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**Targeting of an Expressed Insect Selective Neurotoxin
by its Recombinant Baculovirus:
Pharmacokinetic and Pharmacodynamic Aspects**

E. Zlotkin, S. Maeda, S.G. Kamita, N. Chejanovsky

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Investigators:

(Principal first)

Prof. Eliahu Zlotkin

Prof. Susumu Maeda

Dr. Shizuo George Kamita

Dr. Nor Chejanovsky

Affiliated Institution:

Hebrew Univ. Life Sci. Inst.

UC-Davis Entomology

- " - - " -

Volcani Center, Entomology

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Signature:
Principal Investigator

Signature:
Officer of the Affiliated Institution

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ABSTRACT

Title : Targeting of an expressed insect selective neurotoxin by its recombinant baculovirus: Pharmacokinetic and pharmacodynamic aspects.

Objectives: 1) Clarification of the mode of potentiation of an expressed insect selective neurotoxin (AaIT) by its recombinant baculovirus.
2) In vitro formation and/or modification of neuroactive polypeptides for the design of new improved recombinant baculoviruses.

Results: 1) A combined utilization of bioassays, LM-cytochemistry, the highly resolute EM immunogold and electrical recording from the CNS of baculovirus and AaIT - expressing - recombinant baculovirus infected larvae it has been shown that the recombinant virus potentiates the effect of the toxin. Potentiation is achieved through its continuous expression in the infected tracheal epithelia thus providing a: a. Local supply of freshly produced toxin in the vicinity of its target sites; b. Translocation of the expressed toxin to the insect CNS. The latter exposes the recombinant toxin to new, critical, target sites which are inaccessible through the natural route of scorpion envenomation.

2) Subjecting a recombinant AaIT toxin to a newly designed system of random mutagenesis results in large numbers of new AaIT genes with amino acid substitutions. The new or modified toxin genes were inserted into a linear BmNPV expressed in silkworm cell culture and assayed on blowfly and silkworm larvae. Thus a system for mass formation and screening of neuroactive agents was developed.

Contribution to agriculture: 1) Demonstration of the insecticidal mechanism, capacity and utility of the combination of neuroactive polypeptides and recombinant pathogens.

2) Development of a simple in vitro system for the formation and selection of new neuroactive polypeptides.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The proposed study was aimed to achieve two objectives:

- 1) To clarify the mode of cooperativity between an expressed toxin and its recombinant baculovirus.
- 2) To improve the design of recombinant baculoviruses in order to intensify their insecticidal capacity.

The above two objectives were dealt in our study. The treatment of the first objective was performed by examining the mechanism underlying the toxin "potentiation" phenomenon (see below). This objective, namely the positive cooperativity between the toxin and the virus, has been dealt through the pharmacokinetic approach and has reached an advanced stage. This aspect is presented in a relatively specified and more detailed manner.

The second objective, namely the improved recombinant baculovirus was, as a matter of fact, directed to the improval of the expressed toxin. This aspect was treated by a genetic modification of the toxins' gene through the baculovirus expression system.

RELEVANT DATA (methodology results and discussion)

This final report on project IS-2465-94 is subdivided into two parts: The first includes the detailed presentation of the central and most essential aspect of our study, namely the pharmacokinetic interaction between the virus and the toxin. The second part deals with the genetical (structure/function) analysis of the toxin in order to improve the design of future recombinant baculoviruses.

Part 1: CNS-Oriented Targeting of an Expressed Insect Selective Neurotoxic Polypeptide by its Recombinant Baculovirus.

Methodology

Toxin: AaIT was purified in the lab.

Test animals were, laboratory bred silkworm and blowfly larvae.

Viruses: Two kinds of genetically modified BmPNV silk worm baculoviruses, kindly provided by Prof. S. Maeda (Entomology, UC Davis), were employed in the present study. The first was the strain designated as BmM14, which is polyhedra deficient but mimics the pathology of the wild type virus (WTV) delivered by injection to silkworm larvae. The second was the recombinant virus BmAaIT [2] which is also devoid of polyhedra and expresses the gene encoding the AaIT toxin. Virus propagation, titration and storage were performed in BmN cells [2] according to standard methods [3].

Toxicity assays. In order to monitor the AaIT concentration in the hemolymph of the infected larvae - the contraction paralysis of blowfly larvae (CPU[4]) was employed. Its paralytic potency to silkworm larvae was assessed by the PD₅₀ assay according to [5].

