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PROGRESS REPORT NO.3

DENSOVIRUS AS INSECTICIDE

A RESEARCH PROJECT

U.S.-ISRAEL CDR PROGRAM

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

Submitted by

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Thailand

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PROJECT PROFILE

Country	:	Thailand
Grant No.	:	936-5544-00-G-7016-00
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Project Title	:	Densovirus as Insecticide
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U.S.- ISRAEL CDR PROGRAM

DENSOVIRUS AS INSECTICIDE

THAI GROUP

CONTENTS

1. Introductions..... 1

2. Objectives..... 2

3. Materials and Methods..... 3

4. Results and Discussion..... 8

5. Conclusion.....26

6. Workplan for the Next Period.....27

7. References.....28

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DENSOVIRUS AS INSECTICIDE

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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 to develop as microbial insecticides. Densonucleosis 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 r 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.

OBJECTIVES

The present study is aiming at evaluating the potential of Densoviruses (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 August, 1988 to March, 1989. The specific objectives during this report period include (a) the development of the immunofluorescent technique for the detection and study of the viral proteins synthesis in *Galleria mellonella* and *Heliothis armigera*; (b) the development of insect cell culture for Densovirus production and determination of the insecticidal activity of the DNV host-range mutants; (c) raising GmDNV host-range mutants capable of introducing disease in *Heliothis armigera* and (d) determination the potential hazard of the DNV of *G. mellonella* and GmDNV host-range mutants to silkworm.

MATERIALS AND METHODS

In Progress Report No.2 we indicated to use *Galleria mellonella* Densovirus (GmDNV) as virus source for the experiments we proposed because the Densovirus field strains of *Heliothis* or *Spodoptera* have not been found. The GmDNV will be used to raise the host-range mutant strains capable of introducing disease in *H. armigera* and *S. exigua*. Once the mutants are successfully developed, they will be evaluated for their insecticidal potential.

1. Detection of the DNV by an indirect immunofluorescent technique.

In raising the *Galleria mellonella* DNV host-range mutant, the virus was fed and injected into the larvae of *Heliothis armigera*. To ensure the capability of GmDNV to replicate and cause infection in *H. armigera*, tissues of treated larvae were examined for the presence of the virus by electron microscopic technique. However, this technique is laborious and time consuming. The fluorescent antibody technique is developed for rapid detection of the DNV in *H. armigera* tissues.

Antisera against Densovirus of *G. mellonella* were prepared as described in Progress Report No.2. 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 first injection and one week after the fourth injection). The above-mentioned immune sera will be used in immuno-fluorescent test.

After 5 days of inoculation with GmDNV (injection or feeding), the larvae of *H. armigera* and *G. mellonella* were randomly collected and examined for the localization of the virus. Pieces of tissue and haemolymph of both insect species were smeared in very thin layer on the steriled slides and placed in steriled petri dishes. To fix the samples, the slides were placed in a glass slide bath containing chilled acetone and left at 4°C for at least 10 min. Then soaked slides in PBS^{-} for 30 min in 37°C moist incubator. The immune serum (antibody) was prepared in 1:10, 1:50 and 1:100 dilution in PBS^{-}. After air dried, the samples were covered with 100 μl of each anti-serum dilution and placed in 37°C moist incubator for 30 min. The slides were then washed gently in four consecutive baths at room temperature, two in PBS^{-} followed by two in double distilled water and air dried. A 100 μl of 1:16 dilution of goat anti-rabbit FITC in PBS^{-} was added to each sample and left in 37°C moist incubator for 30 min. After the slides were washed and dried as above, then they were mounted with 50 % of PBS^{-} - 50 % glycerol and sealed with nail polish. Observations were made under dark field illumination by Olympus Vanox fluorescence microscope equipped with UV lamp.

2. Insect cell culture

For virus production and determination of the insecticidal activity of the GmDNV host-range mutant in *in vitro* system, insect cell culture was developed in our laboratory. Insect ovary from pupa, insect fat tissue from larva, embryo from fertilized eggs and hemocytes of *H. armigera*, *S. exigua* and *Bombyx mori* were used to establish the primary cell lines. The

pupae, larvae and fertilized eggs were surface sterilized with 70 % ethyl alcohol. Insect ovaries were removed from the pupae and only the terminal filament parts of the ovaries were used. Insect larvae were dissected under the stereomicroscope and small pieces of fat tissue were collected. Embryos were obtained from fertilized eggs after removal the egg shell in Tyrode's solution. All these specimens were then treated as follow : washed with Hank's balanced salt solution, removed the salt solution, added medium, removed the medium and added fresh medium. The specimen were cut with small needle into small pieces. The tissues and the medium were then transfered into culture flasks and incubated at 27°C in the dark. After one week, the medium was replaced with the new fresh medium.

