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Comparative Replication of *Lymantria dispar* Nuclear Polyhedrosis Virus Strains in Three Continuous-Culture Cell Lines

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We compared the replication of the gypsy moth (*Lymantria dispar*) nuclear polyhedrosis virus in two new cell lines, from embryos and fat body of *L. dispar*, and in a previously available ovarian cell line. Three virus isolates (the Hamden strain [LDP-67] used commercially as GYPCHEK, a plaque-purified clone of Hamden [5-7d], and an isolate from Abington, Mass. [Ab]) were each tested on the three cell lines. The fat-body-derived cell line proved best in terms of occlusion body production for all three virus strains, with the highest yield produced by the Abington strain. On the basis of these results, we conclude that a more efficient in vitro production of gypsy moth virus can be obtained by using the fat body cell line in conjunction with the Abington strain of the virus.

The gypsy moth, *Lymantria dispar*, is the most destructive pest of hardwoods in the northeastern United States. Continuing efforts to develop a means for controlling this insect has centered on a naturally occurring baculovirus. This virus, a multiple-embedded nuclear polyhedrosis virus (MNPV), has been shown to be effective in reducing gypsy moth populations. Challenges to be met with the *L. dispar* MNPV (LdMNPV) include improving its pathogenicity and the production methodology. Recent analysis of viral isolates from different geographic areas (9) has revealed a number of virus strains which are more pathogenic than the strain currently registered by the U.S. Environmental Protection Agency as GYPCHEK. This registered strain, also known as LDP-67 and Hamden, was originally isolated from a gypsy moth population in the Hamden, Conn., area, whereas a strain from Abington, Mass., was more efficacious in bioassays against laboratory-reared gypsy moth larvae.

Production of GYPCHEK involved the large-scale rearing of gypsy moth. This process is labor intensive and requires careful management of the insect colony to ensure optimal production of virus. The development by Goodwin et al. (4, 5) of continuous cell cultures capable of replicating LdMNPV provided a means for in vitro production of the virus. This technique would allow more rigorous control over virus production and could be easily automated. However, the quantities of virus produced in the cell lines described by Goodwin et al. (4, 5) were too small to be economically competitive with in vivo production. Recently, we have isolated several new cell lines from gypsy moth embryos and fat body (8). When production by one strain of LdMNPV in these new lines was studied, significant differences in the susceptibility of the lines to viral infection were found (J. T. McClintock, D. E. Lynn, E. M. Dougherty, and K. Shields, In Vitro Cell. Dev. Biol. **23**:62A, 1987). In the present study, we compare the amount of virus obtained by using three different virus strains, LDP-67 (GYPCHEK, to be referred to here as the Hamden strain [Ha]), a cloned isolate of Hamden (5-7d), and the Abington isolate (Ab), in two of the new lines and one of the lines developed by Goodwin et al. (4, 5).

MATERIALS AND METHODS

Cell cultures. The ovarian cell line (652Y) IPLB-Ld-652Y (5) was grown in IPL-52B (3) containing 9% fetal bovine serum modified by the addition of 0.125 g of phytone peptone (BBL Microbiological Systems, Gaithersburg, Md.) per ml and 0.075 g of liver digest (Oxoid USA, Columbia, Md.) per ml. The fat-body-derived cell line (FB) IPLB-LdFB (8) was grown in BML-TC/10 (2) supplemented with vitamins and trace minerals as listed for IPL-52B (3) and the same levels of fetal bovine serum, phytone peptone, and liver digest listed above. The embryonic line (EIt) IPLB-LdEIt (8) was grown in modified TNM-FH (6) with 9% fetal bovine serum. Each of these media provided optimal cell growth for the respective cell line. All cultures were maintained at 28°C and were subcultured at weekly intervals.

Virus inocula. The inocula for Ha and 5-7d, a plaque-purified clone of Ha (E. M. Dougherty, M. Shapiro, R. Rochford, R. H. Goodwin, A. L. Foehner, and J. R. Adams, J. Ind. Microbiol., in press) were tissue-culture-derived, nonoccluded virus (NOV) which had been passed two and three times in 652Y, respectively. The Ab inoculum was a tissue-culture derived NOV which had been passed three

TABLE 1. Relative sensitivities of gypsy moth cell lines to virus strains

Cell line	Sensitivity[a] to virus strain:		
	Ha	5-7d	Ab
652Y	1	1	1
EIt	0.56	2.61	26.1
FB	0.12	4.65	317

[a] Relative sensitivities were determined by inoculating 96-well plates containing each cell line with dilutions of the virus inocula. The resulting endpoint value for each virus on 652Y cells was assigned a value of 1, and the endpoint values on the other cells were divided by the respective 652Y endpoint to obtain the relative sensitivities listed in this table.

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TABLE 2. Production of NOV in gypsy moth cell lines

Cell line	Amt[a] ($TCID_{50}$/ml) of following virus produced:		
	Ha	5-7d	Ab
652Y	3.83×10^3	2.61×10^5	6.81×10^3
EIt	1.78×10^5	1.00×10^5	1.78×10^4
FB	5.62×10^4	1.21×10^5	4.64×10^4

[a] Supernatants from each cell line were collected 1 week after inoculation with each virus and assayed by the endpoint dilution method on 652Y cells as described in the text.

times in FB. Since Cusack and McCarthy (submitted for publication) have shown that serial passage of a clone of LdMNPV-Ha in vitro leads to reduced occlusion body (OB) production, we used low-passage virus in these studies.