Light microscopy: Fixation was performed by perfusion of 10ml of a standard gluteraldehyde fixative solution through the body cavity of decapitated animals. This was followed by a dorsal cut through the insects cuticle and fixation for an additional hour at room temperature. The VNCs were now dissected and exposed for an additional fixation. At this stage fixative was supplemented with one drop of 30% H₂O₂ per 10ml in the cold (4°C) for 24h. VNC's were dehydrated in ascending concentrations of ethanol and embedded in EPON resin blocks. Sections of 2-5µm from toracic and abdominal ganglia were cut and mounted on gelatin coated glass slides. Sections were etched with methoxide and subjected for immunocytochemistry using the VectaStain ABC kit (Vector laboratories, Inc. Burlingame, CA. USA) with a peroxidase conjugated secondary antibody according to the attached protocol. Visualization was achieved by incubation in diaminobenzidine.

Electron microscopy: VNC's for electron microscopy were processed as described for light microscopy with the addition of a post fixation step using 1% OsO₄ for 45 min. in the dark. Ultra-thin sections (0.1 μ m) prepared from thoracic and abdominal ganglia were collected on nickel grids. The immunogold labeling technique was employed by the post-embedding method [6]. Grids were stained with uranyl acetate and lead citrate and immunogold labeling was viewed using Joel CX100 electron microscope.

Electrical recording: The dissection and processing of VNCs were performed, essentially, according to [7]. Briefly, silkworm larvae were immobilized by cooling, opened along the dorsal midline and pinned upright on a wax block. The gut, fatbody and entire body wall were removed. The intact VNC was bathed with a buffered saline. Extracellular recordings, were made using, teflon pulled, suction electrodes, from the connective between the 7th and the 8th abdominal ganglia. Spontaneous activity was amplified with RC-coupled amplifier (Grass P-15). Data were displayed on a multiple trace storage oscilloscope and stored on a video recorder (Neurocorder, DR-886), with a frequency response of 0-20KHz. The data were later digitized and analyzed on a 486 PC equipped with Computerscope software (Computerscope, R.C. Electronics, Santa Barbara, CA. USA) and then exported as spread sheet files for further analysis.

Results

The potentiation phenomenon: As previously [1] and presently (see below Fig.1 and Table 1) shown, depidopterous larvae infected by a recombinant bavulovirus expressing the insect selective neurotoxic polypeptide (AaIT) reveal the typical paralysis symptoms induced by the native toxin injection (Fig. 1). However, the hemolymph concentration of the recombinant toxin was about two orders of magnitude

lower than that of the native toxin inducing a similar paralysis (the potentiation phenomenon, Table 1).

Our data suggest that the potentiation is a consequence of a virus toxin pharmacokinetic cooperativity which produces a CWS targeted neurotoxicity.

Fig. 1 - Similarity of symptoms induced by the native (a,b) as well as the recombinant (c,d) AaIT toxin to 4th instar silkworm larvae.

a - Larva injected by 5 μ l of distilled water as control. b - Paralyzed larva 5 hours after injection of 3 μ g per 100 mg of body weight of AaIT toxin. c - Larva 50 hours post infection with 10^5 plaque forming units of the recombinant baculovirus - BmAaIT. d - Larva 50 hours post infection with 10^5 plaque forming units of the recombinant baculovirus - BmAaIT.

The recombinant virus mimicks the native toxin by inducing spasmodic contractions, progressive reduction of mobility and finally a complete paralysis in a contracted-shortened body shape (b,d).

Table 1: The potentiation phenomenon: The paralytic potency of the recombinant and native AaIT toxins to lepidopterous larvae.

The insect	Recombinant toxin hemolymph concentration (ng/ μ l) ^(a)	Native toxin hemolymph concentration (ng/ μ l) ^(b)	Potentiation (fold)
<u>Spodoptera littoralis</u> (Egyptian cotton leaf worm)	---	66 ^(c)	---
<u>Heliothis virescens</u> (Tobacco bud worm)	1 ^(d)	70 ^(d)	70
<u>Bombyx mori</u> ^(e) (Silk worm)	1.3	56	43

Remarks: a) The data refer to hemolymph concentration of AaIT in a stage of paralysis as assessed by the aid of the contraction paralysis assay of Sarcophaga blowfly larvae [4]. b) Toxin concentration was calculated from PD₅₀ value and hemolymph volume [5, 8]. c) According to [5]. d) According to [1]. e) We used hemolymph from paralyzed 4th instar silkworm larvae 50 hrs post infection by 10⁵ plaque forming units (pfu) of the recombinant BmAaIT baculovirus.

