

PN-ABM 430

79456

PROGRESS REPORT NO 2

DENSOVIRUS AS INSECTICIDE

A RESEARCH PROJECT

U.S.-ISRAEL CDR PROGRAM

GRANT NO .936-5544-G-00-7016-00

Submitted by

Tipradee Attathom Ph.D.

Project Leader

Department of Entomology, Kasetsart University

Kamphaengsaen Nakornpathom 73140

Thailand

BEST AVAILABLE DOCUMENT

AGENCY FOR INTERNATIONAL DEVELOPMENT
WASHINGTON, D.C. 20523

DATE: _____

1/25/89

MEMORANDUM

TO: AID/PPC/CDIE/DI, room 209 SA-18
FROM: AID/SCI, Victoria Ose *VO*
SUBJECT: Transmittal of AID/SCI Progress Report(s)

Attached for permanent retention/proper disposition is the following:

AID/SCI Progress Report No. _____

C 7 - 150

PR#2

250 of 88

Attachment

BEST AVAILABLE DOCUMENT

Project Profile

Country : Thailand
Grant No. : 836-5544-00-G-7016-00
Program : U.S. Israel CDR Program
Project Title : Denguevirus as Insecticide
Principle Investigators : Dr. Tipvadee Attathom
Department of Entomology, Faculty of
Agriculture, Kasetsart University,
Kamphaengseen, Nakornpathom 73140
: Dr. Jacob Tal
Biology Department, Ben-Gurion
University of the Negev,
Beer-Sheva 84150. Israel.
Organization : Kasetsart University
Co-investigator Mrs. Sudwan Chaeychomsri
Central Laboratory and Greenhouse
Complex, Kasetsart University Research
and Development Institute,
Kasetsart University.
Authorized Officer : Rector, Kasetsart University
Total Project Budget : \$ 149,450
Project Duration : 3 years
Reporting Period : November, 1987-July, 1988
Budget Allocation for this Period: Thai : 1,141,250 Baht,
Israel : \$ 35,951

BEST AVAILABLE DOCUMENT

U.S.- ISRAEL CDR PROGRAM

DENSOVIRUS AS INSECTICIDE

THAI GROUP

BEST AVAILABLE DOCUMENT

Contents

1. Introductions.....	1
2. Objectives.....	2
3. Materials and Methods.....	3
4. Results and Discussion.....	7
5. Conclusion.....	26
6. Workplan for the Next Period.....	27
7. Reference.....	28

BEST AVAILABLE DOCUMENT

U.S.- ISRAEL CDR PROGRAM
DENSEOVIRUS AS INSECTICIDE
THAI GROUP

INTRODUCTION

Species of *Heliothis* and *Spodoptera* complex are major pests that attack at least 30 different important food and fiber crops in Thailand. The extensive and indiscriminate use of pesticides for controlling these two insect species has created many serious problems upsetting the environment and human welfare. Insect viruses due to their specificity and efficiency are receiving considerable attention to develop as microbial insecticides. Denso-nucleosis virus (DNV) is a unique group among the Parvoviridae family. This group of viruses includes at least 10 virus strains that isolated from different insect species. DNV's are extremely stable to the environment and exhibit a narrow species specificity allowing a selective, targeted action against certain insects without harming others. Besides causing disease in insect larvae, densoviruses offer an excellent potential vector to transduce foreign genes into insect cells. They are single-stranded DNA, small and structurally simple viruses which make them amenable to genetic manipulations and the study of their gene expression and regulation relatively easier than that of larger viruses. They are structurally similar to their family members from the mammalian parvoviruses suggesting that they may be found capable of integrating their DNA into their host genome. All these properties make DNV an excellent candidate as biological control agents.

BEST AVAILABLE DOCUMENT

OBJECTIVES

The present study is aiming at evaluating the potential of densovirus (DNV) as microbial insecticide. The overall objectives include (a) The use of Galleria mellonella densovirus (GmDNV) DNA as a model system for the construction of an insect vector, and using it to transport and express foreign genes in insect larvae; (b) isolation of DNVs which can cause disease in the agricultural important pests, Spodoptera and (or) Heliothis; (c) study the genome organization and gene expression of these DNVs as a preliminary step toward utilizing them as insect vectors.

This report covered the research work done by the Thai group during the period of November 1987 to July 1988. The specific objectives during this report period include (a) improvement of the purification procedure in order to obtain the highly purified GmDNV suspension for serology and pathogenicity tests; (b) antisera production and the development of the serological techniques for the detection and study of the viral proteins synthesis in insect cells; (c) determination the insecticidal activity of the GmDNV in laboratory Galleria mellonella culture; (d) host-range mutant study of the GmDNV in Heliothis armigera.

BEST AVAILABLE DOCUMENT

MATERIALS AND METHODS

Unfortunately the densovirus field strains of *Heliothis* or *Spodoptera* have not been found in Thailand. We decide to proceed our work using the *Galleria mellonella* densovirus (GmDNV) to develop the techniques and clarify the hypothesis we proposed. GmDNV host-range mutants capable of infecting *Heliothis* or *Spodoptera* are being raised with the purpose to utilize them as the biological agents for the control of these two noxious insect species.

1. Production and purification of GmDNV

GmDNV was propagated on the greater wax moth, *Galleria mellonella* which were reared successfully on artificial diet in the laboratory as presented in the Progress Report No.1. *G.mellonella* larvae were fed with GmDNV suspended in phosphate-buffered saline. After incubation at room temperature for 6-8 days, diseased larvae were collected and kept frozen until use.

