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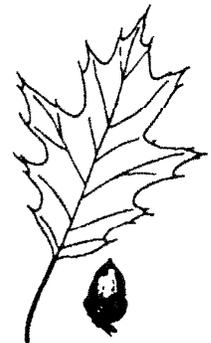
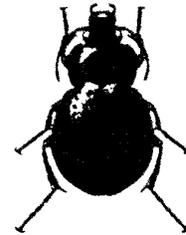
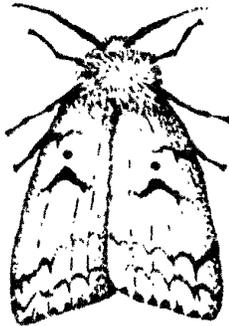
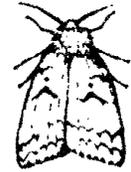
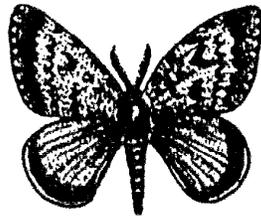
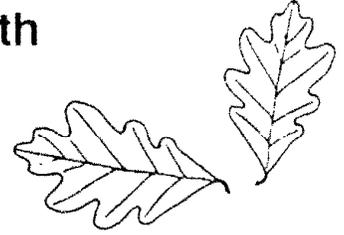
Forest Service

Northeastern Forest  
Experiment Station

General Technical  
Report NE-146



## U.S. Department of Agriculture Interagency Gypsy Moth Research Review 1990



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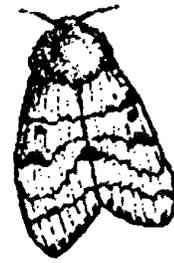
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1990



January 22-25, 1990  
East Windsor, CT

Edited by  
Kurt W. Gottschalk, Mark J. Twery, and Shirley I. Smith

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Animal and Plant Health Inspection Service

Cooperative State Research Service



## FOREWORD

In July of 1989 representatives of Forest Service-Research (FS-R), Animal and Plant Health Inspection Service (APHIS), and Agricultural Research Service (ARS) began regular meetings to discuss opportunities for improving cooperation among the agencies conducting research on gypsy moth. Representatives from the Cooperative State Research Service (CSRS) and Forest Service-State & Private Forestry (FS-S&PF) were added over the next few months. The group is known as the USDA Gypsy Moth Research and Development Coordinating Group and has the following objectives:

- a. To monitor the progress of Service programs and any breakthroughs which may influence USDA policies;
- b. To keep the Services and the Gypsy Moth Working Group appraised of progress in research and methods development;
- c. To identify research and methods development issues and concerns;
- d. To set priorities;
- e. To maximize use of current resources as well as to provide appropriate rationale to justify increased resources.

The Coordinating Group resolved at its initial meeting that a combined interagency review of gypsy moth research and development activities would add immeasurably to better communication as well as provide a comprehensive overview of ongoing research. Members of the Coordinating Group also agreed that a proceedings should be published following the meeting.

These proceedings document the efforts of many individuals: those who made the meeting possible, those who made presentations, and those who compiled and edited the proceedings. But more than that, the proceedings illustrate the depth and breadth of studies being supported by the agencies and it is satisfying, indeed, that all of this can be accomplished in a cooperative spirit.

USDA Gypsy Moth Research and Development Coordinating Group

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T. Hofacker, FS-S&PF

M. McFadden, FS-R, Chairperson

USDA Interagency Gypsy Moth Research Review  
January 22-25, 1990  
Ramada Inn  
East Windsor, Connecticut

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## GYPSY MOTH LARVAL DEFENSE MECHANISMS AGAINST PATHOGENIC MICROORGANISMS

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### ABSTRACT

We investigated the response of gypsy moth, *Lymantria dispar*, larval hemocytes to *L. dispar* nuclear polyhedrosis virus (LdMNPV) administered *per os* and by injection, and to injected hyphal bodies and natural protoplasts of some entomopathogenic, entomophthoralean fungi.

Light and electron microscope observations of gypsy moth larval hemocytes show seven distinct types of circulating cells: prohemocytes, spherulocytes, adipohemocytes, oenocytoids, plasmatocytes, granulocytes, and coagulocytes. Ultrastructural studies show that the latter three cell types predominate. Plasmatocytes and granulocytes engage in phagocytosis, and granulocytes and coagulocytes are involved in hemolymph coagulation. These events are integral components of nodulation, encapsulation and wound healing and together act to protect the host from microbial infection.

