originally allopatric to each other in Palaearctic region (IWATA and NAKANE, 1988), it is very likely that these 2 species have the same physiological characteristics in that both require chilling for pupation.

From this viewpoint, the distribution record of *L. sinensis* in the southernmost part of China (Yúnnán and Guǎngxī) (CHÉN and HUÁNG, 1985) seems to require further taxonomic, climatic and physiological investigations.

All the lyctid species are very easily introduced by humans to new territories, easily establishing themselves insofar as there are no physiological restrictions. This is the case with *L. sinensis*. The physiology described here, however, seems one of the most important factors for the determination of species distribution and spread range. Invasion and establishment of *L. sinensis* in Great Britain (PARKIN, 1932; ALLEN, 1969) are, thus, quite logical, while introduction into subtropical or tropical regions does not seem to lead to this species' establishment.

In conclusion, *L. sinensis* larvae require at least 6 weeks of chilling by 5°C for pupation and subsequent adult emergence.

REFERENCES

- ALLEN, A. A. (1969) *Entomol. Mon. Mag.* **105**: 163.
 CHÉN, Q.-z. and J.-g. HUÁNG (1985) *Illustrated Book of Storehouse Insects*. Science Publisher, Peking, p. 74 (in Chinese).
 CYMOREK, S. (1979) *Document IRG/WP* (193) 1–15.
 IWATA, R. (1981) *Konchû to Shizen (Nature and Insects)* **16**(9): 16–20 (in Japanese).
 IWATA, R. (1988a) *Acta Coleopterol. Japon.* (1): 1–133.
 IWATA, R. (1988b) *Kaoku Gaichû (House and Household Insect Pests)* (35/36): 45–54 (in Japanese).
 IWATA, R. and T. NAKANE (1988) *Konchû to Shizen (Nature and Insects)* **23**(1): 16 (in Japanese).
 KÜHNE, H. (1981) *Mater. Org.* **16**: 141–156.
 PARKIN, E. A. (1932) *Entomol. Mon. Mag.* **68**: 277.

Nuclear Polyhedrosis Virus Infection in a Highly Susceptible Cell Line of *Bombyx mori* (Lepidoptera: Bombycidae) Detected by Enzyme-Linked Immunosorbent Assay¹

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(Received December 23, 1988)

There are a number of cell lines that are susceptible to a nuclear polyhedrosis virus of *Bombyx mori* (BmNPV), but with the exception of *Bombyx* cell lines, BM-N (MAEDA, 1984) and Bm36 (TSUDA, 1985), these lines are not applied to form virus plaques. We have selected a substrate-depending cell line from SES-BoMo-15A (INOUE and MITSUHASHI, 1984, 1988) that is suitable for BmNPV plaque formation. The replication of BmNPV in

this highly susceptible cell line has been investigated by enzyme-linked immunosorbent assay (ELISA).

MATERIALS AND METHODS

Cell line: The cell line, SES-BoMo-15AII (INOUE et al., unpublished), was treated with pancreatin (WEISS, 1982) and subcultured by pipeting. Cells of the 99th subculture were used in the tests.

Virus inoculation: The BmNPV inoculum was obtained from a virus-infected culture medium and had a titer of 1.0×10^6 PFU/ml. Cell cultures of 2.4×10^5 cells/ml were seeded in 50 ml culture flasks (#3013, Falcon Co.) and were maintained at 25°C for 48 hr, at which time a layer of attached cells formed in the flask. Thereafter, the medium (MGM-448 containing 10% fetal bovine serum) was removed and replaced with a 0.5 ml of a ten-fold dilution of BmNPV inoculum. The flasks were rocked several times every 15 min for 1 hr, next the virus suspension was removed by rinsing the cells with CARLSON's solution, and then 5ml of new medium was added to each culture. The cultures were maintained at 25°C. Periodically, the culture medium from one flask was collected,

¹ Appl. Ent. Zool. **24** (4): 480–482 (1989)

centrifuged at 3,500 rpm for 15 min, and the supernatant was tested with indirect ELISA. The cells remaining in the flasks were harvested by treating them with pancreatin and washed three times by centrifugation at 1,000 rpm for 1 min with Carlson's solution, and then smeared on glass slides.

Indirect ELISA: We followed mainly the procedure of KAWARABATA and HAYASAKA (1987). The peroxidase-labeled goat immunoglobulin (IgG) to anti-rabbit IgG was purchased from BIO-RAD Co. (Cat. No. 170-6515) and the assay was performed in a 96-well flat-bottom microtitre plate (Immuno Plate I, Nunc Co.). Each well received 100 μ l of medium collected either from virus-inoculated or non-infected cultures, and the plate was incubated for 1 hr at room temperatures (about 25°C) followed by 4°C overnight. The plate was washed three times, each for 5 min, with phosphate-buffered saline (PBS) containing 0.02% Tween 20 (PBST) and then PBST containing 2% bovine serum albumin was added for 1 hr in order to block the binding of nonspecific antibody on the plastic wells. The blocking solution (PBST-BSA) was removed and 100 μ l of anti-BmNPV serum, at a dilution of 1:10,000 with PBST (TANAI and INOUE, 1988), was added to each well and the plate was held at 37°C for 2 hr. The primary antibody was removed by suction, and the wells were washed three times each for 5 min with PBST, and then each well received 100 μ l of peroxidase-labeled goat anti-rabbit IgG diluted at 1:3,000 with PBST. The plate was incubated at 37°C for 2 hr before the second antibody was removed by suction, and the wells were again washed three times each for 5 min with PBST. Each well received 100 μ l of 2,2-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] (peroxidase substrate kit, BIO-RAD Co.) and the plate was left for 20 min at room temperatures. The reaction was stopped with the addition of 100 μ l of 2% oxalic acid and the reaction color was read by absorbance at 414 nm with EIA Reader (Model 2550, BIO-RAD Co.).