For hemocyte culture, hemolymph was obtained from 70 % ethanol surface sterilized larvae. First pair of the proleg of the larvae were cut and drops of hemolymph were collected in centrifuge tubes cooled in ice bath. The hemolymph was then subjected to low speed centrifugation at 200xg for 3-5 min. The pellet was resuspended in salt solution and subjected to another cycle of centrifugation as above. Fresh medium was added into the tube, the cells and medium were then transfered into culture flasks which were incubated at 27°C in dark place.

The medium used for insect cell culture in our laboratory was Grace's medium (Grace, 1962) supplemented with 10 % heat-inactivated (30 min. at 56°C) fetal bovine serum and the antibiotic (Gentamycin 50 μg/ml). The methods used to establish primary cell lines were those modified from Jones and Cunningham (1961) and Hink and Ignoffo (1970).

3. Raising GmDNV host range mutants

In Progress Report No.2 we demonstrated the effect of GmDNV on the development of *H. armigera*. There was also strong evidences on the presence of Densovirus in *H. armigera* tissues when the larvae were fed or microinjected with GmDNV. However, attempt to isolate GmDNV from *H. armigera* was unsuccessful. Therefore during this report period, several trials on raising GmDNV host-range mutant were performed. Purified GmDNV suspension of 1, 5 and 10 μg in 0.1 x SSC buffer were microinjected into the proleg of third instar bollworm larvae. The tested larvae were reared individually on the artificial diet. Dead larvae were examined by immunofluorescent test as described above for rapid detection of the virus in insect cells. After death, the larvae were collected and homogenized in 0.1 x SSC buffer. The suspension was then filtered through several layer of cheesecloth and finally through membrane filter of 0.45 μm pore size. The virus, if present, in this extract will be considered a possible mutant isolate of GmDNV. In order to determine the domains of this mutant's host-range (an expanded host-range or an altered one), the extract will be further purified and used to inject new larvae according to the above-mentioned procedure.

We also tried to raise the GmDNV mutant isolate by per oral inoculation. The bollworm larvae were fed with the diet contaminated with 1 , 5 and 10 μg of purified GmDNV suspension. Dead larvae were examined and subjected to the same following procedures as described above in microinjection experiment.

4.Potential hazard of the DNV to silkworm

Effects of DNV on mulburry silkworm and eri silkworm were evaluated by bioassay study. GmDNV was purified according to procedure reported in Progress Report No.2. Purified GmDNV in 0.1 x SSC buffer solution (1 x SSC = 0.15 M Sodium citrate) was prepared in five concentrations of 0.001, 0.01, 0.1, 1.0 and 10.0 μg/50 μl. Clean mulburry and castor leavas were cut into small pieces of 1 x 1 inch, 50 μl of each virus concentration was applied on the leaf. Every larvae instar of mulberry silkworm and eri silkworm were tested. The larvae were allowed to feed on these contaminated leaves. Twenty larvae were used for each treatment which consisted of three replications. Fresh leaves were given to the larvae when all of the contaminated leaves were consumed. The 0.1 x SSC buffer was used in the control units. Mortality of the larvae was observed and number of dead larvae was recorded daily until all larvae died or became pupae.

RESULT AND DISCUSSION

1. Detection of the Densovirus in insects by an immunofluorescent technique.

An attempt to use Densovirus in controlling the economic important insect, the cotton bollworm or the beet armyworm is the main objective of this project. Densovirus of *G. mellonella* has been used to cause infection in these two insect species. Since the fluorescein isothiocyanate is the derivatives of fluorescein that has been successfully employed as protein labels, immunofluorescent technique was developed to provide a rapid and precise procedure for diagnostic of virus infection. The capability of GmDNV to replicate and cause cytopathic in *H. armigera* is demonstrated by immunofluorescent technique.

The blood and tissue smears of *G. mellonella* infected with Densovirus exhibited fluorescence. The cells in smears were in the form of brilliant yellow green masses. The brightness of the cells was more pronounced in blood smears than in tissue smears. However, in tissue smears the fluorescent cells were more numerous and in many of them the formation of a fluorescent ring could be observed (Fig.1). In review paper of Kurstak (1972) on small DNA Densonucleosis virus, two patterns for the localization of DNV in infected insect cells were mentioned. The first pattern appears around 4 and 7 hours after DNV infection; it is exclusively cytoplasmic and corresponds to the production of early antigens. The second pattern is intranuclear, corresponding to the production of structural antigens of the DNV and starts close to the nuclear membrane in the form of a fluorescent halo.

