SYNERGISM BETWEEN THE NUCLEOPOLYHEDROVIRUSES OF *AUTOGRAPHA CALIFORNICA* AND *TRICHOPLUSIA NI*

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Summary. – Previous observations on high virulence of *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) and *Trichoplusia ni* single nucleopolyhedrovirus (TnSNPV) acting together led us to test possible synergism between these two nucleopolyhedroviruses (NPVs) on cabbage looper larvae. Because synergism between AcMNPV and the *Trichoplusia ni* granulovirus (TnGV) has been well established before, these two viruses were included in this study as a positive control. Each virus was assayed separately on first-instar cabbage looper and their LC_{50}s were estimated at 2.33, 0.39 and 462 OB/mm^2 diet for AcMNPV, TnSNPV and TnGV, respectively. LC_{50}s of AcMNPV mixed with sub-lethal concentrations of TnSNPV and TnGV increased 8 and 10.7 times, respectively. Synergism between the viruses was analyzed by the ANOVA test for the LC_{50}s, the Plackett and Hewlett's joint-action rate test, and the Tammes-Bakuniak graphic method. All three analyses corroborated the synergism between the viruses. The presence of a putative enhancin in the TnSNPV was analyzed by Southern blot hybridization, using a 1.5 kbp *Kpn*I fragment from the TnGV *vef* gene as a probe. No hybridization was observed. The occurrence of a new putative synergistic factor in TnSNPV is discussed.

Key words: synergism; Trichoplusia ni single nucleopolyhedrovirus; Autographa californica multiple nucleopolyhedrovirus; joint-action ratio; ANOVA test; Tammes-Bakuniak graphic method; viral enhancin

Introduction

Multiple infections are common in insect natural populations. Individuals are exposed to a number of different pathogens and several pathogen species may infect the host simultaneously. Interactions between different pathogens within the same host are poorly understood; however, they are frequently considered competitive (Smith and Holt, 1996). Very few cases of synergistic interaction between pathogens have been documented to date, especially between different baculoviruses. The first thoroughly documented case of a synergistic interaction between baculoviruses has been reported by Tanada (1959), in this case a granulovirus (GV) and a nucleopolyhedrovirus (NPV) of *Pseudaletia unipuncta*, when applied to the host together, showed an enhanced infective activity. Later a protein called "synergistic factor" was identified in the granulovirus (Tanada and Hukuhara, 1971) as the cause of synergism.

Later a similar synergistic factor has been found in the TnGV (Derksen and Granados, 1988) enhancing the activity of AcMNPV. This factor was called "viral enhancing factor" (*vef*) which shared a nucleotide sequence homology of 99.1% with the previously described "synergistic factor" (Corsaro *et al*., 1993). Few years later, similar proteins have been found in a number of granuloviruses (GVs) isolated

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Abbreviations: AcMNPV = *Autographa californica* multiple nucleopolyhedrovirus; GV = granulovirus; LdNPV = *Limantria dispar* nucleopolyhedrovirus; NPV = nucleopolyhedrovirus; OB = occlusion body; R = joint action ratio; TnGV = *Trichoplusia ni* granulovirus; TnSNPV = *Trichoplusia ni* simple nucleopolyhedrovirus; *vef* = viral enhancing factor; vef^{dig} = *vef* gene labeled with digoxigenin

from different lepidopteran species (Corsaro *et al*., 1993; Gotto, 1990; Hashimoto *et al*., 1991; Roelvink *et al*., 1995) constituting a family of enhancing proteins called "enhancins".

Initially, there was evidence that enhancins were exclusively associated with GVs, but Bischoff and Slavicek (1996) have reported the occurrence of an enhancin in the nucleopolyhedrovirus of *Lymantria dispar* (LdNPV) and Li *et al*. (2003) have reported another enhancin in the nucleopolyhedrovirus of *Mamestra configurata* (McNPV). Furthermore, Xu and Hukuhara (1992) have found a synergistic factor with similar activity but different nucleotide sequence in the entomopoxvirus of *Pseudaletia separata*. Even if enhancins have been found in NPVs, there is no evidence of their synergistic effect, as no joint-action bioassays has been carried out to date along with another baculovirus.

In a previous report (Del Rincón-Castro and Ibarra, 1997) the field-collected samples of cabbage looper larvae infected with TnSNPV or AcMNPV or with both viruses showed that the infection was more efficient when both viruses infected the larvae. In the present study a evidence of synergism between these two viruses is presented. Although a putative synergistic factor may be involved in this association, no enhancin was detected by Southern blot analysis.

