNPV AaIT. The experiment ran with only one recombinant NPV at a time in competition with AcNPV.WT. For the AcNPV.AaIT experiment, one group of third-instar *T. ni* was infected with AcNPV.WT and another group with AcNPV.AaIT. For each NPV, the diet in 30-ml rearing cups was surface-treated with 0.1 ml of suspension containing 5×10^6 viral occlusion bodies (OB)/ml water, a dosage intended to infect 100% of the insects. The *T. ni* were infected after the collards grew to a height of approximately 15 cm. One day after infection (i.e., diet-surface treatment), the *T. ni* were released into the greenhouse plots in a randomized block design experiment (each bench = one block replicate with four treatments or plots). Six larvae were placed on each plant with a camel's hair brush, for a total of 96 insects/plot. The 6 larvae per plant assured that sufficient insects would remain after removal sampling to initiate or sustain viral epizootics in the greenhouse microplots. Only 4 larvae were placed on plants in control plots, because the plants could not survive the damage from 6 healthy insects through the course of the experiment. The four treatments of generation-1 larvae released into the plots were: (1) uninfected *T. ni* larvae ("Control"), (2) 100% AcNPV.WT-infected larvae ("WT"), (3) 100% AcNPV.AaIT-infected larvae ("AaIT"), and (4) 1:1 ratio of AcNPV.WT-infected and AcNPV.AaIT-infected larvae ("WT + AaIT").

One week after the release of the insects in Treatments 1–4, and weekly thereafter, healthy second instars were released in all 16 plots, again at a density of 6 larvae/plant or 96/plot (controls, 4/plant or 64/plot). Two days after each release of insects (including the original "treatment" insects), 2 larvae per plant were removed at random. At each time of sampling, insects that reached the pupal stage were removed from the experiment. Sampled larvae were reared individually in 30-ml cups with artificial diet until they died or pupated.

NPV from every larva killed by virus was analyzed to determine whether that insect was infected with AcNPV.WT or AcNPV.AaIT. The dead insects were frozen and held at −20°C until sent by overnight mail from Louisiana to California on dry ice. A DNA–DNA dot-blot hybridization assay was then used to determine the identity of the virus present in each dead insect (Method 1 in Ward *et al.*, 1987). This technique, in which the whole homogenized larva is probed with a high-specific-activity P^{32} DNA probe, was shown to be more sensitive than differential staining, phase-contrast microscopy, or indirect solid-phase radioimmunoassay (Ward *et al.*, 1987). To detect wild-type virus, a fragment of cloned DNA encoding the essential gene ORF 1629 (open reading frame) was used. This probe hybridizes with both of the viruses used in this research. To detect the recombinant virus, the coding sequences for the introduced gene was used as a probe. Thus, this second probe was specific for the recombinant virus.

TABLE 1

Amounts of Wild-Type and Recombinant NPV, Indicated by Bioassay of Vegetation and Soil, Resulting from Epizootics of Wild-Type (AcNPV.WT) or Recombinant (AcNPV.AalT) Virus in *Trichoplusia ni* on Collard Plants in a Greenhouse Microcosm

	Control[a]		WT[b]		AalT[b]		WT + AalT[c]	
Week	WT[d]	AalT[d]	WT	AalT	WT	AalT	WT	AalT
			Mean % mortality[e] (±SE) in larvae on collard leaves					
1	0	0	90.0 ± 5.7	0	15[f] ± 3.7	71.0 ± 7.2	47.8 ± 12.5	44.0 ± 12.8
2	0	0	75.8 ± 13.3	0	0	0.8 ± 1.3	51.8 ± 22.8	3.0 ± 2.1
3	0	0	92.5 ± 3.4	0	0.8 ± 1.3	3.8 ± 2.5	91.5 ± 4.8	0
4	0.8 ± 1.3	0	97.8 ± 1.3	0	0	0	97.0 ± 2.1	0
5	0.8 ± 1.3	0	57.3 ± 9.1	0	0.8 ± 1.3	0	57.8 ± 11.3	0
			Mean OBs/g soil[g] (±SE) after 8 weeks					
	0	0	12060 ± 9209	0	0	263 ± 128	760 ± 188	3 ± 1

[a] Uninfected insects were released weekly onto collards in the greenhouse plots.
[b] All released week-1 insects were infected with AcNPV.WT or AcNPV.AalT. Subsequently, healthy insects were released weekly.
[c] Half of released week-1 insects were infected with AcNPV.WT and half with AcNPV.AalT. Subsequently, healthy insects were released weekly.
[d] Amount of AcNPV.WT or AcNPV.AalT, respectively, detected as disease prevalence in insects on collards or by soil bioassay.
[e] Percentage of insects dying and hybridizing with appropriate DNA probes. Those insects not hybridizing were assumed to be uninfected with AcNPV.WT or AcNPV.AalT. Each mean represents four replications with 32 insects sampled per replication.
[f] Insects identified as infected with AcNPV.WT in this group probably were actually infected with AcNPV.AalT (see text for explanation). If it is assumed that all the insects were infected with AcNPV.AalT, then mean % mortality = 86.0 ± 3.9.
[g] Viral occlusion bodies (OBs)/g soil were calculated from percentages of bioassay mortality according to the following standard curves: AcNPV.WT, $Y = 0.354X + 3.68$; AcNPV.AalT, $Y = 0.408X + 3.38$, where Y = probit % mortality and X = log OB/g soil.