Histopathology. Early histopathological examinations of lepidopterous larvae infected by baculoviruses [17] have shown that hemocytes, tracheal epithelium, epidermis and fat body comprise the virus susceptible tissues which are responsible for its systemic distribution in the body and its mass production. The common manifestations revealed by light microscopy include the occurrence of hypertrophied cells; swollen nuclei massive appearance of the typical polyhedral inclusion bodies and finally disintegration of the entire cell.

Such "classical" histopathology is demonstrated in figure 2 revealing typical cellular hypertrophy and the immunocytochemical manifestation of the recombinant toxin in the fat body (Fig. 2a) and tracheal matrix (Fig. 2b, c).

As shown (Fig. 2c) the recombinant toxin occurs in a very close vicinity of the CNS. The protective sheath of the VNC is composed of two layers, acellular, collagenous neural lamella and the cellular perineurium substantially composed of glial cells. Both layers are heavily tracheolated. Thus, the tracheal system surrounding the VNC is an integral part of the neural protective sheath. This aspect is exemplified in Fig. 2c which reveals that the acellular neural lamella is enveloping the trachea (Fig. 2c). To summarize the present light microscopy immunocytochemistry (Fig. 2) reveals that the recombinant toxin can be transmitted into the CNS via the tracheal system.

Fig. 2: Fat body and tracheal epithelium infected with the recombinant BmAaIT virus expressing the toxin 50-55 hours post infection. The Vectastain ABC reagent (Vector, USA) visualization of the recombinant toxin was obtained with 2-5µm sections of Epon embedded 4th instar silkworm larvae tissues. The peroxidase immunocytochemistry was coupled to the employment of AaIT specific rabbit antiserum.

- a) Virus infected fat body. Notice the hypertrophied nuclei and the vacuolated cytoplasm revealing the presence of the recombinant toxin. The fat body is penetrated by a trachea.
- b) Virus infected tracheae and their hypertrophied epithelium revealing the massive presence of the recombinant toxin.
- c) Tracheation of the CNS. Notice a swollen trachea allocated within the neural sheath (neurolema) surrounding two convectives in close vicinity to an abdominal ganglion.

Legend: C - connective; FC - fatbody cytoplasm; FN - fatbody nucleus; NL - neural lamella; T - trachea; TE - tracheal epithelium; TN - nucleus of tracheal epithelium.

Bars: 10 μ m.

Fig. 2
↓

Recombinant toxin in the CNS: The possibility raised in Figure 2 that the recombinant toxin may be transmitted via the tracheal system into the VNC has been clearly demonstrated by the data presented in Fig. 3. The latter indicate the occurrence of the recombinant toxin in the various cellular elements of the CNS ganglia, namely: membrane of a neural cell (Fig. 3a); Vicinity of small tracheoles allocated in the central part of the ventral ganglia (Fig. 3b) and finally axonal membranes at the neuropyle region (Fig 3c). In all the above cases the occurrence of the immunogold labeling in the ganglia as well as connectives (Fig 3c) of the VNC can be attributed to toxin expression and transfer through the virus infected tracheal epithelial cells (see also Discussion).

Increased excitability of the CNS: The endogenous (spontaneous) electrical activity of an isolated insect ventral nerve cord (VNC) has been used in the past for behavioral and pharmacological studies. In the present work we have decided to take advantage of the availability and technical simplicity of the above preparation. Our approach is based on the assumption that the presence of the recombinant toxin in the insect CNS can be expressed in its extracellularly recorded spontaneous electrical activity.

The data presented in Fig. 4 indicate an obvious impact on the VNC's spontaneous electrical activity through two expressions: Firstly the two fold increase in number of electrical events per unit of time in the VNC derived from the RV infected silkworm larvae when compared to the wild type virus (WTV). It is noteworthy that the VNCs from fully paralyzed larvae (Fig. 4b) reveal 15% higher spontaneous activity than those derived from the partially paralyzed insect (Fig. 4a). Secondly, by the fact that the events recorded from the recombinant virus (RV) infected nerve cords reveal shorter time intervals between them than those of WTV, as shown by the interspike interval

distribution histograms. The latter may suggest a kind of bursting mode of activity induced by the RV infection.