The purification procedure was modified from that reported by Tijssen et.al. (1976). The diseased larvae were homogenized in phosphate-buffered saline containing a few crystals of 1-phenyl-2-thiourea (ca. 0.02 gm per 100 larvae) to prevent melanization using an Ace homogenizer at 1,500 rpm for 5 min. Larvae debris was removed by filtration through two layers of cheesecloth. The filtrate was then centrifuged at 10,000 rpm for 10 min in JA-14 rotor in Beckman J-21 C centrifuge. Lipids were extracted from the supernatant by vigorous shaking with an equal volume of chloroform-n-butanol mixture (1:1) and the phases were separated by centrifugation at 10,000 rpm for 10 min. The aqueous phase was then centrifuged for 1 hr at 16,000 rpm.

The virus was pelleted from the supernatant by centrifugation for 2 hr at 35,000 rpm in RP 50 T-2-203 rotor in Hitachi 85 P-72 ultracentrifuge. The sediment was suspended in 0.5 ml of 0.1xSSC (1xSSC = 0.15 M NaCl plus 0.015 M Sodium citrate) Unsuspended material was removed by centrifugation for 5 min at 10,000 rpm. The supernatant was then centrifuged for 2 hr at 45,000 rpm. The pellet was recovered as partially purified virus in 0.1xSSC buffer.

The virus suspension was further purified by isopycnic centrifugation in CsCl. CsCl of 6.3479 gm was dissolved in 10 ml of 0.1xSSC containing the virus preparation. The density of this solution was 1.40 gm/ml. The uniformly mixed solution were centrifuged in a RP 83 T-143 fixed angle rotor for 48 hr at 50,000 rpm in Hitachi 85 P-72 ultracentrifuge at 20°C. The fraction containing the virus was collected by the syringe and dialyzed against several changes of 0.1xSSC at 4°C.

This purified virus suspension was negatively stained with 1% uranyl acetate and examined on a JEOL-JEM-100 S electron microscope. The UV absorption spectrum of this purified virus suspension was determined by Hitachi Model 200-20 spectrophotometer monitored at 220-320 nm.

2 Serological Study

Antisera were prepared in 2 of the New Zealand White female rabbits. Normal sera were obtained from rabbits by bleeding from the marginal ear vein. Rabbits were immunized by intramuscular injection at two weeks interval for 4 times. Each time 1ml of DNV in 0.1xSSC (contained 375 ug of virus emulsified with 1 ml of Freund complete adjuvant) was applied to the rabbits. First immune sera were taken one week after the third injection. One week after the fourth injection, the immune

sera were collected at weekly interval for 7 weeks by bleeding from the marginal ear vein. The sera were sat at room temperature for 1 hr and overnight in the refrigerator. The top clear sera were then centrifuged at 8,000 rpm for 20 min. The supernatants were collected and added 0.01% sodium azide to prevent bacterial contamination. These antisera were kept frozen for further uses.

Agar-gel double diffusion test was used to determine the titer of the antisera obtained. Agar-gels consisted of 0.75% agarose (w/v), 0.85% NaCl and 0.5% sodium azide (NaN_3) were prepared in plastic petridishes. Purified suspension obtained from the healthy wax moth and normal sera of the rabbits were used as the control. In the experiment, antisera were diluted 1:2, 1:4, 1:8.....to 1:1024 with 0.1 M saline potassium phosphate buffer (PBS) pH 7.4 and the antigen (purified DNV) was diluted 1:10 (contained 1.148 mg of virus) and 1:100 (contained 0.574 mg of virus) with 0.1xSSC. All plates were incubated at 37°C and the reactions were observed for five days.

3. Infectivity of GmDNV

The biological properties of the densovirus of the greater wax moth, Galleria mellonella obtained from Israel were determined. Infectivity tests were conducted using five concentrations of the purified GmDNV as follow : 0.001, 0.01, 0.1, 1 and 10 ug prepared in 0.1xSSC buffer. The tested larvae were the greater wax moth continuously cultured on a synthetic diet in the laboratory. In order to determine the suitable larval instar for viral inoculation, all instars of the larvae were used for this experiment. For each virus concentration, a total of 60 of each larval instar forming 3 replicates of 20 larvae were used for the bioassay.

The virus inoculum, 50ul per larva was applied uniformly on the surface of 1/4-inch cubes of the diet by mean of the Fipetman micropipette. The greater wax moth larvae were then allowed to feed on this contaminated diet individually in the plastic container. Control larvae were treated similarly except that the 0.1xSSC buffer was used as the source of inoculum. All experimental units were kept at room temperature which varied between 27 and 30°C, and examined daily for larval mortality. All test were terminated within 14 days of their initiation since by this time most larvae had either pupated or died.

4. Raising GmDNV host-range mutants

According to the proposed work plan, a second approach to obtain *Heliothis* and *Spodoptera* DNVs will be by introducing GmDNV into heterologous larvae. Microinjection into the proleg of the larvae was used as the mean for virus inoculation. Each injection, 0.8 ul of virus suspension was administered into larvae. Purified GmDNV suspension was prepared into three doses of 1, 5, and 10 ul (in 0.8 ul) with 0.1xSSC buffer. The larvae used in this experiment were obtained from a laboratory stock of *Heliothis armigera* which had been continuously cultured on a synthetic diet. Four hundreds larvae of the fourth instar were used for each virus concentration tested. The control unit was injected with 0.1xSSC buffer. After injection the larvae were raised individually in the plastic cup contained 1/4 inch cubes of the diet and kept at 29°C. Larval mortality was recorded daily. Those individuals surviving the treatment were allowed to pupate and raise to the next generation. Fourth instar larvae of this group (second generation) were injected with the purified GmDNV in the same procedure as described above. with this generation only 100 larvae were used for each virus dose. Information was

collected on the larval mortality as well as the effects of the GmDNV on the development of the survivals.