The releases and sampling were continued for 8 weeks. However, larvae sampled from plants were subjected to DNA probes only through 5 weeks or insect releases, because it was clear at that time that AcNPV.AaIT was no longer infecting insects on the collards (Table 1). After 8 weeks or insect generations, one random soil sample was collected per plot, with a sampler 10 cm diameter and 5 cm deep. Each soil sample was bioassayed by a leaf-disk method with second-instar *T. ni* (Fuxa *et al.*, 1985). Briefly, each soil sample was homogenized in distilled water and centrifuged, and the pellet was resuspended in 0.5% Triton X-100. Fifty larvae were each fed a 5-mm collard leaf disk that had been dipped in the soil suspension and dried. Insects that ate the entire disk within 2 days were transferred to artificial diet. In addition to soil from the control plots, the soil bioassay had its own control treatment. Assay control insects were treated identically except that the leaf disks were dipped only in 0.5% Triton X-100 in distilled water. The soil-bioassay insects were observed daily until they died or pupated. Soil-bioassay larvae killed by NPV, confirmed by detection of OB in tissue smears examined under phase microscopy, were subjected to the DNA probes, as above, to determine which virus had infected individual insects. Standard bioassay curves with known amounts of AcNPV.WT or AcNPV.AaIT in the greenhouse soil (A. Abdel-Rahman, J. R. Fuxa, and A. R. Richter, unpublished data) were used in conjunction with the current soil bioassay results to estimate the number of OB/g of soil. For each standard curve, six viral concentrations were fed on 5-mm diameter leaf disks to second-instar *T. ni*, as above, with an average of 32 larvae/concentration eating the entire disk. The results were subjected to probit analysis, resulting in the standard curves $Y = 0.354X + 3.68$ for AcNPV.WT and $Y = 0.408X + 3.38$ for AcNPV.AaIT, where Y = probit % mortality and X = log POB/g soil.

AcNPV.WT vs AcJHE.SG. The entire experiment was repeated with AcJHE.SG in the place of AcNPV.AaIT and with minor modification of certain procedures. The soil and greenhouse benches were decontaminated prior to the second experiment. Nine random soil samples were collected from each plot to determine viral dispersion patterns and viral population density. Each soil sample was bioassayed by placing a neonatal larva on 6.7 g of a 1:9 (WT:WT) mixture of dried soil sample:artificial diet. Numbers of insects used in this soil assay were the same as in the AaIT experiment. Bioassay insects were observed daily until they died or pupated. A DNA–DNA dot-blot hybridization assay was again used to determine the identity of the virus present in each dead insect except the soil-dispersion-assay insects. Soil-dispersion-bioassay larvae killed by NPV were determined by microscopic

observation. Standard bioassay curves with known amounts of AcNPV.WT or AcJHE.SG in the greenhouse soil (A. Abdel-Rahman, J. R. Fuxa, and A. R. Richter, unpublished data) were used in conjunction with the soil bioassay results to estimate the number of OB/g of soil. For each standard curve, six viral concentrations were fed on soil diet to second-instar *T. ni,* as above, with 40 larvae/concentration. The results were subjected to probit analysis, resulting in the standard curves $Y = 0.342X + 3.41$ for AcNPV.WT, and $Y = 0.249X + 3.90$ for AcJHE.SG, where Y = probit % mortality and X = log OB/g soil.

Calibration. Calibration of dot-blots was accomplished by correlating the P^{32} signal, PSL (radiation dose), with the number of AcNPV.WT OB/larva. Live larvae were infected with AcNPV.WT, and individuals were frozen at 40, 44, 48, 54, 56, 68, 72, 102, 124, or 127 h postinfection. Each of these larvae was then homogenized in 0.25 ml sterile water and blotted on a nitrocellulose membrane. Radioactivity was detected as described for the greenhouse-collected larvae. A Fuji PhosphorImager was utilized for quantification of the radioactive signal. An image from the hybridized nitrocellulose membrane was obtained and analyzed with the Fujix Bas1000 ImageFile Manager softwares, and radiation dose values were obtained for each larva. The number of OBs from each larva was determined by counting the individual OB under a phase-contrast microscope with a hemocytometer after extraction with 0.5% SDS and two washes with 0.5 M NaCl. PSL values were plotted against the number of OBs in corresponding larvae, and a standard curve was obtained using the Delta Graph software. Radiation doses were determined with a PhosphorImager and plotted against the number of OBs for each corresponding larva.

Statistical analysis. Pairs of treatments (within each insect generation or within the soil results) were compared statistically by analysis of variance through the SAS General Linear Models Procedure and the PROC MIXED procedure (SAS Institute, 1996). Pairs of treatments, rather than the entire data set within each insect generation, were subjected to this procedure due to the large number of zeroes in the final data (Table 1). The data for OBs/g soil were transformed to $\log_e$ in order to satisfy normality assumptions for the analysis of variance. Viral horizontal dispersion in soil was subjected to the PROC REG analysis (SAS Institute, 1996). Percentages of mortality from the soil bioassays were converted to OB/g with the standard curves. Viral indices were calculated with Taylor's power law, $\log s^2 = b \log (\bar{x}) + \log a$ (Taylor, 1961), which is the regression of log variance (s^2) on log mean ($\bar{x}$). In this regression, b, the slope, is a species-specific aggregation constant (Taylor, 1961; Elliott, 1979). A slope of 1.0 indicates a random population distribution, whereas a slope >1.0 indicates a clumped distribution and a slope <1.0 indicates a uniform distribution (Elliott, 1979).