Materials and Methods

Viruses and plasmids. The TnSNPV strain used in this study was isolated from an infected *T. ni* larva collected in broccoli fields in Querétaro, Mexico. This strain has been characterized previously (Del Rincón-Castro and Ibarra, 1997). AcMNPV and TnGV were kindly provided by Dr. B.A. Federici (University of California, Riverside, CA). The plasmid p3VEF, containing the *vef* gene from TnGV (Hashimoto *et al*., 1991), was kindly provided by Dr. R.R. Granados, Boyce Thompson Institute, Ithaca, NY, USA.

Occlusion body (OB) purification. To produce primary inocula for bioassays and for DNA extraction OBs of each baculovirus were amplified and purified. Late 3rd instar *T. ni* larvae were inoculated by spreading 2 x 10^7 OBs for TnSNPV and TnGV, and 10^6 OBs for AcMNPV on a Petri dish containing meridic diet (Vail *et al*., 1973). Larvae were incubated at 28 ± 1°C, relative humidity of 70 ± 10%, and 12:12 hrs photoperiod for 5–6 days. OBs were extracted from larvae and purified by sucrose gradient centrifugation according to a procedure described earlier (Derksen and Granados, 1988).

Bioassay procedure. Estimation of LC_{50} (the estimated virus concentration that kills 50% of the tested population) for each baculovirus as well as the virus combinations tested in this study was described in detail by Del Rincón-Castro and Ibarra (1997). The OB concentrations ranged between 0.1671 and 27.85 per mm^2 diet for AcMNPV; between 30.1 and 1,505 per mm^2 diet for TnGV; and between 0.1093 and 3.3 mm^2 diet for TnSNPV. A total of 30 first-instar *T. ni* larvae per concentration were tested in each Petri dish containing the inoculated meridic diet. Bioassays were run under the conditions mentioned above and the mortality was recorded at 7 days post inoculation. The data were subjected to Probit analysis and one LC_{50} value was estimated for each virus.

Evaluation of synergism. To assess synergism effect between AcMNPV and TnSNPV, two series of bioassays were carried out. TnGV was included in the bioassays as a positive control due to its well known synergistic interaction with AcMNPV. In the first series, concentrations below LC_{50} of TnGV (LC_{20}, 166 OB/mm^2) and TnSNPV (LC_{30}, 0.15 OB/mm^2) were mixed with each of the AcMNPV concentrations tested in the bioassay. Further adjustment of the AcMNPV concentrations was necessary, as unexpectedly high mortalities were obtained in this first series of bioassays (see Results). LC_{50} values of AcMNPV alone and in combination with TnSNPV and TnGV, were analyzed by the ANOVA and Tukey's mean tests (SAS Institute, 1992). Synergism was also evaluated by the Tammes-Bakuniak's graphic method (Busvine, 1971; Fuxa, 1979). This method locates the LC_{50}s estimated for the combinations within a coordinate plane. One axis of the plane represents the dose range of one factor (i.e. AcMNPV, Fig. 3A) and another axis represents the dose range of the second factor (i.e. TnSNPV, Fig. 3A). The equitoxic line (straight line between the LC_{50} values of both viruses) (solid line, Fig. 3A) separates the "antagonism zone" from the "synergism zone" in the graph, with a "buffer area" between the fiducial limits (p = 0.95) for each LC_{50} value (dotted lines, Fig. 3A), representing the "additivity zone". The LC_{50} values estimated for the combinations AcMNPV/TnSNPV and AcMNPV/TnGV were located in the plane and the type of interaction was identified according to the zone they were located at.

This diagram is called isobologram and the equitoxic line is defined by: $(z_1/Z_1) + (z_2/Z_2)=1$, where z_1 and z_2 are the quantities of both viruses in the mixture, and Z_1 and Z_2 are the doses of each virus, producing the same effect (i.e. LC_{50}), when tested alone (Busvine, 1971). Any deviation from this assumption constitutes either a synergistic (<1) or an antagonistic (>1) relationship between the viruses.

Finally, the "joint action ratio*"* (R) was calculated according to the Plackett and Hewlett equation (Busvine, 1971): $R = 1\ [1+ (k + 1)^{1/2}]$, where k is estimated from the equations $V_1+kV_1V_2+V_2=1$ and $V_1= z_1/Z_1$, and $V_2= z_2/Z_2$.

R=1 if there is an additive action between the viruses, R>1 if there is synergism between the viruses, and R<1 if there is antagonism between the viruses.

Identification of an ehnancin in TnSNPV. The *Kpn*I fragment of 1.5 kbp was cut out from the plasmid p3VEF, which contains the TnGV *vef* gene and was purified (Hashimoto *et al*., 1991). This fragment was labeled with digoxigenin (vef^{dig}) according to the manufacturer's suggestions (GIBCO-BRL), and used as a probe.