Phagocytosis of non-occluded and occluded LdMNPV took place, but it was an ineffective defense against viral infection. Viral replication was observed in hemocytes within 36 hours, and from then on, dramatic changes occurred in the blood system. During the course of nucleopolyhedrosis, infected larvae experienced significant decreases in total hemocyte count, in percentage of circulating granulocytes, in ability of the hemolymph to coagulate, and in time required for hemolymph to melanize. The blood volume per unit mass of infected larvae was significantly greater than that of controls and wound repair in infected larvae was accomplished in an altered manner.

Gypsy moth larvae were challenged with protoplasts of *Entomophaga maimaiga*, *E. aulicae*, and *E. grylli* and with hyphal bodies of the mite pathogen, *Neozygites* sp. and the Lepidopteran pathogen, *Erynia pieris*. Hemocytes responded to these entomophthoralean fungi in several different ways: these included melanization, phagocytosis, nodulation, encapsulation and lysis of fungal cells. The exact mechanism(s) implemented by the host varied with the fungus but in general, the walled cells of *E. pieris* and *Neozygites* sp. evoked the strongest cellular responses, while protoplasts elicited comparatively weak responses.

This study is an initial step in an investigation of the gypsy moth immune system and of the mechanisms by which this pest protects itself against pathogenic microorganisms.

TEMPORAL ANALYSIS AND SPATIAL MAPPING OF *LYMANTRIA DISPAR*  
NUCLEAR POLYHEDROSIS VIRUS TRANSCRIPTS AND *IN-VITRO* TRANSLATION  
PRODUCTS.

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ABSTRACT

The *Lymantria dispar* nuclear polyhedrosis virus (LdNPV) is being used as a biopesticide against the gypsy moth. We are attempting to enhance the potency of the LdNPV through recombinant DNA technology. As a prerequisite to genetic manipulation, we have characterized LdNPV gene expression in cell culture through the generation of transcription and translation maps. In addition, LdNPV polypeptides expressed in cell culture were identified.

To generate a genomic transcription map, northern blots containing RNAs isolated from 652Y cells 2, 7, 16, 24, 48, and 72 hours after infection with clonal isolate (CI) 5-6, were probed with labelled DNA fragments covering the LdNPV genome. Sixty-six viral transcripts were sized and mapped onto the genome. Fifteen viral transcripts were expressed early in infection, while most viral RNAs were initially detected from 16 to 24 hours post-infection (p.i.), and continued to be expressed until late in infection.

LdNPV CI 5-6 polypeptides, synthesized in 652Y cells, were identified after labelling with 35-S methionine and SDS-polyacrylamide gel electrophoresis. The expression of viral protein occurred in a sequential manner: 4 polypeptides were synthesized from 4 to 16 hours p.i., 24 proteins were initially detected from 12 to 20 hours p.i., and 5 polypeptides were initially detected at 24 hours p.i. These results are in good agreement with the study by J. McClintock et. al. (Virus Res., 5:307-322, 1986).

A translation map of LdNPV CI 5-6 was constructed. Six overlapping DNA fragments encompassing the CI 5-6 genome were used to hybrid select viral transcripts from RNA isolated from infected 652Y cells 7, 16, 24 and 48 hours p.i. The selected transcripts were translated in reticulocyte lysates in the presence of 35-S methionine, the protein products separated by SDS polyacrylamide gel electrophoresis, and the labelled proteins visualized by autoradiography. The approximate genomic location of 89 LdNPV translation products were mapped. No proteins were detected using RNA hybrid selected from cells 7 hours p.i. *In-vitro* translation products were generated using RNAs from cells 16, 24 and 48 hours p.i. Eighteen proteins were expressed from 16 to 48 hours p.i., 5 proteins from 16 to 24 hours p.i., and 32 proteins from 24 to 48 hours p.i. Five proteins were detected only with RNA isolated 16 hours p.i., 22 proteins were specific to RNA isolated 24 hours p.i., and 7 proteins were specific to RNA isolated 48 hours p.i. The genes coding for proteins expressed late in infection were found to be dispersed throughout the LdNPV genome.