RESULTS

The methods in this research were used successfully to induce replicated, 8-week epizootics of AcNPV.WT in 1.35 × 1.35 m microplots in an artificial setting. Insect cadavers were observed in all viral treatment plots. All cadavers in the WT plots exhibited typical signs of nuclear polyhedrosis. These relatively large cadavers virtually became liquified on the leaves, presumably releasing viral polyhedral occlusion bodies. In the AaIT treatment plots (Table 1), the insects died as small larvae and usually fell from leaves onto the soil surface, where they remained intact (did not disintegrate or liquify). In the JHE.SG plots, dead larvae were 10–20% smaller than those killed by AcNPV.WT; some AcJHE.SG-killed larvae remained on the plants and some fell on the soil surface. Larvae killed by AcNPV.WT and AcJHE.SG liquified and disintegrated, although AcJHE.SG-killed larvae took 2 to 3 more days to disintegrate than those killed by wild-type virus.

Calibration of the dot-blot by blotting known amounts of plasmid DNA with wild-type (Fig. 1) and recombinant baculovirus DNA showed that 0.48 ng was the cutoff to score an unknown as positive for AcNPV.WT and AcNPV.AaIT, and 15.6 ng was the cutoff for AcJHE.SG. The calibration curve for detection and quantification of AcNPV.WT, $Y = 1663.2 + 1.974\ X$ ($R^2 = 0.9988$), where Y = radiation dose, PSL and X = number of OBs, confirmed the sensitivity of the DNA–DNA dot-blot assay.

AcNPV.WT vs AcNPV.AaIT. AcNPV.WT out-competed AcNPV.AaIT on the insect host plant in the greenhouse plots (Tables 1 and 2). In the WT treatment (AcNPC.WT only released), AcNPV.WT produced epizootics in *T. ni* for 5 weeks. In the AaIT treatment (AcNPV.AaIT only released), prevalence of AcNPV.AaIT was significantly ($P < 0.01$, Table 2) lower than that of AcNPV.WT in the WT treatment by week 2 (see below for further explanation of generation-1 results), and AcNPV.AaIT prevalence decreased to zero by the fourth week. In the treatment with both viruses released (WT + AaIT), prevalence of AcNPV.WT increased to the point where it was not significantly ($P > 0.05$, Table 2) different from that in the WT treatment by the second week, whereas AcNPV.AaIT prevalence again decreased to zero, this time in the third week. In the WT + AaIT treatment, prevalence of the two viruses was not significantly ($P > 0.05$, Table 2) different in week 1, but prevalence of AcNPV.WT was significantly ($P < 0.05$ in week 2, $P < 0.01$ in weeks 3–5) greater than that of AcNPV.AaIT in weeks 2–5 (Table 2). Prevalence of NPV during weeks 6–8 averaged 82.7% (total $n = 327$) and 74.7% ($n = 302$) mortality

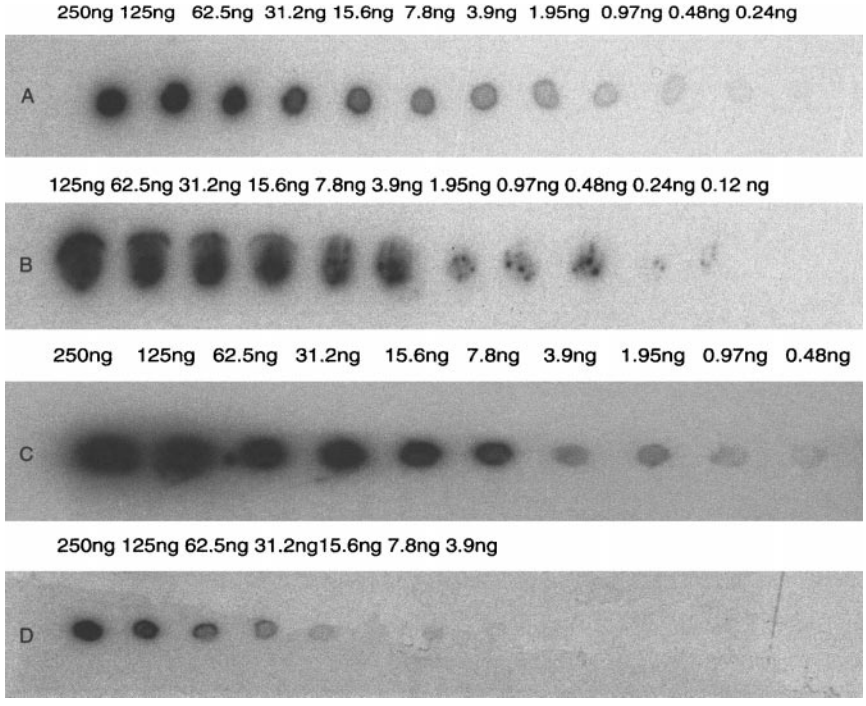

FIG. 1. Calibration of the dot-blot by blotting known amounts of plasmid DNA in the presence of uninfected insect homogenates with P^{32}-labeled wild-type baculovirus DNA (*Kpn*I–*Msc*I fragment of PAcUW21). Blots are for plasmids of (A) AcNPV.WT, (B) AcNPV.WT in the presence of uninfected insect homogenates, (C) AcNPV.AaIT, and (D) AcJHE.SG. The cutoff to score an unknown as positive is 0.48 ng for AcNPV.WT and AcNPV.AaIT and 15.6 ng for AcJHE.SG.

of insects in WT and in WT + AaIT plots, respectively. There was no mortality in control ($n = 362$) or in AaIT ($n = 336$) plots during weeks 6–8.

