BARD

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**Dually Functional Recombinant Baculovirus
Expressing, Both, the Excitatory and Depressant
Insect Selective Neurotoxins**

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הספריה המרכזית
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DUALLY FUNCTIONAL RECOMBINANT BACULOVIRUS EXPRESSING, BOTH
THE EXCITATORY AND DEPRESSANT INSECT SELECTIVE NEUROTOXINS

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AaIT- Anti-insect selective excitatory toxin from the scorpion *Androctonus australis Hector*.

AcNPV = ACMNPV-Autographa californica nuclear polyhedrosis virus = *A. californica* M nucleopolyhedrovirus.

BTI-TN-5B1-4- *Trichoplusia ni* insect cell line.

CPU- contractile paralysis unit.

ED- effective dose.

FPU- fast paralysis unit.

LC- lethal concentration.

LD- lethal dose.

LT- lethal time.

Lqh α IT- anti-insect alpha toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

LqhIT1- anti-insect selective excitatory toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

LqhIT2- anti-insect selective depressant toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

LqhIT3- anti-insect selective excitatory toxin from the scorpion *Leiurus quinquestriatus hebraeus*.

AaIT5- anti-insect selective toxin from the scorpion *Androctonus australis Hector*.

PCR- polymerase chain amplification-based method.

PT- paralysis time.

PU- potency unit.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide electrophoresis.

Sf21- *Spodoptera frugiperda* insect cells.

C) ABSTRACT

The present project is aimed to improve the insecticidal potency of baculoviruses, to American and Israeli lepidopterous pests of *Spodoptera* and *Heliothis* species, by engineering recombinant baculoviruses expressing anti-insect toxins derived from scorpion venom. Through this study were isolated recombinant *Autographa californica* M nucleopolyhedroviruses (AcNPVs) which expressed alpha (Lqh α IT), excitatory (AaIT, LqhIT1 and LqhIT3) and depressant (LqhIT2) anti-insect neurotoxins. Bioassays on *Heliothis* species (*Helicoverpa armigera* and *Heliothis virescens*) were employed to assess the potency of the viruses. The recombinant viruses possessed an enhanced speed of kill compared to wild type AcNPV. Recombinant AcNPVs expressing the depressant toxins emerged as appealing improved baculoviruses. Applied combinations of alpha, excitatory and depressant toxins enhanced their insecticidal activity against blowfly and lepidopterous larvae. Moreover, combined application of recombinant AcNPVs expressing Lqh α IT and AaIT possessed increased insecticidal activity compared to single applications of them.

A reduced growth rate of *H. virescens* larvae was obtained by comparing the larvae infected with recombinant AcNPV expressing AaIT under the control of the AcNPV early *iel* to the very late *p10* promoters. Through this project improved protocols and methods were developed to purify and bioassay the anti-insect toxins and their correspondent recombinant baculoviruses. A novel highly potent anti-insect toxin AaIT5 was isolated and characterized. Finally, the impact of use of recombinant baculoviruses, expressing anti-insect scorpion neurotoxins to non-target insects, was evaluated.

D) Objectives of the original proposal

The present project is aimed to improve the insecticidal potency of baculoviruses, towards American and Israeli lepidopterous pests, by engineering recombinant baculoviruses expressing anti-insect toxins derived from scorpion venom. The feasibility of the approach was previously demonstrated (1, 2), however, the high standards of efficacy established by the use of chemical insecticides motivated us to develop novel approaches to further enhance the speed of paralysis or kill of lepidopterous larvae by recombinant baculoviruses.

The above approaches (p.8 of proposal) included:

- 1) The engineering of orally active AcNPV and SlNPV baculoviruses expressing the excitatory (LqhIT1) and depressant (LqhIT2) anti-insect toxins derived from the Israeli yellow scorpion *Leiurus quinquestriatus haebreus*. This included the construction of various transplacement plasmids with optimal promoters for the expression of the toxins.
- 2) The assessment of the functionality of the recombinant viruses by monitoring the occurrence and activity of the expressed toxin and by evaluating their effect on the intact, and infected insect.

The objectives were achieved by supplying the following elements:

- I. The isolation and characterization of new recombinant AcNPV baculoviruses expressing the excitatory AaIT, LqhIT1 and LqhIT3 (3, 4, 5, 6) the depressant LqhIT2 (5), and the alpha Lqh α IT (7, 8) anti-insect toxins with improved efficacy against lepidopterous larvae. Including new methods of polyhedra production and bioassays (9, 10).
- II. The demonstration that combined application of pairs of the above anti-insect toxins resulted in increased insecticidal potency towards lepidopterous larvae (11).
- III. The demonstration that oral infection of lepidopterous larvae with two recombinant baculoviruses expressing the AaIT and the Lqh α IT anti-insect toxins increased their efficacy towards *H. armigera* larvae (12).