## CONSTRUCTION OF A TRANSFER VECTOR FOR A CLONAL ISOLATE OF LDNPV

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### ABSTRACT

Deoxyribonucleic acid from a clonal isolate of LdNPV (CI A2-1), obtained by *in vivo* cloning procedures, was used to construct genomic libraries in phage (lambda Gem 11) and cosmid (pHC79) vectors. Overlapping clones were selected to generate a restriction enzyme map. The restriction enzyme map, covering about 85% of the CI A2-1 genome, was determined. Efforts are underway to clone and characterize the missing regions.

A BglII fragment (10.5 kbp) containing the entire polyhedrin gene was subcloned into pUC18. This new plasmid was characterized for various restriction enzyme sites and the exact location of polyhedrin coding sequences. Several manipulations of the plasmid DNA were carried out which included: 1) deletion of a 6.2 kbp EcoRI fragment; 2) destruction of an existing HindIII site; 3) deletion of a 211 bp fragment containing sequence coding for N-terminal region of polyhedrin; and 4) replacing it with an oligonucleotide containing sites for NotI, HindIII, BamHI, SmaI, and ApaI enzymes.

The final product, pLdS1, was 6.6 kbp in size. It had 1.5 kbp region containing the promoter for polyhedrin gene and upstream sequences. Immediately following this were sites for NotI, BamHI, HindIII, SmaI and ApaI. The multiple cloning site was sequentially followed by 550 bp sequence coding for the C-terminal region of polyhedrin and 1.8 kbp sequences downstream from the polyhedrin coding region. Analysis of pLdS1 DNA suggests that this will be a suitable transfer vector for introducing foreign genes under polyhedrin gene promoter into CI A2-1.

A reporter gene, betagalactosidase, was subcloned into pLdS1 to produce pLdS1-Bgal. Both pLdS1 and pLdS1-Bgal will be tested by co-transfection experiments to determine whether pLdS1 can be used for introducing foreign genes into CI A2-1.

Using polyclonal antibodies, several cDNA clones corresponding to juvenile hormone esterase (JHE) were isolated from a lambda gt11 cDNA library constructed from poly(A) RNA from 5th instar, day 6 larval fat bodies. These are being analyzed to determine whether any contain the entire coding sequence for JHE. Meanwhile, these clones are being used as probes to isolate the JHE gene from a gypsy moth genomic library constructed in EMBL3 and lambda Gem11 vectors.

REPLICATION AND INCLUSION BODY CHARACTERISTICS OF TWO *LYMANTRIA DISPAR* NUCLEAR POLYHEDROSIS VIRUS PLAQUE VARIANTS.

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ABSTRACT

Propagation of *Autographa californica* nuclear polyhedrosis virus in cell culture results in the generation of a mutant virus, termed few polyhedra. This plaque variant is characterized by a high budded virus titer, the formation of few polyhedral inclusion bodies (PIBs), and the production of PIBs exhibiting a low potency against its natural host. The isolation and study of a few polyhedra variant of *Lymantria dispar* nuclear polyhedrosis virus (LdNPV) could lead to a means of enhancing viral potency against the gypsy moth.

The replication characteristics of two *Lymantria dispar* nuclear polyhedrosis virus (LdNPV) plaque variants were studied in cell culture and *in-vivo*. In addition, the PIBs produced by the variants were characterized with respect to virion number, size, and morphology. The variants, clonal isolates (CI) A2-1 and 5-6, were found to exhibit differences in the number of PIBs generated, the titer of nonoccluded virus (NOV) produced *in-vitro*, the number of virions present within PIBs, and the potency of PIBs against the gypsy moth.

Infection of 652Y cells with CI A2-1 resulted in cells with an average of 51 PIBs per cell (ranging in number from approximately 3 to greater than 50 PIBs/cell), and with the variant CI 5-6, cells with an average of 4.4 PIBs per cell (ranging from approximately 1 to 12 PIBs/cell). The production of NOV in 652Y cells by the plaque variants was markedly different; CIs A2-1 and 5-6 exhibited TCID 50s six days after infection of  $4.1 \times 10^{-4}$  and  $1.6 \times 10^{-7}$ , respectively.