It is virtually certain that all of the insects in week 1 of the AaIT treatment (Table 1) were infected with AcNPV.AaIT. The inocula fed to the week-1 larvae were confirmed by the DNA probes to be the correct NPVs; 100% of the week-1 cadavers in the AaIT plots exhibited symptomatology of infection by AcNPV.AaIT and AcJHE.SG, and there was no evidence of AcNPV.WT epizootics on plants after week 1 or in the soil in the AaIT plots (15% week-1 prevalence of AcNPV.WT should have been more than sufficient to initiate epizootics of this virus with high prevalence rates). However, the shipment of week-1 insects from Louisiana State University to the University of California, Davis was temporarily lost in the mail and remained at room temperature for ca. 1 week. A corollary experiment indicated that the DNA probe for the AcNPV.WT virus hybridized with 100% of samples of insects treated with AcNPV.AaIT, regardless of whether the insects were frozen immediately after death or kept at room temperature for 6 days. On the other hand, the DNA probe for the AcNPV.AaIT virus hybridized with 100% of AcNPV.AaIT-treated insects that had been frozen but with only 88% of those at room temperature. Thus, the actual prevalence rates of NPVs in week-1 insects in the AaIT plots probably were 0% AcNPV.WT and 86% AcNPV.AaIT. The subsequent, occasional detection of single anomalous, AcNPV-WT-infected insects (e.g., in week-4 control and week-3 AaIT plots, Table 1) probably represents minor contamination by AcNPV.WT.

AcNPV.WT accumulated in the soil in greater amounts than AcNPV.AaIT (Table 1). AcNPV.WT in the WT treatment accumulated 46× as many OBs/g soil as AcNPV.AaIT in the AaIT treatment, a difference significant at $P = 0.001$ (Table 2). Similarly, in the WT + AaIT plots, AcNPV.WT accumulated 304× as many OBs/g soil as AcNPV.AaIT, significant at $P = 0.002$ (Table 2). However, the amounts of both NPVs in soil in the WT + AaIT plots (Table 1) were only 1–2% as great as the amounts in the treatments in which the viruses were released individually, although this difference was significant (Table 2) for AcNPV.AaIT ($P < 0.05$) but not for AcNPV.WT ($P > 0.05$).

AcNPV.WT vs AcJHE.SG. AcNPV.WT also outcompeted AcJHE.SG on the insect host plant (Tables 3 and 4). In the WT treatment (AcNPC.WT only released), AcNPV.WT produced epizootics in *T. ni* for 8 weeks. In the JHE.SG treatment (AcJHE.SG only released), prevalence of AcJHE.SG was significantly ($P < 0.05$, Table 4) lower than that of AcNPV.WT in the WT treatment in every week except the sixth. In the treatment with both viruses released (WT +

TABLE 2

Results of Analyses of Variance (General Linear Models Procedure, SAS Institute, 1996) Comparing Certain Treatments within Each Week or within the Soil Bioassay (See Table 1)[a] of the AcNPV.WT versus AcNPV.AalT Experiment

	WT (in WT plots) vs WT (in WT + AalT plots)		WT (in WT plots) vs AalT (in AalT plots)		AalT (in AalT plots) vs AalT (in WT + AalT plots)		WT vs AalT (both in WT + AalT plots)	
Week	F[b]	$P > F$	F[b]	$P > F$	F[b]	$P > F$	F[b]	$P > F$
				Prevalence of virus in larvae on collard leaves				
1	25.97	0.0146	12.82[c]	0.0373[c]	18.15[d]	0.0237[d]	0.07	0.8058
2	3.12	0.1757	81.33	0.0029	2.45	0.2152	13.63	0.0345
3	0.06	0.8159	14002.78	0.0001	6.82	0.0796	1080.29	0.0001
4	1.00	0.3910	16986.78	0.0001	*e*		6272.67	0.0001
5	0.01	0.9284	119.64	0.0016	*e*		78.36	0.0030
				Bioassays of virus in soil[f]				
	8.04	0.0659	137.51	0.0013	28.30	0.0130	96.07	0.0023

[a] WT and AalT = insects infected with AcNPV.WT or AcNPV.AalT, respectively. WT plots, AalT plots, and WT + AalT plots were those in which insects were released which had been infected with AcNPV.WT, AcNPV.AalT, or a 1:1 mixture infected with AcNPV.WT and AcNPV.AalT, respectively (Table 1).

[b] Treatment F; treatment $df = 1$, replication $df = 3$ in every analysis.

[c] Treatment $F = 0.61$, $P > F = 0.491$, when WT and AalT percentages of mortality are combined for AalT infections in the AalT treatment plots (see Table 1 and text for explanation).

[d] Treatment $F = 61.18$, $P > F = 0.004$, when WT and AalT percentages of mortality are combined for AalT infections in the AalT treatment plots (see Table 1 and text for explanation).

[e] Data were all zeroes.

[f] Soil data were transformed to $\log_e$ for analysis to satisfy normality assumptions.