Fig. 3 - Immunogold visualization of the recombinant toxin in an abdominal ganglion of a silkworm larvae 55 hours post infection by the BmAaI'T virus.

- a - Occurrence of the immunogold particles beyond the perineurium on a presumably neuronal cell body membrane or gleal membrane extensions surrounding the neuron. (inset).
- b - The occurrence of the toxin in close vicinity of tracheoles (T) allocated between the neural cell bodies layer and central neuropyle mass. The toxin distribution seems to be limited to the tracheal epithelial cells.
- c - The toxin in neuropyle of the abdominal ganglia. The presence of the gold particles on the axonal membrane (AM) of the neuropyle is obvious.

Legend: AM- axonal membrane; G- gleal cell membrane; M- mitochondria;

NL- neural lamella; NC- neuronal cell nucleus; S- synapse; T- tracheae.

Bars. $\overset{1.0}{\underset{\lambda}{\mu\text{m}}}$; inset $\overset{0.2}{\underset{\lambda}{\mu\text{m}}}$ b. $\overset{2.0}{\underset{\lambda}{\mu\text{m}}}$. Insets $\overset{0.25}{\underset{\lambda}{\mu\text{m}}}$ c- $\overset{0.2}{\underset{\lambda}{\mu\text{m}}}$.

Fig.4 - Spontaneous electrical activity from isolated VNCs derived from RV as well as WtV infected silkworm larvae. The experimental larvae were subdivided into two groups: a. 40-45 hours post infection (hpi) where the RV infected larvae revealed an obvious difficulty in their motion. b. 45-52 hpi where the RV infected larvae were fully paralyzed (Fig 1). At the partial paralysis stage (a) the difference in the total number of events between the RV and WtV (inset) was significantly different ($P=0.0138$, Mann-Whitney unpaired non-parametric test). At the fully paralysis stage (b) the difference was again significant ($P=0.0242$, Mann-Whitney unpaired non-parametric test). The median values of the interspike interval (ISI) distribution histograms was $6.2\pm0.92\text{ms}$ and $10.6\pm1.23\text{ ms}$ in(a) and $5.2\pm0.58\text{ms}$ and 11.6 ± 1.5 in(b) for RV and WtV respectively. All WtV infected larvae were mobile comparable to healthy larvae. Insets represent the difference in total number of events recorded within 5 min in RV as well as WtV infected larvae.

Discussion

The present study demonstrates that the recombinant toxin is: (1) Distributed in the insect body via the common routes for the spreading of the viral infection (see below). (2) Translocated (see below) to the insect CNS and (3) greatly increases its excitability. The above data are examined on the background of previous information concerning the relative tolerance of lepidopterous larvae to AaIT; the impermeability of the insect CNS to the toxin and the process of systemic spreading of the baculovirus in the insect body. It has been previously shown [5] that the tolerance of lepidopterous larvae to the AaIT toxin is achieved by the aid of two mechanisms: Firstly the pharmacokinetic mechanism which includes inaccessibility of the terminal branches of the motor nerves to the toxin and the occurrence of toxin degradation and elimination process in the insect body.

Secondly the pharmacodynamic mechanism as revealed by the fast dissociation of the toxin ($t_{0.5}=7.2\text{sec}$) from lepidopterous neuronal membranes strongly contrasting toxin susceptible insect ($t_{0.5}=5-9\text{min}$)[9]. A tolerance mechanism based on a combination of factors such as metabolic inactivation, accessibility problems and lability of the toxin-receptor complex, may be counteracted by a continuous supply of newly produced toxin in the close vicinity to its target sites. The present study reveals that the recombinant baculovirus serves as a local constant supplier of the AaIT toxin at a cellular distance from its target sites.

