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**Potential of Pest Control by Insect
Immunosuppression**

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Investigators

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Biological control, Insect immunity, polydnavirus, baculovirus *Spodoptera littoralis*, *Heliothis zea*

Abbreviations commonly used in the report, in alphabetical order: AcMNPV, *Autographa californica* multiple nucleopolyhedrovirus; BV, budded virus; CiV, *Chelonus inanitus* polydnavirus; CsV, *Campoletis sonorensis* polydnavirus; PIB, polyhedral inclusion bodies.

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)				
Submitted, in review, in preparation	1	1		2
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings		1		1
Longer Visits (Sabbaticals)				

Description of Cooperation:

The American lab focused in polydnaviruses and the Israeli lab in AcMNPV. We maintained a synergistic cooperation exchanging materials, expertise and data obtained throughout the research project. N.C. visited BAW's Lab to learn manipulation of polydnaviruses and endoparasitic wasps.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

Abstract

Our original aims were to elucidate the mechanisms through which the immunosuppressive insect virus, the *Campoletis sonorensis* polydnavirus (CsV) promotes replication of a well-characterized pathogenic virus, the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in hosts that are mildly or non-permissive to virus replication.

According to the BARD panels criticism we modified our short-term goals (see below).

Thus, in this feasibility study (one-year funding) **we aimed to show that:**

1. *S. littoralis* larvae mount an immune response against a baculovirus infection.
2. Immunosuppression of an insect pest improves the ability of a viral pathogen (a baculovirus) to infect the pest.
3. *S. littoralis* cells constitute an efficient tool to study some aspects of the anti-viral immune response.

We achieved the above objectives by:

1. Finding melanized viral foci upon following the baculoviral infection in *S. littoralis* larvae infected with a polyhedra – positive AcMNPV recombinant that expressed the GFP gene under the control of the *Drosophila* heat shock promoter.
2. Studying the effect of AcMNPV-infection in *S. littoralis* immunosuppressed by parasitism with the Braconidae wasp *Chelonus inanitus* that bears the CiV polydna virus, that resulted in higher susceptibility of *S. littoralis* to AcMNPV-infection.
3. Proving that *S. littoralis* hemocytes resist AcMNPV-infection.
4. Defining SL2 as a granulocyte-like cell line and demonstrating that a *S. littoralis* hemocytic cell line undergoes apoptosis upon AcMNPV-infection.
5. Showing that some of the recombinant AcMNPV expressing the immunosuppressive polydna virus vankyrin genes inhibit baculoviral-induced lysis of SL2 cells.

This information paves the way to elucidate the mechanisms through which the immunosuppressive polydna insect viruses promote replication of pathogenic baculoviruses in lepidopteran hosts that are mildly or non-permissive to virus-replication by:

- Assessing the extent to which and the mechanisms whereby the immunosuppressive viruses, CiV and CsV or their genes enhance AcMNPV replication in polydnavirus-immunosuppressed *H. zea* and *S. littoralis* insects and *S. littoralis* cells.
- Identifying CiV and CsV genes involved in the above immunosuppression (e.g. inhibiting cellular encapsulation and disrupting humoral immunity).

This study will provide insight to the molecular mechanisms of viral pathogenesis and improve our understanding of insect immunity. This knowledge is of fundamental importance to controlling insect vectored diseases of humans, animals and plants and essential to developing novel means for pest control (including baculoviruses) that strategically weaken insect defenses to improve pathogen (i.e. biocontrol agent) infection and virulence.

Achievements

I. *Spodoptera littoralis* responses towards AcMNPV infection

The first aim of our study was to show the involvement of an immune response of *S. littoralis* larvae upon infection with AcMNPV. Preliminary data indicated that *S. littoralis* larvae were completely resistant to oral infection (*via* viral polyhedra, PIB) by AcMNPV but susceptible to infection by injection (*via* budded virus, BV) (1). To this end we utilized a polyhedra – positive AcMNPV recombinant that expressed the GFP gene under the control of the *Drosophila* heat shock promoter (vAchspGFP) (kindly donated by Dr. R. Clem, Kansas State University, USA). Oral infection was performed by inoculating recently ecdysed 4th instar *S. littoralis* larvae with doses of vAchspGFP ranging from 500 to 100,000 PIBs, (2) directly to the mouth and anterior of the midgut using a micromanipulator. This resulted in no larval mortality as expected. To pursue the fate of the recombinant virus in the infected larvae we monitored the appearance of signs of infection and reaction of the host towards the viral challenge. At 24 h postinfection 40-60 % of the larvae showed a high degree of melanization surrounding the columnar epithelial cells of the midgut (Figs. 1 B and D). These corresponded to GFP expression due to the viral infection (Fig. 1 A and C). In contrast no melanization or GFP expression was detected in the midgut of mock-infected larvae (Fig. 1 E and F). At 48h post-infection the percentage of larvae showing viral foci and the extent of foci decreased by 4-6 fold and at 72-96h post infection all foci were cleared, probably due to ecdysis of the larvae to the 5th instar. GFP expression was limited to the midgut cells indicating a severe restriction to the viral infection by the host immune system.