TABLE 3

Amounts of Wild-Type and Recombinant NPV, Indicated by Bioassay of Vegetation and Soil, Resulting from Epizootics of Wild-Type (AcNPV.WT) or Recombinant (AcJHE.SG) Virus in *Trichoplusia ni* on Collard Plants in a Greenhouse Microcosm

	Control[b]		WT[c]		JHE.SG[c]		WT + JHE.SG[d]	
Week[a]	WT[e]	JHE.SG[e]	WT	JHE.SG	WT	JHE.SG	WT	JHE.SG
				Mean % mortality[f] (±SE) in larvae on collard leaves				
1	0	0	96.0 ± 3.0	0	0.5 ± 1.0	93.0 ± 3.0	33.6 ± 5.95	59.4 ± 4.4
2	0	0	38.3 ± 11.8	0	0	11.5 ± 1.8	10.2 ± 3.0	3.1 ± 2.6
3	0	0	72.7 ± 10.3	0	0.3 ± 0.5	26.6 ± 9.7	24.2 ± 5.9	2.3 ± 3.0
4	0	0	83.6 ± 5.3	0	1.8 ± 1.3	57.0 ± 23.9	31.3 ± 13.5	7.0 ± 4.7
5	0	0	82.8 ± 9.4	0	0.5 ± 1.0	67.2 ± 20.7	30.5 ± 15.4	10.2 ± 7.8
6	0	0	28.9 ± 12.1	0	0	44.5 ± 12.6	23.4 ± 11.6	0
8	0	0	79.7 ± 24.3	0.8 ± 1.6	0	29.7 ± 18.5	23.4 ± 5.4	2.3 ± 1.6
				Mean OBs/g soil[g] (±SE) after 8 weeks				
	0	0	1592 ± 1398	0	0	1167 ± 1584	292 ± 447	0

[a] Week 7 is not included due to problems in shipment of the samples.

[b] Uninfected insects were released weekly onto collards in the greenhouse plots.

[c] All released week-1 insects were infected with AcNPV.WT or AcJHE.SG. Subsequently, healthy insects were released weekly.

[d] Half of released week-1 insects were infected with AcNPV.WT and half with AcJHE.SG. Subsequently, healthy insects were released weekly.

[e] Amount of AcNPV.WT or AcJHE.SG, respectively, detected as disease prevalence in insects on collards or by soil bioassay.

[f] Percentage of insects dying and hybridizing with appropriate DNA probes. Those insects not hybridizing were assumed to be uninfected with AcNPV.WT or AcJHE.SG. Each mean represents four replications with 32 insects sampled per replication.

[g] Viral occlusion bodies (OBs)/g soil were calculated from percentages of bioassay mortality according to the following standard curves: AcNPV.WT, $Y = 0.342X + 3.41$; AcJHE.SG, $Y = 0.249X + 3.90$, where Y = probit % mortality and X = log OB/g soil.

TABLE 4

Results of Analyses of Variance (General Linear Models Procedure, SAS Institute, 1996) Comparing Certain Treatments within Each Week or within the Soil Bioassay (see Table 1)[a] of the AcNPV.WT versus AcJHE.SG Experiment

	WT (in WT plots) vs WT (in WT + JHE.SG plots)		WT (in WT plots) vs JHE.SG (in JHE.SG plots)		JHE.SG (in JHE.SG plots) vs JHE.SG (in WT + JHE.SG plots)		WT vs JHE.SG (both in WT + JHE.SG plots)	
Week[b]	F[c]	$P > F$	F[c]	$P > F$	F[c]	$P > F$	F[c]	$P > F$
				Prevalence of virus in larvae on collard leaves				
1	48.07	0.0001	0.03	0.8594	29.40	0.0001	1.78	0.1866
2	10.24	0.0020	14.27	0.0012	0.39	0.5358	0.64	0.4263
3	30.37	0.0001	27.50	0.0001	7.59	0.0074	6.20	0.0151
4	35.47	0.0001	9.13	0.0035	32.36	0.0001	7.60	0.0074
5	35.47	0.0001	3.16	0.0797	42.10	0.0001	5.34	0.0237
6	0.39	0.5358	3.16	0.0797	25.67	0.0001	7.11	0.0095
8	40.96	0.0001	32.36	0.0001	9.68	0.0027	5.76	0.0190
				Bioassays of virus in soil[d]				
	6.70	0.0270	0.51	0.4933	63.42	0.0001	21.75	0.0009

[a] WT and JHE.SG = insects infected with AcNPV.WT or AcNPV.JHE.SG, respectively. WT plots, JHE.SG plots, and WT + JHE.SG plots were those in which insects were released which had been infected with AcNPV.WT, AcNPV.JHE.SG, or a 1:1 mixture infected with AcNPV.WT and AcNPV.JHE.SG, respectively (Table 3).

[b] Due to shipment problem, samples from week 7 had to be deleted.

[c] Treatment F; treatment $df = 1$, replication $df = 3$ in every analysis.

[d] Soil data were transformed to $\log_e$ for analysis to satisfy normality assumptions.