The number of virions present, and size and morphology of PIBs generated by CIs A2-1 and 5-6, were analyzed through electron microscopic examination. CI A2-1 PIBs generated *in-vivo* contained an average of 11.6 virions per square micrometer of PIB surface area, in comparison to 0.09 virions/ $\mu\text{m}^2$  of PIB surface area within CI 5-6 polyhedra. In addition, larvae infected with clonal isolate A2-1 produced an average of  $2.1 \times 10^{-9}$  PIBs per larvae, ranging in size from 1.2 to 2.8  $\mu\text{m}$  in diameter, with an average of 2.0  $\mu\text{m}$ , while CI 5-6 generated an average of  $8.6 \times 10^{-7}$  PIBs per larvae, ranging from 1.9 to 5.8  $\mu\text{m}$  in diameter, with an average of 2.8  $\mu\text{m}$ . The shape of CI 5-6 and A2-1 PIBs produced *in-vivo* and of CI A2-1 produced *in-vitro* were round. In contrast, CI 5-6 PIBs generated *in-vitro* were primarily triangular, rectangular, or trapezoidal in shape. The potency of the variants against second instar gypsy moth larvae was investigated through bioassay. Preliminary data indicate that the LC-50 of CI A2-1 is approximately  $1 \times 10^{-3}$  PIBs per ml of diet. In contrast, the LC-50 of CI 5-6 is greater than  $1 \times 10^{-6}$  PIBs/ml of diet.

The traits of CI 5-6: a high NOV titer, production of few PIBs, and the low potency of the PIBs are consistent with the classification of this virus as a few polyhedra variant. In contrast, the characteristics of CI A2-1: a lower NOV titer, production of many PIBs, and a wild type potency level are consistent with it being classified a many polyhedra wild type virus. The molecular basis for the few polyhedra phenotype of CI 5-6 is currently under investigation. There appears to be a correlation between potency and the number of virions present within PIBs. If such a relationship does exist these studies may yield insights into the process of virion occlusion and present a means of enhancing viral potency.

**AUTOGRAPHA CALIFORNICA NUCLEAR POLYHEDROSIS VIRUS  
REPLICATION IN NON-PERMISSIVE *LYMANTRIA DISPAR* CELL LINES**

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**ABSTRACT**

*Autographa californica* nuclear polyhedrosis virus (AcNPV) the prototypic group A baculovirus, has the widest reported host range of the baculoviruses and is considered to be one of the most virulent baculoviruses studied. The gypsy moth *Lymantria dispar* is not considered a natural host of AcNPV, however. To determine the factors regulating AcNPV restriction in *L. dispar*, *in vivo* and *in vitro* studies on AcNPV replication in gypsy moth cells have been initiated.

*In vitro* studies of AcNPV infection of *L. dispar* cell lines originated from egg, larval and pupal tissue sources revealed a variety of cell responses to infection. Cell susceptibility has ranged from permissive to non-permissive. Two cell lines, IPLB-Ld652Y and IPLB-LdFB have been shown to partially replicate AcNPV (semi-permissive cell lines) and thus are used to study at least one mechanism of AcNPV restriction in *L. dispar* larvae. Synchronous infection of these lines produces no budded virus nor viral occlusion bodies, however, distinct cytopathic effects are observed including nuclear hypertrophy, cell clumping (in IPLB-Ld652Y cells) and very condensed nuclear material (in IPLB-LdFB cells).

Growth rates and mitotic indices of infected cells are significantly inhibited, however, cell viability is not significantly affected initially. The kinetics of viral genome replication are normal (compared with viral DNA replication in permissive cell lines) as measured by DNA-DNA hybridization. Protein synthesis is drastically altered in both systems. Only a few of the early viral proteins are observed before there is a rapid total shut down of both viral and cellular protein synthesis. Analysis of transcriptional events in these systems reveal aberrant viral transcriptional regulation, however, cell translational processes appear normal.

Both *in vitro* systems are serving as models for events which are being monitored simultaneously *in vivo* and may give insight into *in vivo* AcNPV restriction mechanisms in *L. dispar* larvae.

## RESPONSE OF GYPSY MOTH LARVAE TO HOMOLOGOUS AND HETEROLOGOUS NUCLEAR POLYHEDROSIS VIRUS

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### ABSTRACT

The gypsy moth, *Lymantria dispar*, is not particularly susceptible to baculoviruses other than the nuclear polyhedrosis virus originally isolated from the species (LdMNPV). The multiple enveloped nuclear polyhedrosis virus of *Autographa californica* (AcMNPV), a very virulent baculovirus that replicates in a large number of Lepidopteran species, only rarely produces mortality in gypsy moth larvae regardless of the dose ingested.