IV. The isolation and characterization of the anti-insect toxin AaIT5 (13). This included the development of new methods of toxin purification.

V. The demonstration that non-target insects (beneficial, parasitoid and predatory) were not adversely affected by application of recombinant baculoviruses expressing scorpion anti-insect neurotoxins (14, 15).

The above findings established the basis for further development of recombinant baculoviruses as insecticides, including field applications.

During the development of the project it became clear that engineering of recombinant SlNPVs was not an obvious task and although proper transplacement plasmids were prepared we were not able to isolate recombinant SlNPVs. This problem was considered by the investigators and it was concluded that more basic research is required to make progress in that area. An effort to study the future utilization of AcNPV against *S. littoralis* larvae was initiated but it is considered out of the scope of this project (for reference see 16).

E) Description of the research and results

EI. Isolation of recombinant baculoviruses expressing different types of anti-insect scorpion toxins

The cDNAs coding for three different anti-insect scorpion toxins, namely the alpha Lqh α IT, the excitatory LqhIT1 and the depressant LqhIT2 neurotoxins, were cloned into AcNPV transplacement vectors and subsequently the correspondent recombinant baculoviruses were isolated (publication 2 and see below). In addition a synthetic gene was prepared to express the excitatory LqhIT3 toxin, also designed Ptox (4). The details of the cloning procedures for the Lqh α IT toxin and the methodology for the LqhIT3 synthesis are described in publication 2 and in (4).

Hereby we describe in some detail the isolation of recombinant AcNPV's bearing the genes of the excitatory and the depressant anti-insect toxins LqhIT1 and LqhIT2.

The LqhIT1 and LqhIT2 cDNA clones (17) were amplified by PCR utilizing primers corresponding to the cDNA sequence coding for the N-termini of the leader peptides of LqhIT1 and LqhIT2 (17, 18). The three C-terminal aminoacid residues encoded by the LqhIT2

cDNA and absent in the mature polypeptide amino-acid primary sequence (19) were omitted in the primer design. The LqhIT1 and LqhIT2 cDNAs were placed under the control of the viral p10 and polyhedrin very late promoters, respectively, utilizing the basic transplacement plasmid pUW51P2 (described in Fig. 1). Recombinant AcNPV's were isolated as previously described (5).

EII. Expression of LqhIT1 and LqhIT2

The expression of LqhIT1 and LqhIT2 toxins was studied by infecting BTI cells with the recombinant AcNPVs, AcLIT1 and AcLIT2, which bear the respective neurotoxin genes. As can be seen immunoblots of extracts of the infected cells show the presence of the recombinant toxins (Fig. 2 and Fig. 3A and B). A closer examination revealed the presence of two (and some times three) peptides recognized by the anti-toxin antiserum (for example Fig. 3B). [³⁵S]-aminoacid metabolic labeling of the peptides synthesized in the AcLIT2-infected cells also showed a double and sometimes triple band prominent at 48 h p.i. recognized by the anti-LqhIT2 antiserum (Fig. 3A and B). These data suggested that this heterogeneity was due to the presence of unprocessed toxin molecules or, variability at the genetic level due to impurity of the recombinant AcLIT2 used.

In order to distinguish between those possibilities we:

1. Amplified by PCR the LqhIT2 gene present in the recombinant baculovirus genome and sequenced it. The DNA sequence obtained from AcLIT2 indicated that the cloned LqhIT2 encodes a peptide homologous to the mature toxin (Fig. 4).
2. Sequenced the N-termini of the two peptides of the recombinant LqhIT2, recognized by the anti-LqhIT2 antiserum (Fig. 2B), after transfer to a PVDF membrane. The results indicated that the upper band (> 7 kDa) contained the intact leader peptide of the toxin and the lower band (< 7 kDa) represented the mature peptide (Fig. 4).

EIII. Insecticidal activities of the recombinant baculoviruses

The potency of the recombinant and wild-type AcNPV's was compared in bioassays with *Helicoverpa armigera* larvae. The

methodology employed is described in publications 1, 2 and 5. In brief, *H. armigera* larvae (1st. instar) were fed with virus-contaminated synthetic food. 24 h after feeding the larvae were transferred to fresh (non-contaminated food) containers and the indicated times we followed the appearance of intoxication symptoms (paralysis and subsequent death). As can be seen (Fig. 5) PT₅₀ values for AcLIT2 and AcLIT1 are 65 and 75 h respectively. Wild-type AcNPV PT₅₀ values are about 100 h. LD₅₀ values obtained were not significantly different from our published value of 50 (2). Thus, the recombinant viruses AcLIT1 and AcLIT2 showed enhanced insecticidal activity compared to wild type AcNPV. Moreover AcLIT2 expressing a depressant type of scorpion anti-insect neurotoxin showed the shorter time to kill (about a 10-to 15% increase in efficiency compared to every published data on scorpion neurotoxins). The significance of this data will be discussed below.