JHE.SG), prevalence of AcNPV.WT was significantly ($P < 0.05$, Table 4) lower than that in the WT treatment in every week except the sixth. AcJHE.SG prevalence in the WT + JHE.SG plots did not rebound after the second week as did AcJHE.SG in JHE.SG plots. In the WT + JHE.SG treatment, prevalence of AcNPV.WT was significantly ($P < 0.05$) greater than that of AcJHE.SG in weeks 3–6 and 8 (Table 4). Prevalence of AcNPV.WT was significantly ($P < 0.01$) greater in WT than in WT + JHE.SG plots during the entire experiment except week 6. Similarly, prevalence of AcJHE.SG was significantly ($P < 0.01$) greater in JHE.SG than in WT + JHE.SG plots during the entire experiment except week 2. There was no mortality due to NPV in control plots.

AcNPV.WT accumulated significantly more OBs in soil than AcJHE.SG in the WT+JHE.SG plots ($P = 0.0009$) (Table 4). However, in single-virus plots, the accumulated number of AcNPV.WT OBs/g soil was not significantly different from that of AcJHE.SG ($P = 0.4933$) (Table 4). The amounts of both NPVs in soil in the WT + JHE.SG plots (Table 3) were significantly lower than amounts in the treatments in which the viruses were released individually ($P < 0.0001$ for AcJHE.SG, $P = 0.0270$ for AcNPV.WT, Table 4). The slopes of Taylor's power law regressions (Table 5) were not significantly >1 for either AcNPV.WT ($P = 0.481$) or AcJHE.SG ($P = 0.108$). AcNPV.WT and AcJHE.SG at the conclusion of the 8-week experiment were randomly distributed in soil.

DISCUSSION

It is not surprising that AcNPV.WT had higher prevalence rates than AcNPV.AaIT and AcJHE.SG in insects on host plants. AcNPV.AaIT produces only 20%

TABLE 5

Comparison of the Coefficients from Taylor Power Law[a] for Populations of Recombinant and Wild-Type Nucleopolyhedroviruses (NPV) in Soil ($n = 4$ for Each NPV)[b]

Virus	Log a + SE	b + SE	r^2	F	Probability > F	F testing $b = 1$	Probability > F for $b = 1$
AcJHE.SG	2.38 ± 6.65	1.75 ± 0.87	0.668	4.02	0.183	0.75	0.481
AcNPV.WT	−7.92 ± 5.71	3.11 ± 0.75	0.895	17.00	0.054	7.83	0.108

[a] Log $s^2 = b \log(\bar{x}) + \log a$, or the regression of log variance (s^2) on log mean ($\bar{x}$).

[b] Regression and General linear model procedures (SAS Institute, 1996).

(Kunimi *et al.,* 1996) and AcJHE.SG only 34% (Fuxa *et al.,* 1998) as many viral OBs as AcNPV.WT in *T. ni.* Additionally, only 10% of *T. ni* killed by AcNPV.AaIT and 30% of *T. ni* killed by AcJHE.SG disintegrate by day 4 after death, compared to 100% of those killed by AcNPV.WT after 1 day (Fuxa *et al.,* 1998). Thus, it is likely that smaller amounts of the recombinant viruses, particularly AcNPV.AaIT, than wild-type virus are released onto the leaves of host plants (Hoover *et al.,* 1995), where new generations of insects can ingest it and become infected. Thus, in single-virus plots, AcNPV.WT produced continuous epizootics in up to seven recruitment cycles of uninfected insects (Table 3). AcJHE.SG prevalence in weeks 2–7 was generally lower than that of AcNPV.WT (Table 3), and AcNPV.AaIT almost disappeared in the first recruitment cycle of uninfected insects (week 2; Table 1). More importantly, both recombinant viruses were out-competed by AcNPV.WT in the direct-competition plots (Tables 1–4). Thus, AcJHE.SG, the intermediate virus in terms of replication and disintegration, is also intermediate in terms of causing epizootics, but it still cannot compete with AcNPV.WT.

Dynamics of the recombinant NPV were not as easy to predict in soil as on insect host plants. The wild-type NPV could increase to greater levels than the recombinant in soil, because the wild-type virus contaminates leaves and continues to infect and replicate in host insects (Tables 1 and 3). On the other hand, AcNPV.AaIT and perhaps AcJHE.SG could increase to greater levels than wild-type virus in soil, because the wild-type virus in cadavers disintegrating on leaves is more exposed to ultraviolet radiation in sunlight, which inactivates NPVs within hours (Benz, 1987), than recombinant NPV, particularly AcNPV.AaIT, in intact cadavers that have fallen to the soil surface beneath plants (Cory *et al.,* 1994; Hoover *et al.,* 1995). The current results indicate clearly (Tables 1–4) that, if recombinant viruses and wild-type virus are given the opportunity to replicate in several insect recruitment cycles in a greenhouse, then the wild-type virus will increase to greater population densities in soil than the recombinant.

Competition between two species can be defined as an interaction with negative impact on both species (Price, 1997). The interaction between AcNPV.WT and AcJHE.SG fits this definition, because prevalence of both viruses in host insects and in soil was lower in the WT+JHE.SG plots than in the single-virus plots (Tables 3 and 4). Similarly, AcNPV.AaIT and AcNPV.WT both accumulated lower numbers of OB in soil at week 8 in the WT + AaIT plots than in the single-virus plots, though the difference for AcNPV.WT was significant only at $P < 0.10$ (Tables 1 and 2). Fifty percent as many insects infected with each NPV were released at week 1 in the double-virus plots compared with single-virus plots, but there were only 0–18% as many OBs/g soil of all three viruses in double-virus plots as in single-virus plots after 8 weeks (Tables 1 and 3), confirming that interaction negatively impacted populations of both viruses in each experiment.