To further characterize the infection and reaction of the host we performed intrahemocoel injection of BV of vAchspGFP to the proleg of 4th instar *S. littoralis* larvae. In this case we observed the appearance of viral GFP-foci without melanization mainly in the fat body tissue and in the tracheal branches joints at 24h post-infection (Fig. 2 A and B). The infection propagated successfully at 48 h post-infection through the fat body and tracheal branches (Fig. 2 C and D) culminating in a general infection of the larval fat body tissue at 72 h post-infection (Fig. 2 E and F) that later resulted in death, as reflected by the high mortality rate obtained in parallel bioassays of injected BV vAchspGFP (Fig. 3). As expected mock infected larvae survived and showed no sign of GFP signaling (Fig. 2, G and H). A low extent of GFP-signaling (4 to 13%) was observed in the hemocytes of vAchspGFP –injected larvae that peaked at 72 h post infection (Fig. 4).

From these experiments we concluded that the major tissues for propagation of the viral infection were the fat body and the tracheal branches, and that the hemocytes showed a low propensity to infection probably due to a host-reaction against the virus.

II. The *S. littoralis* parasitoid *Chelonus inanitus* enhances AcMNPV infection

C. inanitus is a Braconidae endoparasitic wasp that bears the immunosuppressive polydnavirus CiV. Parasitisation of *S. littoralis* involves immunosuppression by oviposition of the wasp eggs and polydnavirus carried in the calyx fluid into *S. littoralis* eggs (3). We were interested to see what is the effect of AcMNPV infection in *C. inanitus*-immunosuppressed hosts. For that purpose, recently molted 4th instar *S. littoralis* larvae parasitized with *C. inanitus* (kindly donated by Dr B. Lanzrein, Univ. of Berne, Switzerland) were injected with AcMNPV BV. *C. inanitus* parasitized *S. littoralis* larvae became more susceptible to AcMNPV infection: a lower dose of the virus was required to obtain 100 % killing of the population and this process was faster (Table I and Fig. 5). These data support the notion that *S. littoralis* mounts an immune response against AcMNPV and that immunosuppression of the host via *C. inanitus*/ CiV polydnavirus results in enhancement of AcMNPV-pathogenicity towards *S. littoralis*.

III. *S. littoralis* cells as a model to study several aspects of the immune response

S. littoralis SL2 cells react to AcMNPV infection by undergoing apoptosis (4). It has been demonstrated that the hemocytes of *S. littura* (closely related to *S. littoralis*) undergo apoptosis upon AcMNPV infection (). Thus we wanted to find out if SL2 cells share some common characteristics with *S. littoralis* hemocytes. For this purpose we utilized a panel of monoclonal antibodies that were able to recognize specifically hemocytic marker antigens allowing to distinguish between granulocytes, plasmatocytes and spherulocytes (5) that were kindly donated by Prof. M. Strand, Univ of Georgia, USA. By using these approach we found that SL2 cells reacted with the monoclonal antibodies 49B8B10 and 48F2D5 that specifically labeled granulocytes (Fig. 6 and not shown) and not with the monoclonals 42C3G12 that recognized spherulocytes or 49B11C6 and 43E9A10 that label plasmatocytes (not shown) (5). We concluded that SL2 cells share granulocytic characteristics and could be instructive in understanding some aspects of the immune response of *S. littoralis* to AcMNPV infection (see below). Furthermore we obtained a *S. littoralis* hemocytic cell line SIJ2 from Dr. X. Jousset (INRA, Montpellier, France)(6) and challenged it with AcMNPV which resulted in extensive apoptosis (Fig. 7).