EIV. Synergism among scorpion toxins

The scorpion venom gland secretes a cocktail of polypeptidic anti-insect toxins with different pharmacological characteristics (i.e., excitatory, depressant and alpha anti-insect toxins) targeted towards sodium channels in the insect peripheral nervous system (6). Receptor binding experiments indicated that excitatory and depressant toxins were able to bind at the same time to the lepidopterous sodium channel (20). Thus, it is conceivable that combined application of toxins may result in potentiation of the insecticidal effect on the insect target. This hypothesis was tested and indeed, combined application of the following pairs of toxins revealed enhanced insecticidal activity (Table I and publication 3 for details).

As can be seen in the table combined applications of the toxins AaIT + LqhIT2, AaIT + LqhαIT, LqhIT2 + LqhIT3 and LqhIT3 + LqhαIT resulted in 5-fold increase in their potency to lepidopterous larvae.

This suggested that combined application of baculoviruses expressing each a complementary toxin (from above) may yield an increase in their insecticidal efficacy.

Concerted oral application of two baculoviruses, namely, AcAaIT and AcLqhaIT to *Heliothis armigera* larvae, resulted in potentiation of their activity. As can be seen (Fig. 6) low dosage (125 PIBs/ μ l) of viral infections advance the PT₅₀ values from 72 to 62 hours and significantly increase the mortality of the larvae at 72 h post ingestion from 50 to 90%. The effect is less pronounced at 250 PIBs/ μ l.

These results show that combined application of two baculoviruses each expressing a complementary toxin may enable the use of lower doses of recombinant baculoviruses and suggest that engineering baculoviruses able to express two different synergistically acting toxins may be further improve the insecticidal activity of the viruses.

EVI. Timing of the expression of the toxin in the body of the noctuid larvae

The expression of the anti-insect toxins in the above presented baculoviruses was driven by very-late virus promoters. Early studies indicated that the potency of the toxin, the temporal regulation of the promoter and the strength of the promoter may be considered in obtaining baculoviruses with improved insecticidal properties (21). The utilization of the baculovirus promoter *iel* to express early in infection the toxin AaIT is presented in publication 5.

Although the bioassays on *H. virescens* larvae do not show improvement of insecticidal activity of the *iel*-AaIT recombinant compared to the very late driven AaIT in the *p10*-AaIT recombinant, there is some indication that relative growth rates of the *iel*-AaIT-recombinant-infected *H. virescens* larvae were slower than *p10*-AaIT-recombinant-infected larvae, suggesting that expression of AaIT early in infection may enhance the ability of the virus to reduce larval feeding activity (publication 5, table 2). Thus this results indicate that this direction should be further explored.

The search for new anti-insect toxins with enhanced potency is part of the effort to obtain new tools for the future isolation of efficient recombinant baculoviruses.

A new insect-selective scorpion toxin (AaIT5) was purified from the venom of the North African scorpion *Androctonus australis*, and the amino acid sequence was determined by automated Edman degradation and electrospray ionization mass spectrometry. This new insect toxin is composed of 61 amino acids with a molecular mass of 6882 and is very potent against the tobacco budworm, *Heliothis virescens* (100 % lethal dose < 1.8 µg/100 mg body weight), but not toxic to mice by subcutaneous injection. Fig.7 A and B show the profile of elution of the toxin from RP-HPLC columns. The primary amino acid sequence of this new toxin (Fig. 8) has high homology to depressant anti-insect toxins such as *Leiurus quinquestriatus hebraeus* (LqhIT2), *Leiurus quinquestriatus quinquestriatus* (LqqIT2), and *Buthotus judaicus* depressant anti-insect toxin (BjIT2).

EVIII. SlnPV recombinant viruses

An SlnPV transplacement vector was constructed based on the Hind III K-fragment (3.1 Kbp) of the SlnPV genome (Bet Dagan, local isolate). This fragment was cloned into Bluescript, sequenced and found to contain the complete SlnPV-polyhedrin gene which showed 100% homology to the SlnPV-B Moroccan isolate (22). The polyhedrin coding sequences were replaced by the LqhIT2 cDNA sequences, leaving the toxin gene under the control of the polyhedrin promoter. A second transplacement plasmid was prepared, which contained the β-galactosidase gene replacing the SlnPV polyhedrin gene.