Dispersion of pathogen units may affect their probability of encountering members of the host population (Tanada and Fuxa, 1987). Burges and Hussey (1971) hypothesized that pathogens are unevenly distributed in nature. However, populations of AcJHE.SG and AcNPV.WT were randomly distributed over soil. It is likely that disintegration of virus-killed larvae followed by precipitation (watering) caused this dispersion pattern. On collards leaves, dispersion of AcNPV.WT was random, but populations of AcNPV.AaIT and another AcNPV-JHE recombinant were clumped (Fuxa *et al.,* 1998).

An important result of this research was that the methods were used successfully to induce replicated, 8-week epizootics of AcNPV.WT in microplots in an environmentally controlled, artificial setting. This should enable researchers to answer certain ecological questions about NPV epizootics when controlled conditions are necessary or when research in a natural setting is undesirable for regulatory or other reasons.

Our results reduce the level of concern about the possibility of environmental harm due to release of AcNPV.AaIT and AcJHE.SG. The possible harmfulness of recombinant organisms to nontarget organisms in the environment is difficult to predict (Fuxa, 1990), although recombinant viruses have not harmed certain nontarget organisms (Possee *et al.,* 1993; Heinz *et al.,* 1995; McNitt *et al.,* 1995; McCutchen *et al.,* 1996; Treacy *et al.,* 1997; Lee and Fuxa, 2000a). Therefore, the probability that a released recombinant will contact nontarget organisms (exposure assessment) becomes a critical issue in risk assessment. In the case of living organisms, this probability of contact is largely a function of the recombinant's capability to persist, increase in numbers, and move or be transported away from the release site (Fuxa, 1990). The inability of the recombinant virus to compete with wild-type virus in this research increases the probability that populations of the recombinant will decrease after release, which in turn reduces its environmental risk. However, AcJHE.SG and AcNPV.AaIT can be transported by scavenging and predatory arthropods (Lee and Fuxa, 2000a,b).

In addition to risk assessment, our results contribute to current debates concerning virulence in host–parasite coevolution. NPVs have been proposed (May, 1983) as an exception to the long-held theory that parasites evolve to become less virulent toward their hosts (Herre, 1993; Centofanti, 1995; Ewald, 1995). Mathematical modeling (Anderson and May, 1982) has indicated that this trend continues only to a certain point or, in other words, that if a virus becomes too virulent its reproductive success will begin to decrease. Our

data support this last conclusion, because AcJHE.SG and, especially, AcNPV.AaIT, which are more virulent than their parental AcNPV.WT, were out-competed by the wild-type virus in terms of their replication and accumulation in the microcosm miniplots.

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REFERENCES

Anderson, R. M., and May, R. M. 1982. Coevolution of hosts and parasites. *Parasitology* **85,** 411–426.

Benz, G. 1987. Environment. *In* "Epizootiology of Insect Diseases" (J. R. Fuxa and Y. Tanada, Eds.), pp. 177–214. Wiley, New York.

Bonning, B. C., Hoover, K., Booth, T. F., Duffey, S., and Hammock, B. D. 1995. Development of a recombinant baculovirus expressing a modified juvenile hormone esterase with potential for insect control. *Arch. Biochem. Physiol.* **30,** 177–194.

Burges, H. D., and Hussey, N. W. 1971. Past achievements and future prospects. *In* "Microbial Control of Insects and Mites" (H. D. Burges and N. W. Hussey, Eds.), pp. 687–709. Academic Press, New York.

Centofanti, M. 1995. Playing by the rules: How and why organisms turn nasty. *Sci. News* **148,** 382–383.

Cory, J. S., Hirst, M. L., Williams, T., Halls, R. S., Goulson, D., Green, B. M., Carty, T. M., Possee, R. D., Cayley, P. J., and Bishop, D. H. L. 1994. Field trial of a genetically improved baculovirus insecticide. *Nature* **370,** 138–140.

Elliott, J. M. 1979. "Some Methods for the Statistical Analysis of Samples of Benthic Invertebrates." Freshwater Biological Association Scientific Publ. No. 25, Ambleside, UK.

Ewald, P. W. 1995. The evolution of virulence: A unifying link between parasitology and ecology. *J. Parasitol.* **81,** 659–669.

Fuxa, J. A., Fuxa, J. R., and Richter, A. R. 1998. Host–insect survival time and disintegration in relation to population density and dispersion of recombinant and wild-type nucleopolyhedroviruses. *Biol. Control* **12,** 143–150.

Fuxa, J. R. 1989. Fate of released entomopathogens with reference to risk assessment of genetically engineered microorganisms. *Bull. Entomol. Soc. Am.* **35,** 12–24.

Fuxa, J. R. 1990. Environmental risks of genetically engineered entomopathogens. *In* "Safety of Microbial Insecticides" (M. Laird, L. A. Lacey, and E. W. Davidson, Eds.), pp. 203–207. CRC Press, Boca Raton, FL.

Fuxa, J. R. 1991. Insect control with baculoviruses. *Biotech. Adv.* **9,** 425–442.

Fuxa, J. R., Warren, G. W., and Kawanishi, C. 1985. Comparison of bioassay and enzyme-linked immunosorbent assay for quantification of *Spodoptera frugiperda* nuclear polyhedrosis virus in soil. *J. Invertebr. Pathol.* **46,** 133–138.