To confirm the replication ability of the densovirus of the wax moth in the heterologous host, H. armigera, electron microscopic study was performed. Dead larvae from the microinjection tests were collected and pieces of tissue were dissected. Ultrathin sections were prepared following the method described in Progress Report NO.1 (Material and Method, Section 4, Pathology of the GmDNV). Observations were made with a JEOL-JEM-100S electron microscope operated at 80 KV.

RESULTS AND DISCUSSION

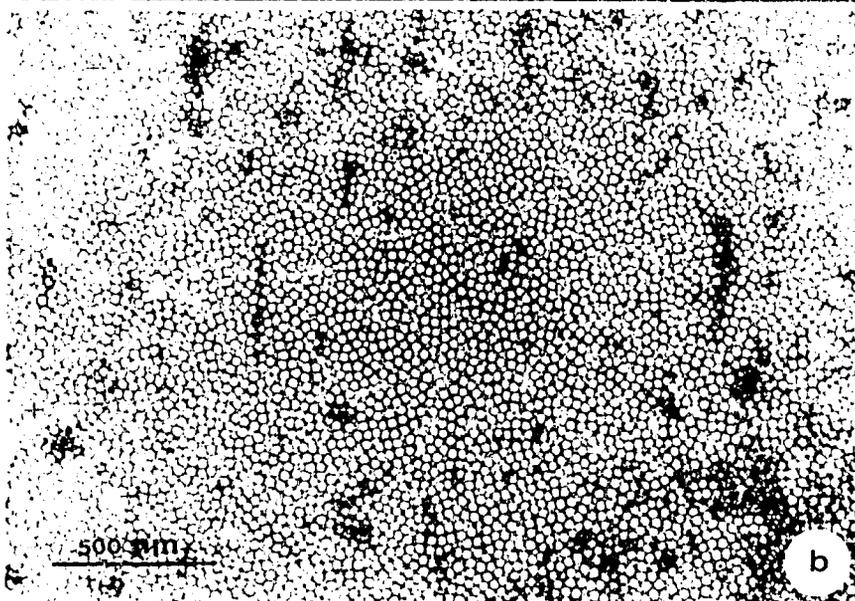
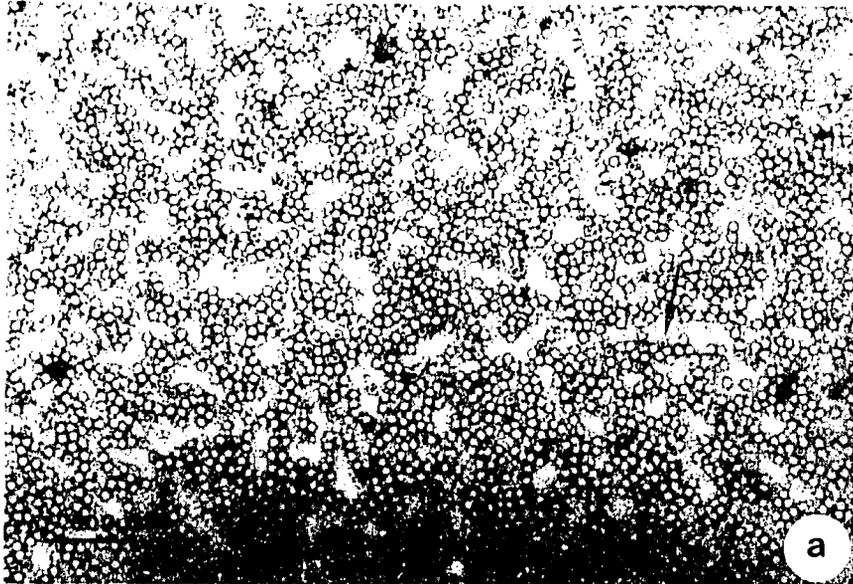
1. Purification of GmDNV

Differential centrifugation provided partially purified virus suspension. Further purification by sedimentation equilibrium centrifugation in CsCl tremendously improved the purification procedure. After the equilibrium centrifugation, two white bands were observed in the centrifuge tubes at 2.1-2.6 cm and 4-4.4 cm below the meniscus. These two bands were collected separately and preparations negatively stained with 1% uranyl acetate were observed under the electron microscope. The first band next to the meniscus consisted of small, spherical complete virus particles about 23 nm in diameter, empty virus particles with stain penetrated and many small globular structures (Fig.1a) The majority of the particles found in the second band were complete virus particles (Fig.1b). This result was similar to that observed by Kelly et al. (1980), and indicated that isopycnic centrifugation in CsCl was effectively separated the complete virus particle from other components. In the Progress Report No.1

Fig.1. Purified densovirus of the greater wax moth, Galleria mellonella after isopycnic centrifugation in CsCl.

- a). Band I, top component consists of complete virus particles, empty virus particles (arrow) and small globular structures.
- b). Band II, bottom component consists of complete virus particles.

BEST AVAILABLE DOCUMENT



BEST AVAILABLE DOCUMENT

the small globular structures identified as the capsomers from disrupted virus particle (Nakagaki and Kawase 1982), the long-therad-like structure and the empty virus particles were observed in all partially purified virus suspensions. All the structures were eliminated and the highly purified virus suspension was obtained after isopycnic centrifugation in CsCl.