The second role that the recombinant virus (RV) fulfills in potentiating its expressed toxin is by providing the toxin with new critical, CNS allocated target sites (Fig. 2 and 3) which are inaccessible to the toxin through the natural route of scorpion envenomation. The accessibility of the, virus targeted, recombinant toxin to the CNS obtains a special significance with the background of two pieces of previous information. The first is the fact that the insect's CNS (as exemplified on VNC) is impermeable to the native toxin. This aspect was demonstrated through electrophysiological as well as microscopical approaches. D'Ajello et al [10] have shown that the AaIT when applied to the isolated cockroach VNC in concentration exceeding by 3 to 4 orders of magnitude the paralytic concentration for the intact insect [11] were ineffective. Furthermore VNCs dissected from fully paralyzed insects (by injection) revealed a normal electrophysiology [10]. The impermeability of the VNC to the toxin was also demonstrated by assays of microscopical radioautography on intact nerve cords derived from insect injected by the radioiodinated AaIT toxin [11,5]. However, once accessibility is provided by artificial means, the AaIT is able to affect the insect's CNS. Such an effect was demonstrated by electrophysiology on isolated CNS's derived giant axons [12,13]; binding assays (with CNS derived insect

synaptosomes [14,15]) and microscopical autoradiography (with damaged, desheated VNCs [11,5].

The above information suggests that the virus mediated occurrence of the recombinant toxin in the insects CNS should possess a functional meaning. This expectation was verified by the data presented in Fig. 4. The latter revealed a significant increase in the spontaneous electrical activity in the VNCs derived from recombinant virus infected larvae which seems to be proportional to the degree of paralysis (Fig.4a,b). We assume that the increased excitability of the infected VNC is a reflection of the well known excitatory action of the AaIT toxin shown by its induction of repetitive firing ins insect axons [12,16]. The similarity in the symptoms of paralysis revealed by toxin injected and RV infected larvae (Fig.1) follows from the fact the toxin induced activation of skeletal muscles is derived from its stimulation of the motor nerves (presynaptic) [16]. Thus, one can expect same symptoms of paralysis regardless whether the motor nerve is activated at its ganglionic segment or the terminal branches. It is also noteworthy that the mechanism, which enables the recombinant toxin to affect the CNS, should similarly amplify its peripheral action.

The next question refers to the manner by which the recombinant toxin potentiated its expressed toxin. In other words, how does the virus perform the localized toxin delivery and its translocation? This question directs our attention to the process of systemic host infection produced by the budded form of a baculovirus [18, 2].

A previous immunohistochemical study was aimed to reveal the temporal infection sequence of the Autographa californica baculovirus (AcMNPV) in certain lepidopterous larvae. It has been shown that the initial infection occurred in the midgut epithelial

columnar as well as regenerative cells. Systemic infection was attributed to free virus and diseased hemocytes, supposed to spread virus among the sensitive tissues [17,18]. However, a more recent study by Engelhard et al [19] employing AcMNPV containing a lac-Z reporter gene, revealed that the major conduit for viral systemic infection, enabling its rapid spreading throughout the host insect, is supplied not by the hemocytes, but rather by the tracheal system which is also accessible to external infection [20]. The suitability of the tracheal system to guide the recombinant virus and its expressed neurotoxin follows from the fact that: 1. The tracheal system is distributed among the various tissues, is intimately associated with individual cells and is flexibly expanding and adapting itself to the growing organism by the aid of the tracheoblasts. 2. The tracheoblasts cross the tissue protective extracellular matrices (basal lamina) allowing the infecting virions to circumvent these barriers [19]. The tracheal epidermis forms a kind of syncytial continuity, through its lymph and cytoplasmic extensions, enabling the fast spreading of the virus [19].

The data presented in Figure 2 and 3 exemplify the role that the infected tracheal system fulfills as a vector of the recombinant toxin. This is revealed by the presence of the toxin in and around the infected tracheal epithelial cells in the vicinity and inside the VNC (Fig. 2). The highly resolutive EM immunogold method demonstrates toxin distribution via tracheoles (Fig.3b) inside the VNC ganglia (Fig.3) and their axonal membranes (Fig. 3c).

To summarize the potentiation of the recombinant toxin is a consequence of its translocation (motion, spreading) in the body of the baculovirus diseased insect. The above translocation is performed through a) the infection of existing tracheal epithelia; b) the branching and spreading of newly formed tracheae from infected tracheoblasts and c) the local release-excretion of the toxin from the infected tracheal epithelium to

their close surrounding in either the peripheral or the central nervous systems. Thus, the pharmacokinetic cooperativity between the recombinant virus and its expressed toxin forms a new mode of CNS targeted neurotoxicity which is produced neither by the native toxin nor by the wild type baculovirus.