Heinz, K. M., McCutchen, B. F., Herrmann, R., Parrella, M. P., and Hammock, B. D. 1995. Direct effects of recombinant nuclear polyhedrosis viruses on selected non-target organisms. *J. Econ. Entomol.* **88,** 259–264.

Herre, E. A. 1993. Population structure and the evolution of virulence in nematode parasites of fig wasps. *Science* **259,** 1442–1445.

Hoover, K., Schultz, C. M., Lane, S. S., Bonning, B. C., Duffey, S. S., McCutchen, B. F., and Hammock, B. D. 1995. Reduction in damage to cotton plants by a recombinant baculovirus that knocks moribund larvae of *Heliothis virescens* off the plant. *Biol. Control* **5,** 419–426.

Kunimi, Y., Fuxa, J. R., and Hammock, B. D. 1996. Comparison of wild type and genetically engineered nuclear polyhedrosis viruses of *Autographa californica* for mortality, virus replication and polyhedra production in *Trichoplusia ni* larvae. *Entomol. Exp. Appl.* **81,** 251–257.

Laird, M., Lacey, L. A., and Davidson, E. W. (Eds.). 1990. "Safety of Microbial Insecticides." CRC Press, Boca Raton, FL.

Lee, Y., and Fuxa, J. R. 2000a. Ingestion and defecation of recombinant and wild-type nucleopolyhedroviruses by scavenging and predatory arthropods. *Environ. Entomol.* **29,** in press.

Lee, Y., and Fuxa, J. R. 2000b. Transport of wild-type and recombinant nucleopolyhedroviruses by scavenging and predatory arthropods. *Microb. Ecol.* **39,** 301–313.

Maeda, S., Volrath. S. L., Hanzlik, T. N., Harper, S. A., Maddox, D. W., Majima, K., Hammock, B. D., and Fowler, E. 1991. Insecticidal effects of an insect-specific neurotoxin expressed by a recombinant baculovirus. *Virology* **184,** 777–780.

May, R. M. 1983. Parasitic infections as regulators of animal populations. *Am. Sci.* **71,** 36–45.

McCutchen, B. F., and Hammock, B. D. 1994. Recombinant baculovirus expressing an insect-selective neurotoxin: Characterization, strategies for improvement, and risk assessment. *In* "Natural and Engineered Pest Management Agents" (P. A. Hedin, J. J. Menn, and R. M. Hollingworth, Eds.), pp. 348–367. Am. Chem. Soc., Washington, DC.

McCutchen, B. F., Betana, M. D., Herrmann, R., and Hammock, B. D. 1997. Interactions of recombinant and wild-type baculoviruses with classical insecticides and pyrethroid-resistant tobacco budworm (Lepidoptera: Noctuidae). *J. Econ. Entomol.* **90,** 1170–1180.

McCutchen, B. F., Choudary, P. V., Crenshaw, R., Maddox, D., Kamita, S. G., Palekar, N., Volrath, S., Fowler, E., Hammock, B. D., and Maeda, S. 1991. Development of a recombinant baculovirus expressing an insect-selective neurotoxin: Potential for pest control. *Bio/Technology* **9,** 848–852.

McCutchen, B. F., Herrmann, R., Heinz, K. M., Parrella, M. P., and Hammock, B. D. 1996. Effects of recombinant baculoviruses on a nontarget endoparasitoid of *Heliothis virescens. Biol. Control* **6,** 45–50.

McNitt, L., Espielie, K. E., and Miller, L. K. 1995. Assessing the safety of toxin-producing baculovirus biopesticides to a non-target predator, the social wasp *Polistes metricus* Say. *Biol. Control* **5,** 267–278.

Omenn, G. S. 1986. Controlled testing and monitoring methods for microorganisms. *In* "Biotechnology Risk Assessment" (J. Fiksel and V. T. Covello, Eds.), pp. 144–163. Pergamon, New York.

Possee, R. D., Hirst, M., Jones, J. D., Bishop, D. H. L., and Cayley, P. J. 1993. Field tests of genetically engineered baculoviruses. *Br. Crop Protect. Council* **55,** 23–36.

Price, P. W. 1997. "Insect Ecology," 3rd ed. Wiley, New York.

SAS Institute. 1996. SAS 6.11 for Windows. SAS Institute, Cary, NC.

Stewart, L. M. D., Hirst, M., Ferber, M. L., Merryweather, A. T., Cayley, P. J., and Possee, R. D. 1991. Construction of an improved baculovirus insecticide containing an insect-specific toxin gene. *Nature* **352,** 85–88.

Tanada, Y., and Fuxa, J. R. 1987. The pathogen population. *In* "Epizootiology of Insect Diseases" (J. R. Fuxa and Y. Tanada, Eds.), pp. 113–187. Wiley, New York.

Taylor, L. R. 1961. Aggregation, variance, and the mean. *Nature* **189,** 732–735.

Treacy, M. F., All, J. N., and Kukel, C. F. 1997. Invertebrate selectivity of a recombinant baculovirus: Case study on AaHIT gene-inserted *Autographa californica* nuclear polyhedrosis virus. *In* "New Developments in Entomology" (K. Bondari, Ed.), pp. 57–68. Research Signpost, London.

Ward, V. K., Fleming, S. B., and Kalmakoff, J. 1987. Comparison of a DNA–DNA dot-blot hybridization assay with light microscopy and radioimmunoassay for the detection of a nuclear polyhedrosis virus. *J. Virol. Methods* **15,** 65–73.