IV. Elucidation of CsV immunosuppressing functions

We completed sequence analyses of the CsIV genome and is in final stages of sequence analyses of 2 others (MdBV and HfIV). Two other genomes are in earlier stages of analysis (TrIV and GfIV). Sequence and functional analyses suggest that two viral gene families are involved with immunosuppressive function. One viral gene family, the viral ankyrin or vankyrin genes (Fig. 8),

has similarities to inhibitors of $\text{Nfk}\beta$, specifically cactus. This family of seven genes encodes only the inhibitory ankyrin repeat domains and lacks the regulatory domains found on cactus and other cellular I- κ Bs (Fig. 9). The structure of these genes suggests the hypothesis that the vankyrin genes are irreversible inhibitors that block immune cell signal transduction and thereby prevent effective cellular immune responses to viral infection. Interestingly members of the gene family are differentially expressed with some predominantly expressed in hemocytes and other expressed predominantly in fat body cells (Figs. 10 and 11). We studied the vankyrin genes by constructing baculovirus expression vectors expressing each of the 7 vankyrin genes. Interestingly, the recombinant viruses have dramatic and differential effects that correlate with their expression patterns (Fig. 12). Baculoviruses expressing vankyrin genes (such as vAcPank3) that are expressed in hemocytes cause cell lysis at 4 days post-infection as do most recombinant or wild type viruses. However, those viruses that encode vankyrin genes that are expressed in the fat body have dramatic delays in cell lysis in SF9 cells and inhibited apoptosis of SL2 cells (Fig. 13). There was no difference in levels of virion production and recombinant protein was expressed at high levels in infected cells indicating that there was no impact on virus replication itself. These data indicated that vankyrin genes disrupt cellular signalling of insect anti-viral immune responses.

A preliminary bioassay showed that the infectivity of vAcPank3 to *S. littoralis* larvae was 10 fold higher than wild type AcMNPV (Table II).

In summary, we concluded that the *S. littoralis* system represent an usefull model to elucidate various aspects of the role of the lepidopteran immune system in aborting the baculovirus infection (e.g. AcMNPV), and the mode of action of polydna viruses suppressing the host responses. Moreover immunosuppression of the host could enhance baculovirus infectivity.

Significance of main achievements

The knowledge acquired in this study will enable to elucidate the mechanisms through which the immunosuppressive polydna insect viruses, such as the *Campoplex sonorensis* polydnavirus (CsV) and the *Chelonus inannitus* PDV (CiV), promote replication of a well-characterized pathogenic virus, the *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) in lepidopteran hosts that are mildly or non-permissive to virus- replication and contribute to identify CsV and CiV genes involved in the above immunosuppression (e.g. inhibiting cellular encapsulation and disrupting humoral immunity).

Agricultural impact

Our findings and the information we expect to gain pursuing our research are of fundamental importance to controlling insect vectored diseases of humans, animals and plants and essential to

developing novel means for pest control (including baculoviruses) that strategically weaken insect defenses to improve pathogen (i.e. biocontrol agent) infection and virulence.

Details of cooperation

The American lab focused in polydnaviruses and the Israeli lab in AcMNPV. We maintained a synergistic cooperation exchanging materials, expertise and data obtained throughout the research project. N.C. visited BAW's Lab to learn manipulation of polydna viruses and endoparasitic wasps. Common experiments were performed in Kentucky using AcMNPV and Spodopteran (*S. exigua*) larvae parasitized with endo-parasitic wasps. Discussions were maintained personally and using electronic mail. Polydna viruses, recombinant baculoviruses, antibodies and cell lines were exchanged. Data analysis was performed at different stages of the project personally and via e-mail. Host lepidopteran insects were not exchanged because they are not indigenous to both locations. B.A.W laboratory isolated CsV and viral DNA, constructed CsV plasmidic libraries and transferred appropriate CsV reagents to NC. CsV-infections and analysis of recombinant AcMNPV infections of *H. virescens* larvae were performed by the American investigator. NC's laboratory performed AcMNPV infections in *S. littoralis*.

Appendix

List of Publications

Responses of *Spodoptera littoralis* to infection by the *Autographa californica* nucleopolyhedrovirus: suppression and submission Rivkin H., Webb B. A. and N. Chejanovsky (in preparation).

IKB-related vankyrin genes in the *Campoletis sonorensis* ichnovirus exhibit temporal and tissue-specific expression in parasitized *Heliothis virescens* lepidopteran hosts. J. A. Kroemer and B.A. Webb (in preparation).

A

B

C

D

E

F

Fig. 1. Oral infection of *S. littoralis* larvae with vAchspGFP. Midgut mounts of infected larvae. Left pannels GFP fluorecence. Right pannels binocular picture showing brown patches of melanization in the infected larvae (A-D) . C and D a delicate scrapping on the midgut surface showing the clear correlation between the melanine patches (arrows) and the viral GFP-expressing infected cell. E, F mock-infected larvae.