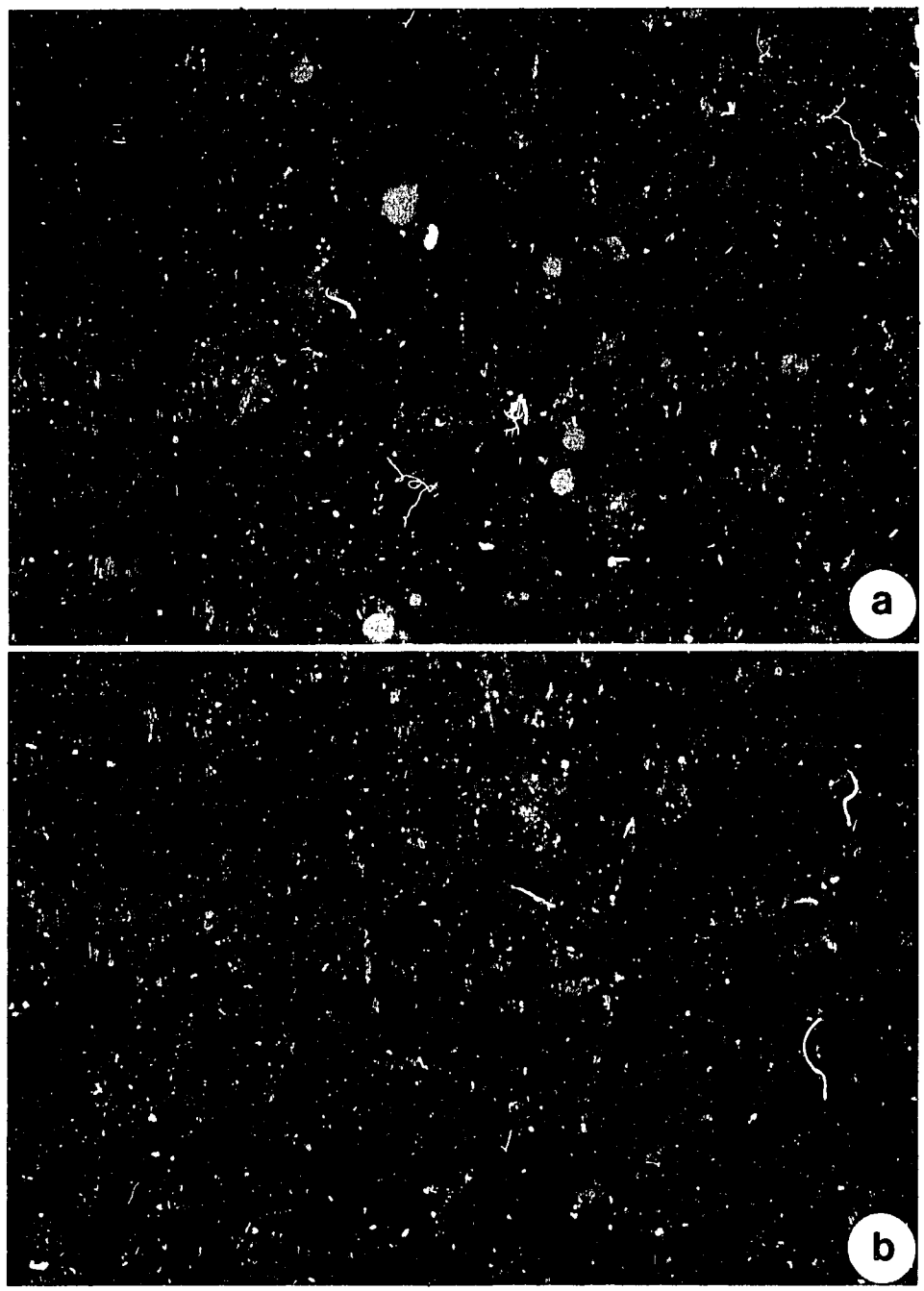
The time of appearance of the second pattern varies according to the virus titer, the temperature, and the type of infected cells. The intranuclear localization of antigens gradually embodies the whole nuclear surface, then extends into the cytoplasm. In the later stage of infection, the whole cell may exhibits fluorescence. We observed the blood and tissue smears at the very late stage of infection, therefore the cells appeared as compact fluorescent masses. The fluorescent material filled the whole area of the nucleus which became hypertrophic.

In blood and tissue smears of *H. armigera* infected with GmDNV, the fluorescence not as prominent as those derived from *G. mellonella*. This is not surprising since *G. mellonella* is the virus's original host. In this study we observed fluorescent granules of various sizes in the nuclei of some cells (Fig.2). We can not draw a conclusion at this stage that GmDNV can replicate in cells of *H. armigera*. However, the difference between the uninfected controls and the infected smears was quite definite. This gave us a resonable evidence that the fluorescent materials are the protein of the virus which was stained specifically. It is possible that GmDNV is present or even replicates in the cells of *H. armigera* but not in sufficient amount to be observed as a fluorescent mass. Of special interest is the finding that fluorescence was observed only in the cells of *H. armigera* infected with GmDNV by injection. Negative results were obtained in feeding experiments. This study indicated that the presence of GmDNV in *H. armigera* can be rapidly detected by immunofluorescent technique hence facilitate the study of raising GmDNV host range mutant. Paraffin sections

Fig.1. The haemolymph and tissue smears of *Galleria mellonella* infected with Densonucleosis virus exhibited fluorescence when stained specifically by immunofluorescent technique.