The ultraviolet absorption spectrum of those two bands were shown in Fig. 2. The spectrum characteristic of a nucleoprotein with the ratio of absorbance at 260 nm to that at 280 nm = 1.60 and 1.62 for the first and the second bands respectively. Kelly et al. (1980) reported the extinction coefficient for densovirus isolated from Galleria mellonella was $5.49 \pm 0.32/\text{mg per ml}$ at 260 nm which was the same value of that isolated from the butterfly, Junonia coenia. From this extinction coefficient value it was found that DNV obtained from this purification procedure was about 15 mg per 500 wax moth larvae (average of six experiment). The present modification of the virus purification procedure provided satisfactory results on both virus purity and virus yield.

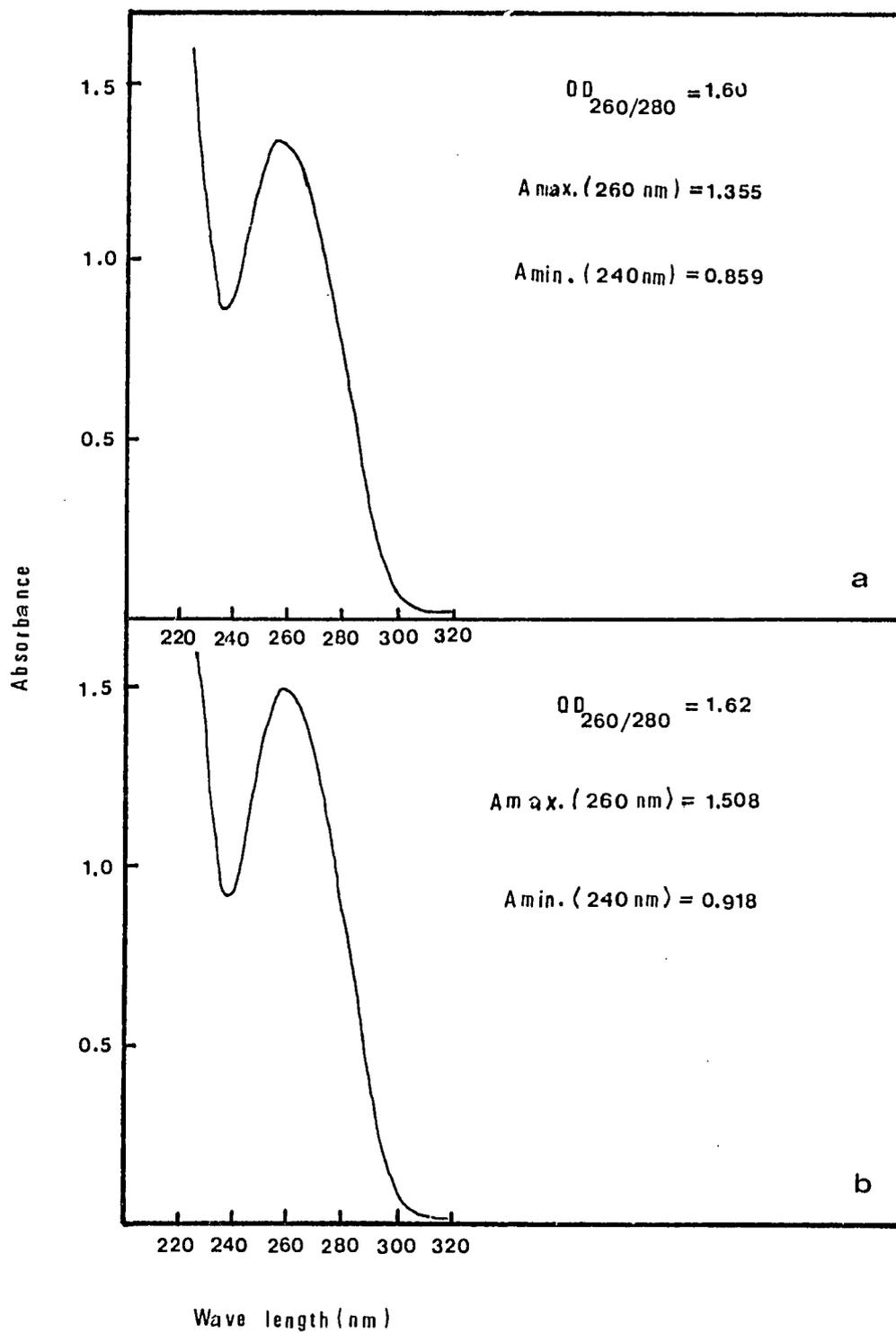
2. Serological study

Titer and purity of the antisera produced in two New Zealand White female rabbits were determined. These two rabbits provided an equal quality of the immune sera. Agar-gel double diffusion test demonstrated that the immune sera with highest titer were those bled from the marginal ear vein in the 8th week of the injection and bleeding schedule (eight weeks after the first injection and one week after the fourth injection).

Fig.2. Ultraviolet absorption spectra of the purified
denguevirus of the greater wax moth, Galleria
mellonella

a). Band I, top component.

b). Band II, bottom component.



BEST AVAILABLE DOCUMENT

When 1:10 and 1:100 diluted virus suspension were tested against the antisera, the precipitin bands were formed with the antisera diluted up to 512 times. However reaction was noted when the immune sera diluted up to 64 times were tested against the purified suspension obtained from the healthy wax moth. No reaction occurred when the virus was tested against the normal serum.