Southern blot analysis was carried out by digesting 1 µg of genomic DNA of AcMNPV (negative control), TnGV (positive control) and TnSNPV (test) with *Hind*III. The digested DNA was subjected to electrophoresis in agarose gels and blotted to nylon membranes (Hybond N^+). The membranes were subjected to hybridization at high stringency conditions (65°C) overnight, using the fragment vef^{dig} as a probe.

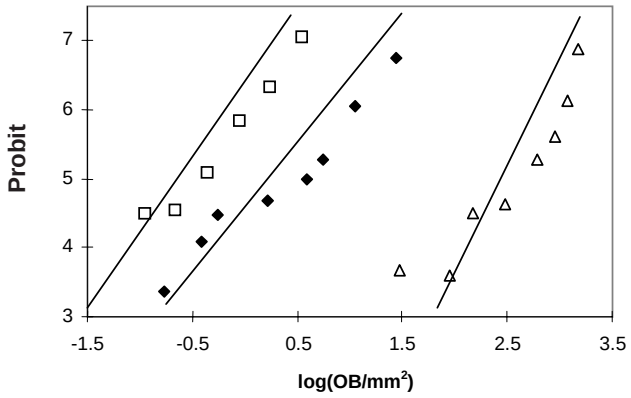

Fig. 1

Dose-mortality regression lines calculated from the Probit analysis of bioassays on cabbage looper first-instar larvae

AcMNPV (■), TnSNPV (□), and TnGV (Δ) tested separately.

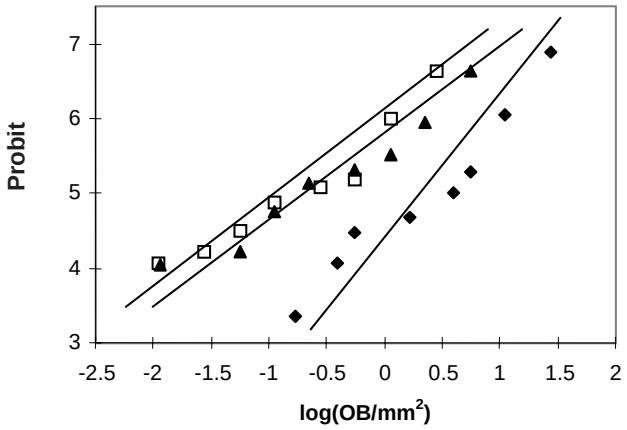

Fig. 2

Dose-mortality regression lines calculated from the Probit analysis of bioassays on cabbage looper first-instar larvae

AcMNPV tested separately (■) and in combination with TnSNPV (▲) and TnGV (□).

Results

Virulence of individual viruses

Before any synergistic relationship between baculoviruses can be established, the level of virulence of each virus alone should be estimated. For this purpose, bioassays performed on cabbage looper 1st instar larvae with AcMNPV, TnGV, and TnSNPV alone, were carried out, and LC_{50} values were estimated at 2.33, 462, and 0.395 OB/mm^2 diet, respectively. Fig. 1 shows the comparison between the dose-mortality regression lines, estimated by the Probit analysis. The shift of the TnSNPV regression line to the left of the graph indicates that this virus was more virulent (5.9 times) than AcMNPV; however, the shift of the TnGV regression line to the right of the graph was not related to its virulence, as no comparison could be done between the LC_{50} values of NPVs and GVs due to great difference in the number of virions occluded in each OB.

Virulence of combinations of viruses

Mortalities between 52% and 100% were obtained in the first series of bioassays, when AcMNPV concentrations were combined with the LC_{20} and LC_{30} of TnGV and TnSNPV, respectively. These results could not be used for statistical analysis of LC_{50} by the Probit analysis (Ibarra and Federici, 1987). Consequently, the dose range of AcMNPV was adjusted to lower levels. In a second series of bioassays, LC_{50}s were adequately estimated and regression lines were calculated from dose-response relationships (Fig. 2). The combination AcMNPV/TnSNPV showed an LC_{50} of 0.29 OB/mm^2, while the combination AcMNPV/TnGV showed an LC_{50} of 0.218 OB/mm^2. These results indicate that AcMNPV alone was 8 times and 10.7 times less virulent than those combinations, respectively. The two regression lines estimated for the combinations were very similar (close to each other), and they differed significantly from the AcMNPV regression line if applied alone (Fig. 2). Statistical analysis (the ANOVA and Tukey's tests) corroborated such a significant difference ($p < 0.001$) between the LC_{50} values of the combinations and that of AcMNPV applied alone.