S. littoralis SL2 cells (16) were transfected with the latter plasmid and SlnPV DNA using either calcium phosphate or lipofection techniques. Despite the fact that we obtained expression of β-galactosidase by transient transfection of SlnPV-infected SL2 cells, we were unable to isolate viable SlnPV-β-galactosidase recombinants. Moreover, direct attempts to isolate SlnPV-LqhIT2 recombinants did not succeed. These results

suggested that SLNPV-recombinants were unstable and probably did not replicate well in our system. Thus, we concluded that more basic research is required in order to make progress in this area.

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EIX. Safety considerations

The awareness of possible damage to the environment by the use of recombinant baculoviruses expressing anti-insect scorpion neurotoxins motivated us to assess the impact of their application on non-target insect populations specially predators of lepidopterous larvae, endoparasitoids and beneficial insects (publications 6 and 8). The data indicate that no adverse effects were observed on lepidopteran predators *Chrysoperla carnea* Stephens and *Orius insidiosus* (Say), endoparasitoids *Microplitis croceipes* and beneficial insects *Apis mellifera* L.

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F) Description of the cooperation

The American and the Israeli teams cooperated at different levels by sharing:

1. Materials such as plasmid constructs, antibodies, cDNA clones and recombinant viruses.
2. Methodologies such as performance of bioassays on blowfly and lepidopterous larvae, maintenance of viral polyhedra and toxin purification methods.
3. Information regarding toxin expression, potency of the recombinant viruses and combined application of toxins and baculoviruses.
4. Publications.

This cooperation was implemented by e-mail and fax communication, the visits of N. Chejanovsky, M. Gurevitz and E. Zlotkin to Davis, of R. Herrmann to Bet Dagan and the participation of N. Chejanovsky and H. Moskowitz in the "Development of Recombinant and Wild Type and Viruses for agricultural Pest Control" symposium organized by Bruce D. Hammock in Hawaii (December 17-22, 1995). Moreover, this project encouraged us to pursue our cooperation to develop new strategies to enhance the potency of baculoviruses, based in the results of this project, in a new project supported by BARD.

G) Evaluation of results and conclusions

Isolation of recombinant baculoviruses expressing scorpion neurotoxins belonging to the different anti-insect groups revealed that the depressant toxins exhibit enhanced insecticidal effect. In this context we accomplished the isolation and characterization

of the new anti-insect toxin AaIT5 which showed sequence similarity to depressant scorpion toxins. This toxin is a good candidate for further cloning into baculoviruses.

Synergism among scorpion toxins was demonstrated by combined application of them. Further investigation revealed that combined application of two recombinant baculoviruses expressing an excitatory and an alpha anti-insect toxins, respectively results in potentiation of their insecticidal activity. More studies are required to evaluate the effect of combined applications of baculoviruses expressing excitatory and depressant toxins. Also, this studies suggest that further improvement could be achieved by expressing two pharmacological complementary toxins by one recombinant baculovirus.

Data obtained by expressing AaIT under the control of an early promoter suggest that studies in this direction should be continued. It is also conceivable that some improvement in the baculovirus' efficacy could be achieved by expressing two synergistically acting toxins, each under the control of differentially temporally regulated baculovirus promoters. The present research yielded as well information about the sequence of expressed toxins, improved methodology for their isolation and baculovirus bioassays. Also, the impact to the environment was assessed and the results obtained encourage the use of baculoviruses for the control of insect pests. Moreover, we achieved the objectives of providing new recombinant baculoviruses against *Spodoptera* and *Heliothis* pests. At this point, the accumulated data allow us to formulate improved strategies for further improvement of baculoviruses. These strategies will focus on:

1. Further diminishing the time require to paralyze the pest.
2. Lowering the dose of recombinant baculovirus required.
3. Preserving a clean environment

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Patents

Inventors: B.D. Hammock, H. Moskowitz, R. Herrmann. Insect Control with Multiple Toxins.

Disclosure submitted to the University of California Office of Technology Transfer on 5/12/94. Case # 94-142-1 UC. Subsequently submitted: Pending patent application 5/8/95. Serial No. 08/435,040.

Inventors: B.D. Hammock, R. Herrmann, H. Moskowitz. Combined application of recombinant and wild type viruses is cost effective and reduces the input of recombinant viruses into the environment. Technology Transfer on 9/12/95. Case # 96-085-1. Not submitted for patent consideration.