a). The hemocytes were stained specifically as brilliant yellow green masses. (x 200)

b). The formations of a fluorescent halo were observed prominently in tissue smears. (x 200)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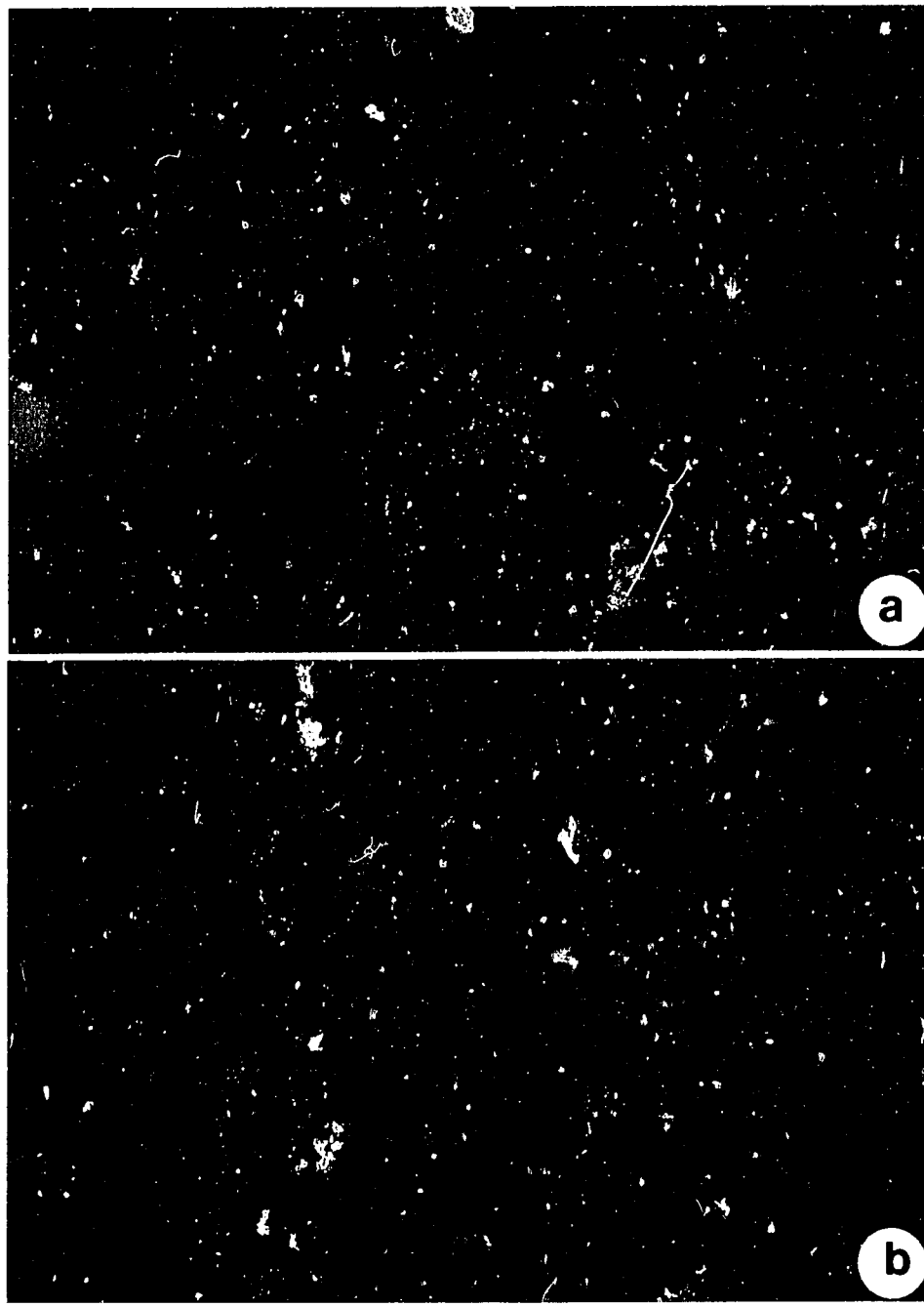
12

Fig.2. The haemolymph and tissue smears of *Heliothis armigera* infected with Densovirus of *Galleria mellonella* showing different size of small, yellow green and red fluorescent granules.

a). The haemolymph smear. (x 200)

b). The tissues smear. (x 200)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of the tissue of *G. mellonella*, *H. armigera* and *S. exigua* infected with GmDNV are being investigated by immunofluorescent technique to determine the cytopathic effect of GmDNV on these three insect species.

We, however, did not exclude the possibility of other type of fluorescence that can be observed under the microscope, for example the nonspecific staining or the autofluorescence. Bigazzi and Tilton (1980) suggested that different kinds of immunofluorescent reactions can be discriminated by the use of the proper controls. Autofluorescence is identified in substrates incubated with phosphate-buffered saline alone, whereas non-specific staining may be observed in those treated with phosphate-buffered saline and conjugated alone.Differentiation of different types of fluorescence in our specimens is under investigation in order to confirm the positive results of our experiments.