Endpoint dilution assays. The virus inocula in each cell line were assayed by the endpoint dilution method. Tissue culture 96-well plates (Becton Dickinson Labware, Oxnard, Calif.) were seeded with 2×10^3 cells per well in 0.1 ml of medium. Serial 10-fold dilutions of each virus were added to the cells (0.1 ml per well, 12 wells per dilution), and the plates were incubated at 28°C. Wells were scored positive for infection if OBs were present by 2 weeks, and the data were analyzed by the method of Karber as described by Finney (1) to provide estimates of the 50% endpoint tissue culture infectious dose ($TCID_{50}$). Comparative sensitivities to the viruses were determined by arbitrarily assigning 652Y assays a value of 1 for each virus and dividing the endpoint value obtained in the other lines by the value obtained in 652Y. For consistency, nonoccluded virus produced in each line was assayed in 652Y.

Infection studies. Virus replication was assayed by initiating 24-well plates (Becton Dickinson) with each cell line, with 7.5×10^4 cells per well in 0.5 ml of medium. Four wells of each line were inoculated with each virus at a multiplicity of infection of 1 $TCID_{50}$ per cell of each virus as determined by the endpoint dilution assay in the homologous cell line. NOV production was determined by collecting media from each well at 1 week postinfection, centrifuging ($100 \times g$ for 15 min) to remove cells, and assaying by using the endpoint dilution method and 652Y cells (1×10^4 cells per well). OB production was determined at 1 week postinfection by suspending the cells in each well in 0.5 ml of sterile distilled water, adding sodium dodecyl sulfate to a final concentration of 1%, and allowing the OBs to sediment overnight. The number of OBs in a random area of each well was counted with an inverted phase-contrast microscope. The data obtained in each of these studies were analyzed by analysis of variance and, when appropriate, by Duncan's mean comparison test.

RESULTS AND DISCUSSION

The LdMNPV strains showed different levels of replication in the various cell lines. Relative sensitivities to the three virus strains were determined by endpoint dilution analysis in each line and are summarized in Table 1. The greatest difference occurred in FB (the fat-body-derived line), which was 300-fold more susceptible to the Ab virus than the 652Y cells were. In this study, the same virus dilutions were used with each cell line. The 652Y endpoint dilution assay indicated that the inocula contained 1.21×10^5 $TCID_{50}$/ml, while the FB assay indicated that they contained 3.83×10^7 $TCID_{50}$/ml, or 317 times as much. Since these values were from the identical dilutions, the differences must be due to different susceptibilities of the two cell lines. A previous study (7) showed similar differences among cell lines from different insect species which are susceptible to the *Autographa californica* MNPV. Additionally, Goodwin et al. (4) showed differences in the abilities of other gypsy moth cell lines to support the replication of several different viruses. Possible explanations for these results include different numbers or types of virus receptor sites on the cells, variation in mechanisms of adsorption or uncoating of the virus, or the presence of cellular defense mechanisms in the 652Y cell line. We have no evidence to support any of these possibilities, and no explanations have been made of why insect species or tissues differ in susceptibility to different viruses. The fact that the three virus isolates differ in relative susceptibility indicates that viral components are also involved.

Because of the results obtained in the sensitivity experiment, the virus inocula used in subsequent studies were adjusted so that each cell line received an equivalent challenge of virus. Productivity was measured by two criteria, NOV production and OB production. NOV production was relatively low in each virus-cell combination (Table 2). The maximum amount was obtained in 652Y cells infected with 5-7d, and the minimum amount was obtained in the same line infected with Ha.

The differences in OB production between the various combinations of cells and virus were significantly greater than was observed for NOV production (Table 3). Ab virus produced more OBs in each line, and FB cells produced the greatest quantity of each virus. This study demonstrated the importance of developing new cell lines and searching for new virus isolates for use in insect virus production. The GYPCHEK strain of virus (Ha) produced about 1.4×10^5 OB per ml in the previously available cell line (652Y). The new line, FB, produced 50-fold more Ha virus under the same conditions. Additionally, when the more productive virus isolate (Ab) was used, OB production was enhanced threefold in FB cells. Overall, these data indicate that the FB-Ab combination is 150 times more efficient at producing virus than the 652Y-Ha combination is. These results will be important in the possible in vitro commercial production of gypsy moth virus. Shapiro et al. (9) have also shown that the Ab virus is more infectious to gypsy moth larvae. These data suggest that in addition to being more economical to pro-

TABLE 3. Production of gypsy moth virus OBs in gypsy moth cell lines

Cell line	No.[a] of OBs of following virus produced:		
	Ha	5-7d	Ab
652Y	6.90×10^4 [h] $\pm 1.2 \times 10^4$	4.36×10^5 [g] $\pm 1.3 \times 10^4$	5.99×10^6 [bc] $\pm 1.9 \times 10^6$
EI5	6.33×10^5 [f] $\pm 1.3 \times 10^5$	9.09×10^5 [e] $\pm 1.5 \times 10^5$	6.64×10^6 [b] $\pm 9.4 \times 10^5$
FB	3.51×10^6 [d] $\pm 9.3 \times 10^5$	4.25×10^6 [cd] $\pm 8.7 \times 10^5$	1.06×10^7 [a] $\pm 2.0 \times 10^6$

[a] Cells were lysed 1 week after inoculation with each virus, and the number of OBs was counted as described in the text. Values are mean number of OBs per well ± 1 standard deviation based on four replicates of each cell line-virus combination. Means followed by the same letter are not significantly different ($P \leq 0.05$) as determined by Duncan's mean comparison test.

duce, less virus may be needed to obtain effective control of the pest.

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