We were unable to obtain a LC<sub>50</sub> for AcMNPV in first instar gypsy moth larvae, and observed only occasional mortality among larvae fed concentrations of polyhedral inclusion bodies (PIB) exceeding 10<sup>9</sup> PIB per cup. This dose was more than six million times the dose required for a LC<sub>50</sub> with the homologous virus.

The pathogenicity of AcMNPV for gypsy moth was not enhanced when PIB were produced *in vivo* in alternate hosts or *in vitro* in susceptible gypsy moth cell lines, or when PIB were fed to larvae in combination with inactivated homologous virus. Alkali-disrupted AcMNPV PIB were not infectious *per os* or when injected into the hemocoel of gypsy moth larvae, although when injected into *Trichoplusia ni* larvae, 100% died of nucleopolyhedrosis.

Infections were readily established in gypsy moth larvae when extracellular non-occluded AcMNPV, produced in TN-368 cells *in vitro*, was injected into the hemocoel, but only low yields of PIB were obtained from cadavers and lethal times varied from 9 to 20 days.

The pathway of infection and histopathological effects of AcMNPV on *L. dispar* tissues were aberrant compared to what is observed during LdMNPV infection. Larvae infected with AcMNPV often had ruptures in the cuticle, presumably the result of dense accumulations of fibrous material that were observed in infected epidermal cells. Midgut cells, while not obviously infected, gradually degenerated and the midgut became grossly distended. Hemocytes became infected but little budding of non-occluded virus was seen. Other tissues in the body cavity were infected in a haphazard fashion. Pericardial and circumesophageal nephrocytes did not replicate AcMNPV, but accumulated electron-dense granules and underwent a significant color change. It is unclear whether the changes in nephrocytes are merely a form of necrosis due to an aberrant infection or whether these cells play a role in insect defense.

DETECTION OF *LYMANTRIA DISPAR* NUCLEAR POLYHEDROSIS VIRUS IN  
INFECTED LARVAE USING A DNA HYBRIDIZATION ASSAY

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ABSTRACT

The incidence of nuclear polyhedrosis virus in gypsy moth populations is currently measured by rearing field-collected larvae until death. Dead larvae then examined microscopically to confirm the presence of virus. Beside from being quite tedious, this method has several inherent difficulties and inaccuracies. In order to circumvent some of these problems, we have developed a DNA hybridization assay using radio- and digoxigenin-labeled viral probes to identify virus infected larvae using slot blot vacuum filtration and whole larval squashes. These methods are less tiresome and give more definitive results which are comparable with mortality data obtained from laboratory experiments as well as field collected larvae.

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GENETICALLY-ENGINEERED BACULOVIRUS PESTICIDES  
AND THEIR ENVIRONMENTAL SAFETY

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ABSTRACT

Baculoviruses such as the *Lymantria dispar* nuclear polyhedrosis virus (LdMNPV) are ecologically attractive alternatives to chemical insect pesticides but have a slow rate of control. To overcome this we have developed and are field testing an environmentally acceptable strategy which can be used for the introduction and expression of pesticide-enhancing genes by baculoviruses. The field release data will be used to construct environmentally-safe, viral pesticides which have improved pesticidal properties and which will not persist in nature.

The model virus for this study has been the *Autographa californica* nuclear polyhedrosis virus (AcMNPV). Similar genetic constructions are being performed with the LdMNPV. Genetic alterations to the polyhedrin region of the LdMNPV will provide a phenotypic and genomic marker for release studies. The markers will provide a method to study the epidemiology of the LdMNPV in nature. In addition the genetic alterations are being made in such a way as to allow for the insertion and expression of foreign pesticidal genes.

An important ecological consideration involving the release of a genetically altered LdMNPV is the possibility of vertical transmission and persistent infections. In an effort to document and assess the extent of persistent virus infections, we have conducted experiments designed to induce productive LdMNPV replication in "persistently" infect gypsy moth larvae. A polyhedrin-minus mutant of the AcMNPV has been injected into gypsy moth larvae in an attempt to induce LdMNPV productive replication. We will report on the extent and nature of polyhedra produced in challenged, laboratory reared larvae.