2. Insect cell culture

Early insect cell culture media consisted of balanced salt solutions supplemented with 5-50 % v/v insect haemolymph to provide factors for growth. Melanization was often a problem. Modern tissue culture media, Grace's medium supplemented with 10 % heat activated fetal bovine serum was used in our laboratory to established *H.armigera*, *S. exigua* and *B. mori* cell culture (Fig.3). Antibiotics are not normally used for routine passage but can be added to experimental cultures as required. Penicillin, streptomycin and the antimycotic fungizone are used by many investigators. However, we used antibiotic gentamycin of

15

Fig. 3. Culture flask contained Grace's medium for insect tissue culture.

a). Cultures of cells derived from fat tissue explant of *H. armigera* larvae.

b). Cultures of cells derived from embryos of fertilized eggs of *B. mori*.

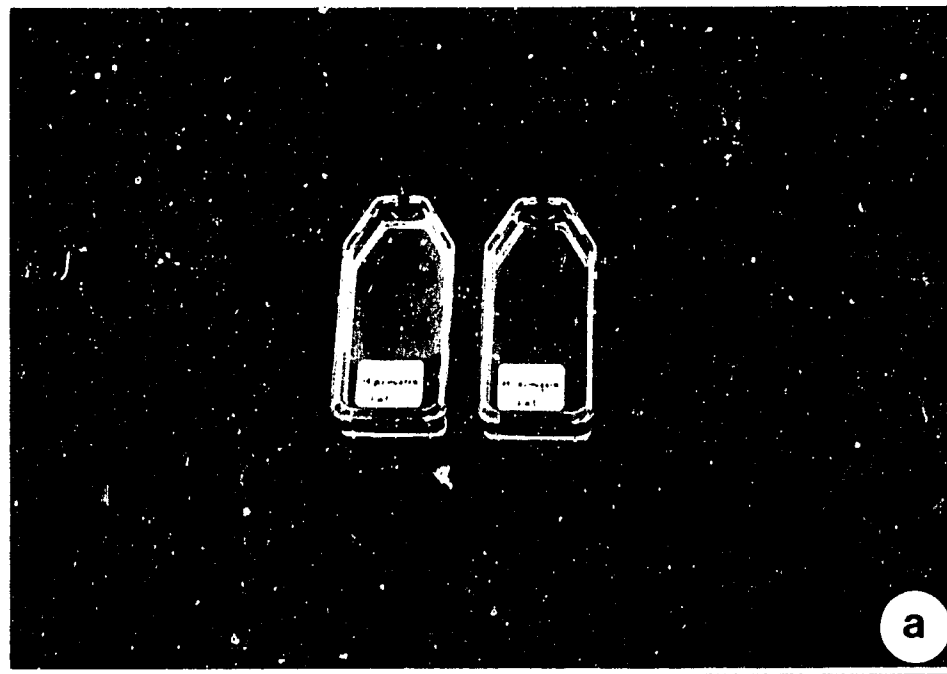
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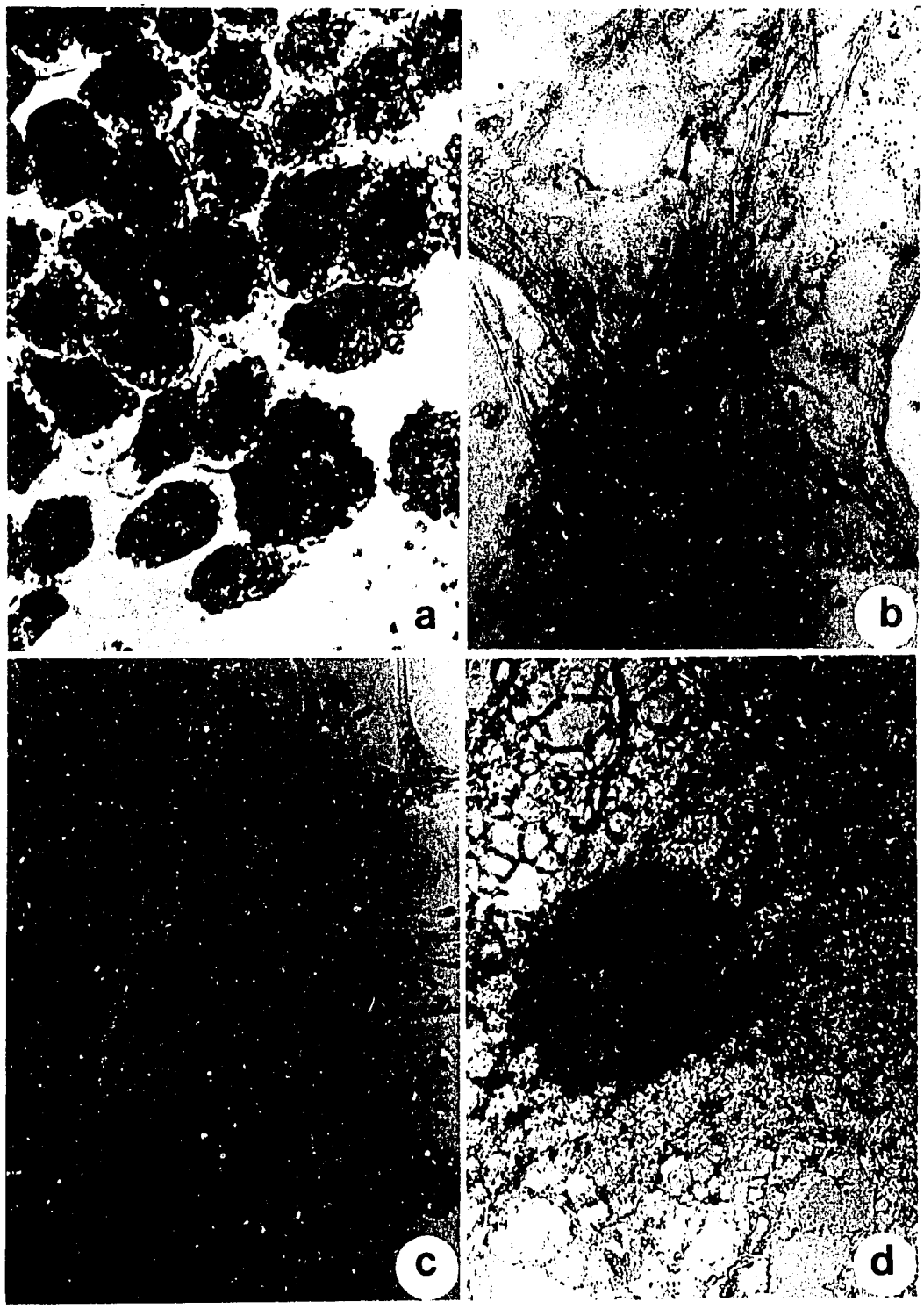
Fig.4. Cultural characteristic of cells derived from two types of tissue isolated from *Heliothis armigera* and *Bombyx mori*