The validity of the pharmacokinetic cooperativity concept.

In our project silkworm larvae and the respective baculovirus preparation served as a model for the study of the virus-toxin interaction offering the conclusion on pharmacokinetic cooperativity. The validity and generality of the above conclusion were most recently established by different (a) approach; (b) baculovirus (AcNPV); (c) lepidopterous insect; (d) expressed toxin and (e) different promoters.

Briefly, by the aid of the Reverse Transcriptase in-situ PCR technique it has been shown that the agroeconomically important *Heliothis virescens* larvae, infected by Autographa californica nuclear polyhedrosis virus expressing the insect selective depressant neurotoxin LqhIT₂ (AcNPV-LqhIT₂) reveals a pattern of distribution of the depressant toxin mRNA (transcript) which is extremely resembling the immunocytochemical distribution of the recombinant excitatory AaIT neurotoxin (see above Fig.2). The obvious occurrence of the LqhIT₂ transcript is revealed in the infected swollen tracheal epithelia surrounding the ventral nerve cord of Heliothis larvae- beyond the barrier of the acellular collagenous neural lamella (data not shown).

To summarise the basic information concerning the insecticidal mechanism of the recombinant baculovirus, which was revealed by infected silkworms, can be implied and extended to other recombinant baculoviruses, which are agroeconomically important.

Part 2: Improvement of Recombinant Virus Technology: Formation and Identification of Recombinant Neurotoxins with an Increased Potency.

Dr. Amit Lotan, who obtained a BARD postdoc fellowship in the laboratory of Prof. Maeda, on the present project on virus toxin interaction, is involved in this aspect.

Methodology (the developed system)

In Prof. Maeda's group was developed a procedure based on two steps which enables a large scale mutagenesis and screening of AaIT toxin. The first step is a construction of an AaIT gene which contains, at its c-terminal region, two unique restriction sites enabling the subcloning of randomly prepared oligomers. The second step included the construction of a linearized Bm NPV virus which is devoid of an essential DNA segment. The above linearized and crippled virus recovers its functional circular form when co-transfected with a certain transfer vector which carries the essential missing segment of DNA and one of the various forms of the AaIT. Thus, the above forms could have been detected and assayed avoiding the time consuming, conventional plaque, assay. This was the principle, more specifically:

A new AaIT gene containing AflI and XbaI restriction sites, which flank a 97 bp region corresponding to the C terminal 32 amino acids of the AaIT toxin was constructed. The new AaIT gene was subcloned into a newly constructed transfer vector, pBB30, which has the advantage of being small enough to facilitate cloning efficiency. Modified AaIT

genes were prepared by annealing single strands of 3'-5' and 5'-3' oligomers homologous to the DNA synthesis was performed with an unbalanced mixture of phosphoramidite bases (95% of homologous base, 1.67% of each other three bases). The annealed oligomers were ligated into the AflI and XbaI sites of a plasmid, pBB30' containing the AaIT gene. The resulting construct was transformed into E. coli cells, and plasmid DNA was extracted, Analyses of 91 modified AaIT clones were performed by sequencing and 57 genes showed various nucleotide substitutions. Deletions and insertions of nucleotides were also found in 14% of the oligomers, resulting in amino acid deletion and insertion, as well as frame shifts and early termination's.

To generate large numbers of recombinant viruses with modified AaIT genes a new BmNPV virus having a high recombination rate was constructed. Using site directed mutagenesis, we placed a unique Bsu36I site into an essential gene, gene 1629, without changing the reading frame of the gene. A second Bsu36I site was placed downstream of the polyhedrin promoter. The new virus, named BmNPV MABB, when linearized by Bsu36I was unable to recover by itself due to partial deletion of the 1629 gene. Cotransfection of linearized BmNPV MABB and pBB30 had a recombination rate of 100%, and enabled us to select recombinant viruses without having to do plaque assays.

To summarize, a recombinant baculovirus carrying mutated AaIT genes was constructed.

Results and Discussion

1. Evaluation of the insecticidal/ behavioral effects of the mutated

AaIT on infected larvae: Mutated AaIT genes were contransected with the linearized BmNPV MABB into silkworm Bmm cell culture and incubated for 4 days. 20 μ l (1000-10⁷ PFU/ml) from each tissue culture dish containing a mutant AaIT recombinant virus was injected into a fourth instar silkworm larvae. The larvae were observed for 2-5 days post injection to monitor the effect of the modified toxins on silkworm behavior and mortality.