Fig. 1 Schematic representation of the transplacement vector pAcÜW51P2

Unique cloning sites, EcoRI BglII and BamHI, at the p10 and polyhedrin promoters, respectively, are indicated.

Fig. 2. Expression of the excitatory (LqhIT1) and the depressant (LqhIT2) anti-insect scorpion toxins in insect cells infected with recombinant baculoviruses. Immunoblot analysis of protein extracts of BTI-TN-5B1-4 cells infected with AcLIT1 and AcLIT2 (MOI of 10) bearing the LqhIT1 and LqhIT2 genes, using anti-LqhIT1 and Anti-LqhIT2 sera, respectively. LqhIT1 and LqhIT2, marker toxins. AcNPV, wild type virus. M protein markers. Arrows point to toxin bands.

Fig. 3. Time-course expression of LqhIT2 in AcLIT2-infected cells.

BTI-TN-5B1-4 cells were infected with AcLIT2 or AcNPV as in Fig.2. At 2h before the time points (indicated at the bottom of the figure) [³⁵S]-methionine/cysteine mixture was added and the cells were incubated for 2h and harvested. Cell extracts were subjected to PAGE and subsequently transferred to a nitrocellulose membrane to perform immunoblot analysis and further exposure to X-Ray film.

A. 35S-labeled polypeptides. B. Immunoblot.

Fig. 4. Insecticidal activity of the recombinant baculoviruses expressing LqhIT1 and LqhIT2. *H. armigera* larvae (1st. instar) were fed with virus-contaminated, AcLIT1, AcLIT2 and AcNPV, synthetic food (4000 PIBs/μl). 24 hours later the larvae were transferred to uncontaminated food. Paralysis of the larvae was recorded at the indicated times.

Fig. 5 Amino-acid sequence of the N-terminus of the recombinant LqhIT2.

nLqhIT2 = translated cDNA sequence.

rLqhIT2 >7kDa = recombinant LqhIT2 polypeptide higher of 7 kDa.

rLqhIT2 <7kDa = recombinant LqhIT2 polypeptide lower of 7 kDa.

Fig. 6 Combined application of baculoviruses expressing the anti-insect toxins AaIT and LqhαIT. *H. armigera* larvae were fed with mixtures of polyhedral inclusion bodies from recombinant viruses expressing AaIT and LqhαIT. Paralysis of the larvae were recorded as described in Fig. 4.

A. $125 \text{ PIBs}/\mu\text{l AcAaIT/Lqh}\alpha\text{IT} = 62.5 \text{ PIBs}/\mu\text{l AcAaIT} + 62.5 \text{ PIBs}/\mu\text{l AcLqh}\alpha\text{IT}$. B. $250 \text{ PIBs}/\mu\text{l AcAaIT/Lqh}\alpha\text{IT} = 125 \text{ PIBs}/\mu\text{l AcAaIT} + 125 \text{ PIBs}/\mu\text{l AcLqh}\alpha\text{IT}$

Fig. 7. Chromatograms of RP-HPLC purification of *A. australis* venom. (A) Crude venom of *A. australis* (Sigma Chemical Co.) was purified using C4 RP-HPLC column (Vydac 0.46 =B4 25 cm) equilibrated with 5% acetonitrile containing 0.1% TFA, with a gradient of 1.0 %/min at a flow rate of 0.6 ml/min. (B) Fraction 5 separated in Fig. 1-A was lyophilized and dissolved in the deionized water, then loaded again onto the C4 RP-HPLC column (Vydac 0.46 =B4 25 cm). The column was equilibrated with 95% acetonitrile containing 0.1% HFBA, with gradient of 1 %/min at a flow rate of 0.6 ml/min.

Fig. 8. Comparison of primary sequences of the new toxin AaIT5 with known depressant insect toxins. X are unidentified amino acids in the direct sequencing.

Fig. 1

Fig. 2

Fig. 3

Fig. 5

A

B

Relative Absorbance at 214 nm

Relative Absorbance at 214 nm

Fig. 7

AcITS	Total Sequence
DGYIKRRHDGCKVTCLINDNYCDTECKXEGGSYGYCYSVGFACWCEGLPDDKAWKSETNTCX	
DGYIKRRDGCKVACLIGNEGCDKECKAYGGSYGYCWTWGLACWCEGLPDDKTWKSETNTCG	LqhIT2
DGYIRKRDGCKLSCLFGNEGCKECSYGSYGYCWTWGLACWCEGLPDEKTKWKSETNTCG	LqqIT2
DGYIRKKDGCKVSCIIGNEGCRKECVAHGGSGFYCWTWGLACWCENLPDAVTWKSSTNTCG	BjIT2