a). Cells derived from fat tissue explant of *H.armigera*. Cells grew in cluster and exhibited normal characteristic of spherical in shape. (x 435)

b). Cells derived from embryos of fertilized eggs of *B. mori*. Cells in fibroblast-like appearance were observed (arrow). (x 218)

c). Cells derived from embryos of fertilized eggs of *B. mori* showing filamentous network appearance of cell growth. (x 218)

d). Relatively large and vacuolated cells derived from the embryos of fertilized eggs of *B. mori*.(x 218)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50 μg/ml which gave us satisfactory result in avoiding contamination in our insect cell culture.

One month after culturing according to the procedure described in Materials and Methods, the fat cells from fat tissue explant of *H. armigera* divided and formed a cluster of cells. Cells exhibited a normal characteristic of spherical in shape. (Fig.4a) Some of these cells were attached to the culture flasks and seemed to start the monolayer formation while many cells were unable to attach. In *B. mori* cultures, cell growth was observed only in the cultures derived from embryo from fertilized eggs of the insect. The migration of cells began in one week after culturing. The cells were proliferated and attached to the culture flask. There were many characteristics of cell growth observed in the cultures. Some cells were fibroblastic (Fig.4b) and some were in filamentous networks (Fig.4c). Most of the cells were in large size and vacuolated (Fig.4 d).

At this stage we only succeeded in culturing fat cells of *H. armigera* and embryo of *B. mori*. Attempt to establish cell culture from other kinds of tissue explant for example ovary or hemocytes were unsuccessful. The cultivation of insect cells in artificial medium always take long period of growth. Many trials have been made to cultivate the cells and establish the insect cell line in our laboratory. However, in some trials, the cells grew normally for a certain period of time and then the cell growth was slow down and stopped. In other trials the cells are continuing to proliferate. Melanization is still one of the problems that has to be solved. Goodwin (1975) isolated several cell lines from the pupae of the Lepidoptera and suggested the

improvement of primary culture survival by controlling the total tissue volume per unit medium volume, and by the addition of glutathione to prevent melanization and to improve cell adherence to the substrate. Primary culture tissue disruption can be improved by substituting collagenase for trypsin. Five cell lines from pupal ovaries of 2 species of *Heliothis* were established using a mammalian cell culture medium, TC-199 MK (McIntosh and Ignoffo, 1983). More recently Inoue and Mitsuhashi (1988) succeeded on establishment of a continuous cell line obtained from embryos of silkworm, *Bombyx mori* using MGM-448 medium with 10 % fetal bovine serum (FBS) as well as in MM medium with 3 % FBS. Although we are able to culture certain types of insect cell, the cultural characteristic were unsatisfactory. The medium composition and the cultural technique suggested in previous reports will be taken into consideration and practiced to achieve the establishment of insect cell line in our laboratory. Pathology of GmDNV in *in vitro* system is under investigation.

3. Raising GmDNV host-range mutant

Several trials on raising GmDNV host-range mutant that have been done during this report period were the continuous and repeating work in order to obtain the mutant isolate capable of producing disease in *H. armigera*. All observations have confirmed the result reported in Progress Report No.2. that are : 1) GmDNV has some effects on the development of *H. armigera*. When the cotton bollworm larvae were injected and fed with GmDNV, they either died,unable to moult or pupate or produced nonviable eggs.