Thirty-three modified AaIT toxins with at least one amino acid substitution were tested. Thirteen AaIT mutations resulted in no change or a slightly reduction in the typical contraction paralysis of the silkworm. Surprisingly, the rest of the mutations modify the mode of action of AaIT, from a contractile paralysis to a flaccid paralysis.

The extensive mutagenesis of the AaIT gene and the proper system to express the mutant toxins for testing their activities, bring to light valuable information concerning the structure and the function relationship of the AaIT insect selective neurotoxin. To summarize:

- a. Deletion of the C-terminal region maintains toxicity. However, this modification changes the excitatory reaction of the toxic towards a depressant action.
- b. Modification or deletions in the disulfide bridging does not destroy toxicity, however, it shifts the excitatory action of the toxin towards a depressant action.
- c. Substitution of the reactive lysine residues do not destroy toxicity.

2. **Isolation of the recombinant AaIT from silkworms** : For further analysis of the mutated AaIT and its mode of action the recombinant AaIT was purified from infected silkworm. Fifth silkworm instar and cocoons were infected with 20 μ l of recombinant BmNPV containing AaIT gene. Hemolymph was extracted from the silkworm and diluted in 3-4 extraction volume of buffer (10 mM Ammonion Acetate PH=7, 1mM EDTA). The expression level of active AaIT was tested by injecting the hemolymph of the infected silkworm into blow fly larvae and by western blot analysis, using an anti AaIT antibody. One unit of activity was defined as the amount of hemolymph needed to contract 50% of the injected blow fly larvae. It was discovered that more hemolymph and more AaIT can be extracted from cocoons than from fifth instar larvae. In order to receive the maximum yield of AaIT from the cocoons, they should be infected three days post pupation. Testing the concentration of the AaIT in the cocoons at 2,3,4,5 days post infection revealed the highest concentration (0.35 μ l hemolymph/unit) after 4 days. The active hemolymph was extracted in 50% acetonitril (ACN) and centrifuged for 10 min. at 2000xg. The supernatant which contained the soluble AaIT was diluted with water to a final concentration of 10% ACN and directly applied to a RP-HPLC C4 column. The AaIT was eluted from the column with a small volume (2ml) of 80% ACN. The eluant was lyophilized and applied to a reverse phase column (vydac C4, 4.6x250mm) equilibrated in buffer A (5% ACN, 0.1 TFA). Buffer B was 95% ACN, 0.1% TFA. The toxin was eluted using a linear gradient of 0-60%B within 70 min. at a flow rate of 0.6 ml/min. Absorbency was monitored at 215 and 280nm. Active AaIT was eluted from the C4 column after 44 min as confirmed by injection to blow fly larvae and Western blot analysis.

3. **Structure and function analysis of the modified AaIT:** a. Comparing the activity unit of WT and modified AaIT on blow fly larvae: Blow fly larvae are very sensitive to the WT AaIT (ED₅₀ of 5ng/larva). To compare the affective dose of the mutated AaIT to the WT toxin we injected the isolated toxin, which was purified from silk worm, into blow fly larvae. Testing 4 mutant with substitutions ins Lys No. 50,51 revealed the same contraction in the same doses as the WT. Chemical modification on the Lysine residue of the AaIT reduce its toxic activity. It was suggested that Lys 50,51 might play an important rule in the active site of the AaIT. Thus, those results demonstrated that: a. Even if site 50,51 is the active site of the AaIT toxin, it is no depend on the existence of the two Lys. b. Substitution of AA on AaIT toxin for the study of it's structure function relationship is more accurate and sensitive than the AA chemical modification. Substitution of Cys in site 63 into a Tyr reveal flaccid behavior of silkworm 70 hour post injection. Testing this modified AaIT on blow fly larvae demonstrate no activity at any dose up to 5ug/larvae (1000 fold of the WT AaIT). This observation indicated that shift of the excitatory action of the toxin towards a depressant in silkworm involve in extensive reduction of the toxic towards a depressant in silkworm involve in extensive reduction of the toxic activity towards blowfly. Aminoacid substitution of Cys63 leave the AaIT with an odd number of Cys. Therefore we suggests that the modified AaIT with only 3 Cysteine bonds (instead of 4 in the WT) might fold in different way. Surprisingly the new molecular structure maintain depressant activity, which selectively affects silkworm and causes no effect on blowfly.