Based on the results obtained there are common antigen present in the purified virus suspension and the suspension purified from the noninfected larvae. This suggest that ultrastructures frequently observed in purified virus suspension may be the remnant of the host cell components. to clarify the purity of the antisera produced, immunosorbents were performed. The antisera were absorbed with the suspension of the noninfected cellular materials and incubated at 37° C for 1 hr. the antisera were then purified by centrifugation at 8,000 rpm for 10 min. This absorbed antisera proved to be more specific to their homologous virus antigens. Although the present antisera contained some nonspecific antibodies, the immunosorbent can be used to remove those antibodies. However the purity of the antibodies and their specificity are essential for further serological studies. Tiissen and Kurstak (1974) reported several methods used for the purification of immunoglobulin. When it is ready for the immunoassays for example the ELISA and the immunoperoxidase assays, the quality of the present antisera will be verified.

BEST AVAILABLE DOCUMENT

ELISA and immunoperoxidase techniques will be used as the rapid and sensitive method to identify the field strains densovirus of Heliothis armigera and of Spodoptera exigue if they exist. They will also be used to distinguish the original GmDNV and the host range mutant GmDNV. Papers reported by Kurstak et al.(1977); Rose and Bigazzi (1980); Kurstak and Tijssen (1981) and Tijssen et al.(1982) were studied and evaluated extensively. Modification procedures from those reported are being investigated and developed.

3. Infectivity of GmDNV

The densovirus used in all experiments at present is the densovirus of the greater wax moth, Galleria mellonella (GmDNV) obtained from Dr. Jacob Tal, the co-principle investigator from the Ben-Gurion University of the Negev, Israel. Since large amount of GmDNV was needed for experimental used, effective mass propagation of this virus is essential. Information on an effective dose of virus inoculum, the suitable larval instar for viral application and the optimal condition for the viral incubation period will facilitate the production procedure of this virus. Moreover the biological properties for example the pathogenicity, host range etc. are also important.

Infectivity tests demonstrated that densovirus is highly pathogenic to G. mellonella. It can cause disease to all instar of the larvae, (Table 1). However younger larvae are more susceptible than the older larvae. The concentration of the virus used to induce disease can be as low as 0.001 ug. Over 90% mortality was observed to all larval instar when using the virus up to 10 ug except the fifth instar larvae. Those fifth instar larvae who survived the treatment either died in pupation or emerged into adults with malformation. The LD50 (lethal dose), the suitable dose and the suitable larval instar for inoculation are under statistically analyzed. Further experiments will be made to

TABLE 1. Percent mortality of the greater wax moth , Galleria mellonella infected with different doses of G.mellonella densovirus.^{1/}

Dose (ug)	No. of larvae treated ^{2/}	Instar I ^{3/}		Instar II		Instar III		Instar IV		Instar V	
		No. res.	% cor. mor.	No. res.	% cor. mor.	No. res.	% cor. mor.	No. res.	% cor. mor.	No. res.	% cor. mor.
0.001	60	39	53.33	33	40.00	32	41.69	45	37.50	12	11.11
0.01	60	42	64.70	53	60.56	37	54.88	46	66.64	27	38.89
0.1	60	56	83.25	57	87.50	54	80.00	39	41.66	24	8.30
1	60	57	94.44	53	87.73	49	81.65	13	7.82	13	17.50
10	60	60	100.0	59	97.93	59	97.93	57	93.75	35	56.13

1/ Percent mortality corrected by Abbott's formula.

2/ Surface diet contamination , 50 ul per larva.

3/ 3 replications of 20 larvae each.

determine the optimum condition for the progress of the disease and the incubation period of the virus. These informations are necessary for the better understanding of the densovirus which will bring about a wise use of this virus for insect pest control.

4. Raising GmDNV host range mutants.

Larval mortality was observed after microinjection the purified GmDNV into the fourth instar larvae of Heliothis armigera. Percent mortality of 18.75 was recorded in the first treated generation after receiving 10 ul of the purified GmDNV. However percent mortality increased to 34.33 when the second generation of the same bollworm population was subjected to the same treatment (Table 2 and 3). Larvae that survived the first treatment, pupated and developed into adults. Enough progeny were provided for the second treatment. Although the percent mortality increased in the second treatment, several larvae survived and undergo pupation. Some of the pupae were malformed, adults that were able to emerge, laid eggs as normal but the eggs had never hatched. This result demonstrated the effect of GmDNV on the development of the bollworm. Hence GmDNV host range mutants capable of growing and introducing disease in bollworm are of possible to raise in this laboratory.

In general autonomous parvoviruses display narrow species specificity (Siegl, 1965). As long as these viruses are transferred via the normal route of infection, this specificity is maintained. The species specificity of the densoviruses differs from each other. The host range of GmDNV is restricted to G. mellonella but Junonia DNV infects various Lepidopteran larvae and not that of G. mellonella (Longworth, 1978). To confirm the species specificity of the GmDNV studied, the purified GmDNV was applied to the fourth instar larvae of H. armigera via both feeding and microinjection. Observation of the

TABLE 2. Effect of densovirus of Galleria mellonella on the first ^{1/} treated generation of American bollworm, Heliothis armigera.

Dose (ug)	No. of larvae ^{2/} treated	No. of larvae responding	No. of ^{3/} pupation	% corrected ^{4/} mortality
1	400	157	183	7.14
5	400	231	169	17.64
10	400	245	155	18.75

1/ Microinjected treatment.

2/ Fourth instar larvae.

3/ Emerge into adults, mate and lay viable eggs.