Garzon and Kurstak (1976) reported earlier that infection of the key organs of *G. mellonella* by the DNV inhibited the moulting and the metamorphosis of the larvae. The similarity of the results may suggest the possible infection of GmDNV in *H. armigera*. GmDNV of higher concentration produced more effect on *H.armigera* larval development than the lower concentration. The effect was more intense on the following generation of the treated larvae when they received the similar treatments. The result suggested that repeated application of virus to successive generations of insect might be able to induce infection in that insect population. 2). GmDNV are present in *H. armigera* tissue when this virus was administered into this insect species. Results from immunofluorescent study demonstrated some fluorescent materials in the nuclei of *H. armigera* cells injected with GmDNV (Result and Discussion 2). Although it is not obvious that this virus can replicate in the cells of *H. armigera*,those fluorescent materials were believed to be viral protein that stained specifically. This suggested the possible existing of GmDNV in *H. armigera* cells.

The most reliable evidence was the electron microscopic observation of ultrathin sections of the infected bollworm tissues. In Progress Report No.2, we reported the presence of the GmDNV particles arranged in cluster or paracrystalline array in *H. armigera* cells. This report confirmed the previous study that GmDNV was found in *H. armigera* tissues. Although not frequently, a considerable number of virus particles were found at one site of observation. They arranged randomly in large mass (Fig.5a) or in crystalline array (Fig.5c). Groups of virus particle that

22

Fig.5. Electron micrographs of *Heliothis armigera* cells infected with Densovirus of *Galleria mellonella*.

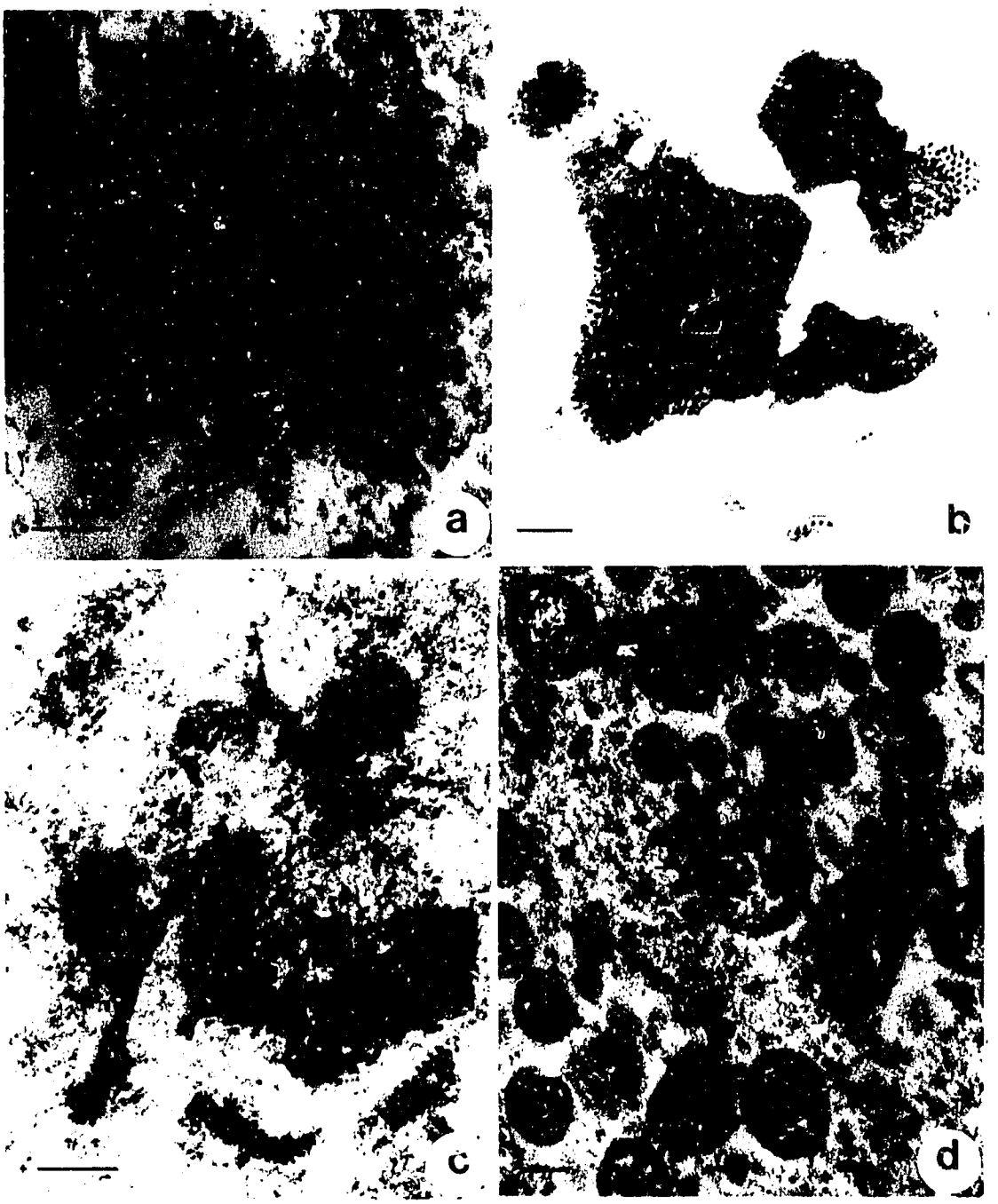
a). The virus particles aggregated randomly in large mass.