Fig2. Budded virus infection of *S. littoralis* larvae with vAchspGFP. Right pannels, whole body mounts of infected larvae, left GFP fluorescence. A and B, C and D, E and F, 24, 48 and 72h postinfection. G and H, mock infected larvae. 7

Fig. 3. Time-mortality curve of 4th instar *S. littoralis* larvae injected with 2.10^4 BV of vAchsp70GFP

Fig.4. Percentage of hemocytes showing GFP-expression in 4th instar *S. littoralis* larvae injected with 2.10^4 BV of vAchsp70GFP. Cohorts of 10 caterpillars were sacrificed at each time-point for hemolymph collection. The per cent GFP-positive hemocytes in each sample was determined and plotted as mean + SD.

Table I: Pathogenicity of AcMNPV to *S. littoralis* larvae parasitized by *C. inanitus*

Treatment	BV Dose injected	Mortality (%)
AcMNPV	5.10^1	15.0
AcMNPV	5.10^2	52.0
AcMNPV	5.10^3	61.9
AcMNPV + <i>C. inanitus</i>	5.10^1	56.0
AcMNPV + <i>C. inanitus</i>	5.10^2	80.9
AcMNPV + <i>C. inanitus</i>	5.10^3	95.2
<i>C. inanitus</i>	-----	45.0

S. littoralis eggs were exposed to *C. inanitus* and transferred to individual caps. AcMNPV BV-injections were performed with recently ecdysed 4th instar larvae.

Fig.5. Time-response of *C.inanitus*-parasitized *S.littoralis* larvae to AcMNPV-infection. Larval death of 30 cohorts per sample challenged with the indicated BV doses (the experiment was repeated in triplicates).

Fig. 6. SL2 cells express a granulocyte marker. Micrographs of SL2 cells incubated with the anti-granulocyte monoclonal antibody 49B8B10 (A and B). A Texas-Red fluorescent antibody served as secondary antibody. C and D, control cells incubated only with the secondary antibody.

1 2 3 4 5 6

Fig. 7. AcMNPV induces apoptosis of the *S. littoralis* hemocytic cell line SIJ2. Lanes 1 and 2, oligonucleosome ladder from P1 cells infected at m.o.i. of 1 and 10 with AcMNPV. Lanes 3 and 4, control AcMNPV-infected permissive SF9 cells at the above m.o.i. Lanes 5 and 6, mock-infected P1 and SF9 cells, respectively.

Fig. 8: Protein sequence alignment of the CsIV *vankyrin* gene family. A. The seven *vankyrin* proteins show considerable sequence divergence between members of the gene family. Black residues indicated a divergence from the calculated consensus sequence. B. The P-ank-2 and P-ank-4 proteins share the highest sequence identity (84.4%) followed closely by the P-ank-1 and I²-ank-3 sequences (83%). The P-ank-4 and I²-ank-3 exhibit the lowest sequence identity (44.4%). C. A phylogenetic tree constructed from the consensus sequence suggests that *vankyrin* genes (with the exception of P-ank-2 and P-ank-4) are more closely related between segments than within segments indicating the potential that genes between segments arose during a recent intersegmental duplication event.

Fig. 12: Baculovirus Expression of CsIV vankyrin proteins. All CsIV vankyrin proteins were produced in a recombinant baculovirus and crude extracts (10 mL) from Sf9 cells were assayed for reactivity with anti-V5 anti-P-ank-1 and anti-P-ank-4 antibodies (Ab). The seven recombinant proteins reacted strongly with the V5 Ab. The anti-P-ank-1 Ab reacted strongly with recombinant P-ank-1 protein and weakly with recombinant I²-ank-3 protein. The anti-P-ank-4 Ab reacted strongly with recombinant P-ank-2 protein and weakly with recombinant P-ank-4 protein.

Fig. 13. Sf9 (Panel A) and SI2 (Panel B) cells exhibit differential morphological effects when exposed to recombinant AcMNPVs expressing CsIV vankyrin proteins. Cells infected with recombinant viruses expressing FB-specific P-ank-1 and I²-ank-3 proteins (asterisks) are more stable and resemble non-infected cells at 4d pi. Cells exposed to recombinant viruses expressing the remaining CsIV vankyrin proteins undergo lysis by 4d pi and resemble cells infected with wild-type AcMNPV. 40X objective magnification.

Table II. Infectivity of recombinant AcMNPV bearing the immunosuppressive CsIV gene P-ank-3.

Treatment	Dose (BV injected)	Mortality (%)
AcMNPV	10 ³	38.4
AcMNPV	10 ⁴	50.0
AcMNPV	10 ⁵	92.0
vAcPank3	10 ³	68.0
vAcPank3	10 ⁴	96.0
vAc Pank3	10 ⁵	92.0

4th instar *S. littoralis* larvae were injected with the above indicated doses of BV.

References

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