4/ Corrected by Abbott's formula.

TABLE 3. Effect of densovirus of Galleria mellonella on the second ^{1/} treated generation of American bollworm, Heliothis armigera.

Dose (ug)	No. of larvae ^{2/} treated	No. of larvae responding	No. of ^{3/} pupation	% corrected ^{4/} mortality
1	100	41	59	20.27
5	100	43	57	12.31
10	100	56	44	34.33

1/ Microinjected treatment.

2/ Fourth instar larvae, progeny of the first treated bollworm population.

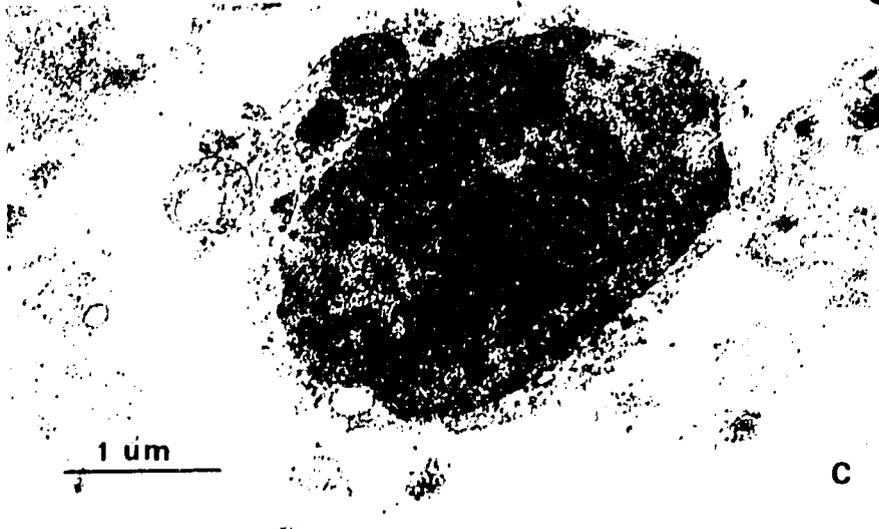
3/ Emerge into adults, mate and lay nonviable eggs.

4/ Corrected by Abbott's formula.

infected tissues revealed the presence of the virus particles arranged in cluster or paracrystalline array characteristic of the densovirus in infected cells. In homologous host, G. mellonella, this virus multiplied in almost all of the insect tissues with the exception of the midgut (Kawase, 1980) and the virus particles were found in both nuclei and cytoplasm of the infected cells (Progress Report No.1). It was observed that mitochondria of infected cells of G. mellonella undergo hypertrophy and lose their cristae characteristic. The virus particles frequently arranged in paracrystalline array in close association with the altered mitochondria (Fig. 3). After peroral infection GmDNV into the heterologous host, H. armigera, the virus was found to multiple in midgut and other tissues as well (Fig 4). This result differs from that reported by Kawase (1985). In mid gut tissue, the virus was found in goblet cells and epithelial cells. The virions arranged in large masses and in paracrystalline array (Fig.4). Cytopathic effects observed in fat cells similar to those in infected G. mellonella. The mitochondria had clearly swollen, lost their cristae and contents. The virions appeared in group close to the infected mitochondria. It was suggested that the virions may finally fill the empty mitochondria forming membrane-bounded large mass of virus particles (Fig.4). By microinjection, GmDNV was observed in fat cells. No virus-like particles was found in the cells of mid gut. The infected nuclei of fat cells were obviously degraded. The areas of virogenic stroma occurred as patches where the virions were formed (Fig.5). An increase in the number of free ribosomes and the formation of microbody-like structure in infected cells were also observed. The virions arranged in group as masses.

Fig.3. Electron micrographs of Galleria mellonella cells infected with densovirus of G. mellonella.

- a). Mitochondria swollen and degraded.
- b). Virus particles filled in degraded mitochondria and arranged in close association with mitochondria.
- c). Virus particles arranged in paracrystalline array in infected cell.