Cell staining: Smear cells were fixed with acetone for 10 min, then received 2% bovine serum albumin, and kept for 30 min at room temperatures before the fluid was aspirated in order to prevent non-specific reactions. The primary antibody at 1,000 fold dilution was added to the slides to react with the antigens for 45 min at 37°C, then the slides were rinsed with PBS three times each for 5 min and followed with the addition of 1,000 fold

Fig. 1. BmNPV antigen in the culture medium of SES-BoMo-15AII cells detected by indirect ELISA. Non-specific control data were deducted from these absorbances. Histogram represents the percentage of infected cells.

dilution of the second antibody. After incubation, the slides were rinsed as described above with PBS. Firstly, diaminobenzidine-4HCl of 0.04% concentration was added to the slides, followed by 0.03% hydrogen peroxide in 0.05 M Tris-HCl buffer. After 10 min, the slides were rinsed with PBS and mounted with cover slips.

RESULTS AND DISCUSSION

1. Detection of BmNPV antigen in culture medium

A slight, positive reaction of a blue-green color caused by 2,2-azino-di-[3-ethylbenzthiazoline-6-sulfonic acid] was observed in non-infected control medium. The absorbances of the control medium at 414 nm were 0.098, 0.099, 0.116, and 0.125 at 12 hr, 24 hr, 48 hr and 96 hr, respectively. A much stronger reaction was observed in the virus-infected medium. The absorbance data, from which the non-specific control data had been deducted, are shown in Fig. 1. The increase in virus antigen in the culture medium was very gradual during 12 to 48 hr, and after 48 hr, it increased markedly.

2. Detection of BmNPV antigen in cells

Cells of the non-infected control were not colored as in the case of the virus-infected cells which were detected easily because of the brown color of DAB reaction (Fig. 2). The number of infected cells in the samples of 12, 24 and 48 hr post virus inoculation (p.i.) was counted at random, and the percentages of infected cells in the samples were 20.8%, 50.8% and 91.3%, respectively (Fig. 1). Very small polyhedra were observed in the nuclei at 24 hr p.i. and mature-sized polyhedra at 48 hr p.i. (Fig. 2 c, d). The cytolysis of the infected cells

Fig. 2. SES-BoMo-15AII cells stained by the indirect peroxidase method. a: control, cells unstained, b: 12 hr p.i., infected cells with the brown color of DAB reaction, c: 24 hr p.i., small polyhedra present in nucleus, d: 48 hr p.i., mature-sized polyhedra present in nuclei. Bar shows 20 μ m.

were first observed in cultures at 48 hr p.i.

These results of increase in virus antigen in the culture media, when compared with the percentage of virus-infected cells, suggest that the increase in virus antigen during 12 to 48 hr is due to viruses budded from cells, and after 48 hr, the increase is mainly due to viruses from cell lysis.

MCCARTHY and HENCHAL (1983) had reported that antiserum against the nucleocapsids obtained from occluded viruses by detergent (Nonidet-P40) treatment exhibited a strong reaction not only with non-occluded viruses in the tissue culture medium but also with occluded viruses. VOLKMAN (1983) showed that the budded and occluded phenotypes of *Autographa californica* NPV had immunologically cross-reacting structural proteins. The antiserum that we had used in the present study was against the purified occluded virus, but it reacted also with the non-occluded virus in the hemolymph of diseased larvae as indicated in the Western blot test (TANAI and INOUE, 1988). We conclude that antigens detected in the present study are involved with occluded and non-occluded viruses.

ACKNOWLEDGEMENT

We thank to Dr. Y. TANADA, Professor Emeritus of the University of California, for his valuable suggestions and discussion.

REFERENCES

- INOUE, H. and J. MITSUHASHI (1984) *J. Seric. Sci. Jpn.* **53**: 108–113.
- INOUE, H. and J. MITSUHASHI (1988) *Appl. Ent. Zool.* **23**: 8–14.
- KAWARABATA, T. and S. HAYASAKA (1987) *J. Invertebr. Pathol.* **50**: 118–123.
- MAEDA, S. (1984) *J. Seric. Sci. Jpn.* **53**: 547–548.
- MCCARTHY, W. J. and L. S. HENCHAL (1983) *J. Invertebr. Pathol.* **41**: 401–404.
- TANAI, K. and H. INOUE (1988) *J. Seric. Sci. Jpn.* **57**: 265–269.
- TSUDA, K. (1985) Doctoral thesis in Univ. of Kyushu.
- VOLKMAN, L. E. (1983) *J. Virol.* **46**: 221–229.
- WEISS, S. A., D. PELOW, S. S. KALTER, and J. L. VAUGHN (1982) *In Vitro* **18**: 298.