b). Groups of particle were confined in membrane-like boundary.

c). The virus particles arranged in paracrystalline array (arrow).

d). Microbody -like structures (arrow) containing virus particles in infected cells.

Bar = 200 nm.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were confined in membrane-like boundary were also observed (Fig.5b). The virions appeared in close association with mitochondria and finally they might filled the mitochondria. The cytopathic change frequently observed in infected cell was the microbody like structure contained numerous virus particles Fig.5d). It is difficult to interpret this cytopathic change occurred in infected cells. The virions may be confined in the microbody and be destroyed later as one of the defense mechanism of host cells to viral infection. Microbody like structure contained no virions can be observed in healthy cells. However, microbodies of different sizes and with or without virions inside were found much more frequently in infected cells.

With a combination of electron microscopic observations and immunofluorescent studies, it was beleived that GmDNV was present and probably could be replicated in *H. armigera* tissues.

4. Potential hazard of GmDNV to silkworm.

The results obtained from this experiment can not be analysed. We faced the problem in trying to inject the mulberry and eri silkworm with GmDNV. In oral inoculation, the virus was applied on the leaves which were cut into small pieces. The leaves dried quickly and the silkworm refused to feed on. Due to problem of handling young larvae in microinjection tests, many of them died. In both experiments, approximately 10 percent of the control units were died, therefore the results are not reliable and can not make any conclusion. We are trying to modify the method of inoculation and handle the tested insect properly.

From the experiments we did not see any obvious symptom of DNV infection in both mulberry and eri silkworm. The host range of *Galleria* DNV is restricted to *G. mellonella* (Kawase, 1985). It is believed that as long as this virus was transferred via the normal route of infection, this specificity is maintained. However, in our study (raising GmDNV host-range mutant) we found that GmDNV was present and possible replicated in *H. armigera*. We must find out whether this phenomenon could occur in silkworm, since it is the economic important insect in Thailand. The experiments to determine the potential hazard of GmDNV to silkworm are in progress.

CONCLUSION

Immunofluorescent technique has been developed for rapid detection of Densonucleosis virus of *Galleria mellonella* (GmDNV) in *Heliothis armigera* cells. Although we can not draw a conclusion at this stage that GmDNV can replicate in cells of *H.armigera*, we observed fluorescent granules of various sizes in the nuclei of hemocytes and fat cells from tissue smears of *H. armigera*. Electron microscopic observations of ultrathin sections of the bollworm tissues revealed the presence of GmDNV in both cytoplasm and nuclei of infected cells. The virus particles arranged in patterns characteristic of Densovirus normally infected in its original host. Successful in isolating GmDNV from *H.armigera* tissues can be the solution to convince the capability of GmDNV in producing disease in *H. armigera*.

Primary cell culture derived from fat tissue explant of *H. armigera* and embryos from the fertilized eggs of *B. mori* were cultivated in our laboratory.Although the cells divided and grew, the cultural characteristic was not in satisfactory condition. The medium composition and the culturing technique will be modified and improved. The pathology of GmDNV in *in vitro* system is under investigation.

Research work concentrated on raising GmDNV host-range mutant are underway. We expected to be able to isolate this mutant and determine its potential in controlling the cotton bollworm and the beet armyworm.

WORK PLAN FOR THE NEXT PERIOD

Most of the work reported herein have not been completed, they will be continued to the next period. The workplan for the next report period can be summarized as follows :

1. Comparative study will be performed to determine the insecticidal activity of the GmDNV mutant isolate (if successfully raise) against *H. armigera*, *S. exigua* and its original host, *G. mellonella*. The comparative study includes the bioassay study, the histo and cytopathology study.

2. Biological properties of the GmDNV mutant isolate will be determined in comparison to the original isolate. The properties studied include : infectivity, host range, and viral transmission.

3. Tissue or cell culture derived from insect ovary, hemocytes and embryo from fertilized eggs will be investigated continuously to determine the suitable cell for GmDNV replication. This *in vitro* system once established will be used to facilitate many parts of the work proposed in this project.

4. Serological study will be explored to determine the best technique for rapid detection of the Densovirus replication in both *in vivo* and *in vitro* system. We will also determine and compare the serological characteristic of the GmDNV mutant isolate and the original isolate. The immunofluorescent technique has already been developed in our laboratory. We are aiming at developing the ELISA technique, the immunoperoxidase and the immunoelectron microscopic technique in our laboratory.

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