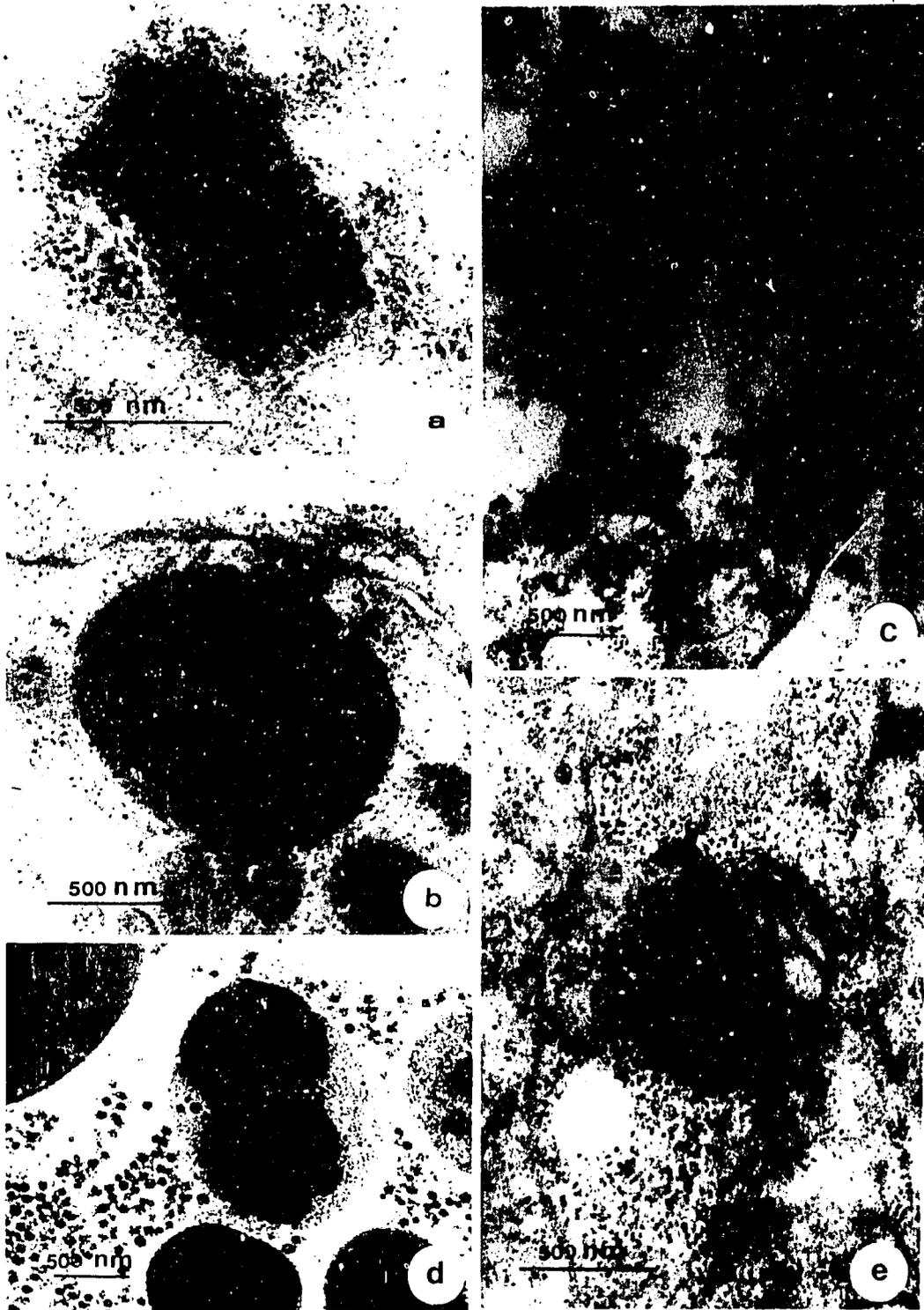
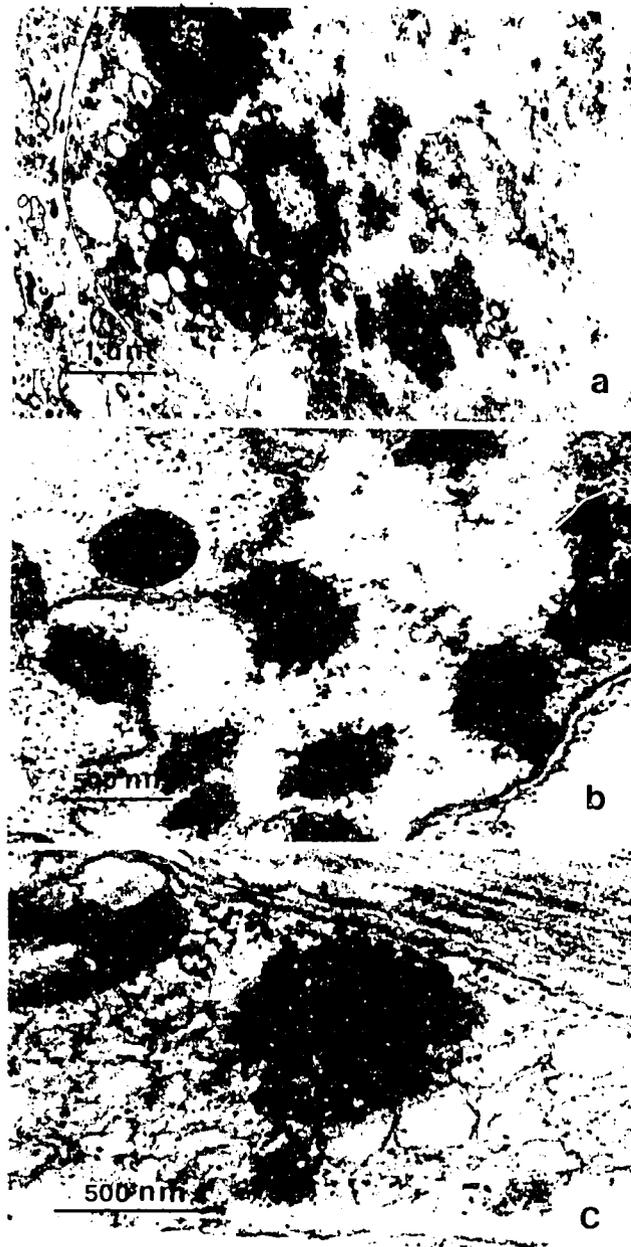


Fig.5. Electron micrographs of Heliothis armigera fat cells infected with densovirus of Galleria mellonella by microinjection.

- a). Electron dense area in infected nuclei where the virus particles are replicated.
- b). Virus particles enclosed in microbody-like structure.
- c). Group of virus particles in infected fat cell.



BEST AVAILABLE DOCUMENT

This study demonstrated that GmDNV can be multiplied in tissues of H. armigera. Host-range mutants are being raised by isolation GmDNV from H. armigera and re-injected into H. armigera. Mutant isolated will be determined its stability, tissue specificity. Immunofluorescent techniques described by Sinha (1964) and Magureanu (1965) are being investigated to rapidly identify the presence of GmDNV in tissue smear of infected H. armigera.