The screening of the mutant demonstrated that mutations in the AaIT gene could lead to the loss of it's toxic activity or to shift the toxin from an excitatory toxin to

depressant. The above raised the possibility that these effects may occur due to an incomplete, truncated expression. To compare the recombinant toxin's size to that of the scorpion derived toxin, the exact molecular mass of the isolated toxins were determined using an Electrospray Mass Spectrometer. Isolated AaIT from scorpion venom gland and recombinant AaIT isolated from silkworms has the same molecular weight (7824 dalton). These results indicated that the WT AaIT is expressed in an accurate manner in baculovirus system, in silkworm. Testing the Mass of the isolated Cys63 modified AaIT (expected to be 7864) displayed no translated products around this site.

Finally, to summarize a new approach to increase the diversity of scorpion toxins by random mutagenesis was developed. The new synthetic scorpion toxins were directly assessed as potential pesticides against their target, the lepidopteran larvae. The technology enabled us to evaluate the pesticide potential of tens of scorpion toxins in parallel, without their prior isolation. The results demonstrate that one AA substitution can increase the selectivity of the toxin toward lepidoptera and to change the mode of action of the toxin. It is necessary to study the structure and function relation of those modifications to analyze their environmental impact and use them for the development of more potent selective bioinsecticides.

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EVALUATION OF RESEARCH ACHIEVEMENTS

Our study has undoubtedly provided information which:

- (1) Clarifies the mechanism of toxin potentiation by its recombinant virus.
- (2) Supplies a biotechnological system which enables a modification of clonable insect selective neurotoxins and their choice, selection.

There is no doubt that the main goals of the proposed study were achieved - but each of the above two main aspects has a drawback:

- (1) The present pharmacokinetic clarification of the toxin-virus cooperativity represents a successful, useful treatment of the so called "vector hypothesis" according to which the baculovirus serves as a transporter and local supplier of the expressed toxin. However, such information does not exclude the complementary possibility which has been formulated in the original proposal by the term the "nervous system hypothesis". This working hypothesis (which was only superficially treated in our study - see 1st annual report) - claims that the haculoviral infection may sensitize the nervous system to the toxin. Our preliminary data (1st report) indicate that diseased larvae by the WT virus are more sensitive to the paralytic effect of the toxin but the mechanism, on the cellular - sub-cellular level was not treated. We believe that the "nervous system hypothesis" plays only a secondary role in the "toxin potentiation" phenomenon - but its clarification could have provided far reaching implications on the approach and performance of the integrated pest control, based on the combination of pathogens and insecticides.
- (2) The second drawback concerns our objective to "improve the design of the recombinant virus". In other words, the modified and preferable forms at the AaIT toxin - were not processed; in the present study, to construct an agrotechnically field functional construct of a new recombinant virus. However, it would be fair to admit that the system for obtaining expression and selection of toxin mutation is an achievement of an agrotechnical value by itself (see below).

DESCRIPTION OF COOPERATION

In the first glance it seems that the Israeli and American partners have strictly divided the tasks between them: the Israelis dealt with the virus toxin interactions and the Americans, with the structure of the recombinant toxin. However, as a matter of fact, each of the two partners was involved in each of the two aspects of the study, namely: The American have provided the Israelis with the essential components of the experimental design - the WT and recombinant viruses, the proper cell lines and the silkworm test animals. The Israelis, on the other hand have provided the American laboratory with the native AaIT toxin, and the proper bioassays to monitor its function and the function of the mutated recombinant forms of the toxin.

CONCLUSIONS

Agriculture has benefited from this project:

Firstly by the demonstration of the insecticidal mechanism, capacity and utility of the combination of neuroactive polypeptides and recombinant pathogens.

Secondly by the development of a simple in vitro system for the formation and selection of new neuroactive polypeptides.

LIST OF PUBLICATIONS

L. Fishman, R. Hermmann, D. Gordon and E. Zlotkin (1997). Insect tolerance to a neurotoxic polypeptide: pharmacokinetic and pharmacodynamic aspects. J. Exp. Biol. 200, 1115-1123.

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