Evaluation of synergism

A distinctive synergism was proved for both combinations using the Tammes-Bakuniak graphic method. Fig. 3A shows that the LC_{50} estimated for the combination AcMNPV/TnSNPV was located in the area of "synergism", below the "additivity" zone defined by LC_{50} fiductial limits. On the other hand, the combination of AcMNPV/TnGV, used as a positive control, also showed a distinctive synergism between these viruses as expected (Fig. 3B). The LC_{50} estimated for this combination was also located in the "synergism" zone of the graph.

Additionally, the mathematical approach of the equitoxic line, defined by $(z_1/Z_1) + (z_2/Z_2)=1$, showed a substantial deviation from additivity, as $(z_1/Z_1) + (z_2/Z_2)$ was equals to 0.5 and 0.45 for the combinations AcMNPV/TnSNPV and AcMNPV/TnGV, respectively, indicating a synergism between these viruses. Furthermore, the joint action ratio (R) defined as $R = 1\ [1+ (k + 1)^{1/2}]$ was estimated at 2.19 and 2.59, for the combinations AcMNPV/TnSNPV and

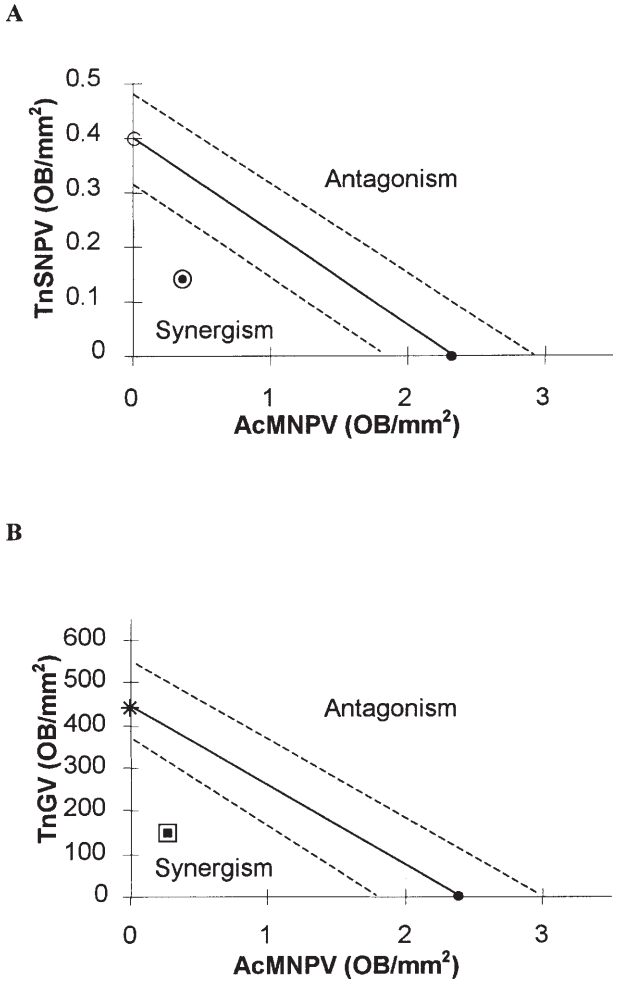

Fig. 3

The Tammes-Bakuniak graphic method used to establish the type of joint-action effect of the combinations AcMNPV/TnSNPV (A) and AcMNPV/TnGV (B)

The straight line (isobole) between the LC_{50} values of AcMNPV (●), TnSNPV (○) and TnGV (*), acting separately, describes an additive interaction between both factors. Broken lines connect the LC_{50} fiducial limits. Both LC_{50} values were estimated for the combinations AcMNPV/TnSNPV (◉) and AcMNPV/TnGV (▣) fall in the "synergism" area of the graphic.

AcMNPV/TnGV, respectively, thus corroborating synergistic association between these viruses.

Detection of an enhancin in TnSNPV

When the digested genomes of AcMNPV, TnGV and TnSNPV were hybridized with the *Kpn*I fragment of the TnGV *vef* gene, only the positive controls (TnGV and the plasmid p3VEF) showed a distinct hybridization signal. A 6.9 kbp band from the TnGV genome showed a clear