BEST AVAILABLE DOCUMENT

CONCLUSION

Modification of the purification procedure enable us to obtain sufficient amount of densovirus of Galleria mellonella (GmDNV). The purified virus was considered in good quality for serological, infectivity and host-range mutants studies. Antisera were produced successfully in high titer and was subjected to some purification methods to improve the purity. Work has been done rather slow at this stage but many techniques that has never been established before in this laboratory are under extensively investigated and developed. ELISA, immunoperoxidase and immunofluorescent are being developed for the study of virus identification, viral synthesis and viral replication etc.

Results from this study gave strong evidences that GmDNV can replicate in Heliothis armigera. The presence of this virus in H. armigera cells and the cytopathic effects observed in the cells suggested that host-range mutants can be raised successfully. Attempts have been make to determine the stability, specificity and pathogenecity of the mutant isolates.

Eventhough the primary cell culture of H. armigera failed to establish at the first effort, trials will be made again. We also investigate the possibility of obtaining cell line of this insect from elsewhere to accomplish the work plan as was proposed.

BEST AVAILABLE DOCUMENT

WORKPLAN FOR THE NEXT PERIOD

1. Raising GmDNV host-range mutants capable of introducing disease in H. armigera will be continued. Mutants isolated will be examined to determine the domains of their host range (expanded host range or an altered one). Tissue specificity and pathogenicity of the mutant viruses will also be determined.

2. Replication of GmDNV infectious clone (construction by the Israeli group and send to Thailand) in Galleria larvae. Micro and cytopathological studies of this clone will be investigated in comparison to the mutant isolate.

3. ELISA and immunoenzymatic assays will be developed to determine the serological relationship between isolates. These methods will also be utilized as rapid tools for diagnostic of field strains DNV of H. armigera which are still being explored continuously.

4. First attempt to establish insect cell cultures was unsuccessful. Training in Japan by one of our colleagues this year will make possible for the establishment of insect cell cultures in our laboratory. Attempt will probably focus on obtaining cell line from elsewhere instead of commence with primary cell culture. At present studies on viral DNA synthesis and viral replication using immunofluorescence techniques are being developed only in in vivo system.

5. Culture of silkworm, Bombyx mori are readily established in our laboratory. The potential hazard of the DNV of G. mellonella and GmDNV host-range mutants to the silkworm will be determine by both feeding and microinjection experiments.

- Bigazzi, P.E. and Tilton, R.C. 1980. Immunofluorescence and immunoenzyme procedure. In Methods in immunodiagnosis. (N.R. Rose and P.E. Bigazzi, eds.), pp. 161-202. A Wiley Medical Publication. John Wiley and Son. New York.
- Kawase, S. 1985. Pathology associated with densovirus. In Viral insecticides for biological control. (K. Maramorosch and K.E. Sherman, eds.), pp. 197-231. Academic Press. New York.
- Kelly, D.C., Moore, N.F., Spilling C.R., Barwise, A.H and Walker, I.O. 1980. Densonucleosis virus structural proteins. J. Virol. 36(1) : 224-235
- Kurstak, E., Tijssen, P., and Kurstak, C. 1977. Immunoperoxidase techniques in diagnosis virology and research : Principle and application. In Comparative diagnosis of viral disease. (E. Kurstak and C. Kurstak, eds.) Vol. 2, part B, pp. 403-448. Academic Press. New York.
- Kurstak, E. and Tijssen, P. 1981 Animal parvoviruses : Comparative aspects and diagnosis. In Comparative diagnosis of viral diseases. (E. Kurstak and C. Kurstak, eds.) Vol. 3, pp. 3-65 Academic Press. New York.
- Longworth, J.F. 1978. Small isometric viruses of invertebrates. Adv. Virus Res. 23 : 103-157
- Magureanu, E., Husetescu, M. and Grobnicu M. 1965. Histological study of adenovirus type 14 development in cell cultures. J. Hyg. Camb. 63 : 99-104.

- Nakagaki, H. and Kawase, S. 1982. Capsid structure of the densovirus of the silkworm, Bombyx mori. J. Seric. Sci. Jpn. 51(5) : 420-424.
- Siegl, G. 1983. Biology and pathogenicity of autonomous parvoviruses. In The Parvoviruses. (K.I. Berns, ed.) pp. 279-349. Plenum Press. New York.
- Sinha, R.C. and Reddy, D.V.R. 1964. Improved fluorescent smear technique and its application in detecting virus antigens in an insect vector. Virology 24 : 626-634.
- Tijssen, P. and Kurstak, E. 1974. Basic techniques in advanced immunochemistry towards the use of enzymatically active Fab fragments as tracers. In Viral Immunodiagnosis. (E. Kurstak and R. Morisset, eds.), pp. 125-138 Academic Press. New York.
- Tijssen, P., van Den Hurk, J. and Kurstak, E. 1976. Biochemical, biophysical and biological properties of densovirus. I. Structural proteins. J. Virol. 17 (3) : 686-691.
- Tijssen, P. and Kurstak, E. 1981. Biochemical, biophysical and biological properties of densovirus (Parvovirus). III. Common sequence of structural protein. J. Virol. 37 : 17-23.
- Tijssen, P., Su, D.M. and Kurstak, E. 1982. Rapid and sensitive heterologous enzyme immunoassays for densovirus (Parvoviridae). Archives of Virology 74, 277-291.

BEST AVAILABLE DOCUMENT