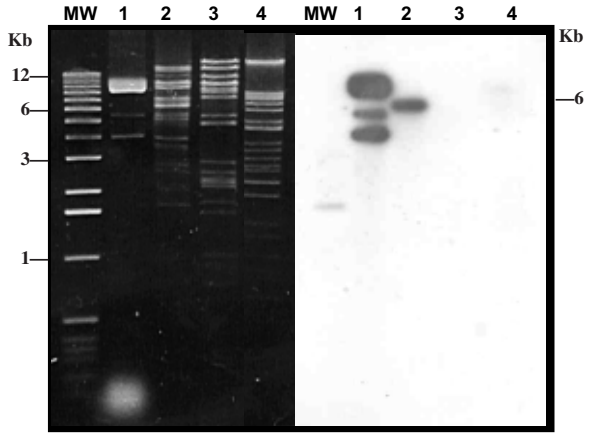

Fig. 4

Southern blot analysis *Hind*III-digested viral genomes using the *Kpn*I fragment of the TnGV *vef* gene as a probe

Agarose gel (left) and blot (right). Molecular size marker (lanes MW); p3VEF plasmid (lane 1); TnGV genome (lane 2); TnSNPV genome (lane 3); AcMNPV genome (lane 4).

hybridization with the probe (Fig. 4), corresponding to the *Hind*III fragment that contains the *vef* gene of this virus (Hashimoto *et al*., 1991). As expected, the digested AcMNPV genome showed no hybridization and, interestingly, the TnSNPV genome displayed no hybridization signal, too (Fig. 4). Because the TnGV *vef* gene and the putative TnSNPV enhancin gene may present low homology between each other, the same Southern blot analysis was carried out under less stringent conditions; however, the same results were obtained (data not shown). These results indicate that TnSNPV contains no enhancin family gene in its genome.

Discussion

This report shows conclusive evidence about synergistic relationship between AcMNPV and TnSNPV. Multiple approaches used to measure the joint activity of both viruses corroborated such a relationship. It is important to notice that most of the reports on synergism between baculoviruses have used less strict procedures to attest synergism, as some even lack measuring the effect of the viruses applied alone (Hukuhara *et al*., 1987; Tanada and Hukuhara, 1971). However, the synergism shown in those reports has been generally supported later by the cause-effect evidence. Synergism between GVs and NPVs has been well documented and based on the presence of enhancing proteins in the GVs (Derksen and Granados, 1988). However, in spite of the discovery of enhancins in NPVs (Bischoff and

Slavicek, 1996; Li *et al*., 2003), this is the first report showing evidence of a synergistic relationship between two NPVs.

The synergism between AcMNPV and TnSNPV was well established, as the comparison of the LC_{50} values and the Probit regression lines of both viruses acting alone and in combination, showed a significant difference. Furthermore, the Tammes-Bakuniak's graphic method, the deviation from mathematical definition of the equitoxic line, and the joint action ratio (R), all indicated a synergistic relationship between these viruses. Besides, the adequacy of these approaches was corroborated by the relationship between AcMNPV and TnGV, whose synergism has been well established for. More techniques that measure the joint action between lethal factors are available in the literature (Busvine, 1971; Tabashnik, 1992); also the experimental design may vary substantially (Tanada and Hukuhara, 1971; Xu and Hukuhara, 1992), but the evidence accumulated in the present report seems to be sufficient to demonstrate the synergism between AcMNPV and TnSNPV.

It is worth mentioning that the combination AcMNPV/TnGV was always more virulent than the combination AcMNPV/TnSNPV. That observation was evident as the mortality obtained with the combination of the LC_{30} of TnGV with the AcMNPV concentrations was so high that the LC_{30} had to be lowered to the LC_{20}. Still, the mortality remained so high that the concentration range of AcMNPV was lowered. The difference in the virulence of both combinations may suggest the involvement of a different synergistic mechanism between AcMNPV and TnSNPV, rather than the presence of a *vef* gene in TnSNPV. Interestingly, a dominance of AcMNPV polyhedra was observed in the bioassay cadavers in both combinations. Similar observations have been reported before (Tanada, 1959; Tanada and Hukuhara, 1971), suggesting that the virulence of AcMNPV was evidently potentiated by its synergistic partner.

Because the plausible explanation for the synergism between AcMNPV and TnSNPV was the presence of a protein from the enhancin family in the latter virus, the detection of a *vef* gene was attempted but no hybridization signal was observed. This result proved the absence of this gene in TnSNPV. Because homology levels between enhancin genes vary, low stringency conditions of hybridization have been used before to detect this gene in some GVs (Granados and Corsaro, 1990). However, in spite of using low stringency conditions to detect a putative *vef* gene in the TnSNPV genome, no hybridization signal was observed. These results indicate that TnSNPV may contain a different synergistic factor that enhances the virulence of AcMNPV, unrelated to the GVs enhancins; however, further studies are required to unveil the nature of this factor, its genetic background, as well as the mechanism by which it enhances the pathogenic potency of AcMNPV.

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