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COMPARISON OF CELL LINE MAINTENANCE PROCEDURES ON INSECT CELLS USED FOR PRODUCING BACULOVIRUSES

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SUMMARY

A gypsy moth cell line, IPLB-LdEIta, maintained under various conditions was tested for susceptibility to and productivity of two baculoviruses, the *Autographa californica* nucleopolyhedrovirus (AcMNPV) and *Lymantria dispar* nucleopolyhedrovirus (LdMNPV). The results suggest that cells maintained in serum-containing medium (modified TC100) were more susceptible (on the basis of titers in an endpoint assay) to LdMNPV than cells maintained in a serum-free medium (ExCell™ 400). Such a difference was not apparent with AcMNPV. Similarly, little difference existed in the proportion of cells containing occlusion bodies (OBs) a wk after inoculation with AcMNPV (i.e., the percent infected) in any LdEIta strains, although one combination of cells and medium (cells maintained in ExCell 400 but infected in TC100) showed a lower percent infection with LdMNPV. Even though the percentage of cells infected varied little, the number of OBs produced varied by 3 logs with AcMNPV and 11/2 logs with LdMNPV. In each case, cells normally grown in ExCell 400 and infected in the same medium produced the lowest number of OBs. However, productivity was improved when cells normally grown in ExCell 400 were infected in TC100. Even more interesting was that cells normally grown in TC100 produced more AcMNPV OBs when infected in ExCell 400 medium. This suggests that changing culture medium (regardless of the normal maintenance medium) can stimulate virus production. In addition to examining virus productivity in LdEIta cells in both serum-containing and serum-free media, I also tested a strain maintained at low temperature (17° C) for over a yr. This maintenance protocol was not detrimental for LdMNPV productivity and was slightly stimulatory for production of AcMNPV.

Key words: gypsy moth cell culture; baculovirus; nucleopolyhedrovirus; virus productivity.

INTRODUCTION

Insect cells have been used for studying insect viruses for over 30 yr and for 15 yr as a tool in the baculovirus expression vector system (BEVS) (Smith et al., 1983). Since the goal of the expression vector system is to produce recombinant proteins, considerable effort was made to develop serum-free and low protein media for growing insect cells (Wilkie et al., 1980; Mairorella et al., 1988) which has led to several commercial media producers listing serum-free insect cell culture media among their products. These media are effective for growing cells, viruses, and recombinant proteins (King et al., 1992; Chen et al., 1993; Deutschmann et al., 1994; McKenna et al., 1997).

My laboratory has developed numerous new cell lines, including an embryonic line from gypsy moth (IPLB-LdEIta; Lynn et al., 1988) that is capable of replicating both the *Autographa californica* nucleopolyhedrovirus (AcMNPV) and the *Lymantria dispar* nucleopolyhedrovirus (LdMNPV). AcMNPV is the principal virus used in BEVS and LdMNPV is a prime candidate for control of the gypsy moth. The LdEIta line is an effective alternative for use in BEVS (Betenbaugh et al., 1991; Hink et al., 1991) as well as for studies on LdMNPV (Lynn et al., 1989; Horton and Burand, 1993; Park et al., 1993).

When the first commercial serum-free insect cell culture medium was marketed (ExCell™ 400), I tested its ability to support growth of several cell lines maintained in my lab, including LdEIta. LdEIta cells adapted quickly to the new medium and obviously grew better in the serum-free formula than in serum-containing medium (TC-100, unpublished observation). Since then (Dec. 1988), the line has been maintained continuously in ExCell 400. When the suggestion was made that long-term passage of cells in serum-free could lead to reduced virus replication, a sample of the LdEIta line was thawed from liquid nitrogen storage and has been maintained in modified TC100 (Lynn, 1988) for nearly 5 yr.

During recent attempts to develop new lines, I have begun using a lower temperature incubator (17° C) for maintaining some cells. The original purpose for this was based on evidence that some cells maintained at lower temperatures remained susceptible to one type of baculovirus, granulovirus (GV) (Winstanley and Crook, 1993). In addition, I have found that many insect cell lines can be maintained on a less frequent subculture interval (typically at 3 wk compared to a 1-wk interval for cells maintained at 26° C), thus reducing the amount of effort required for maintaining less frequently used lines. A strain of the LdEIta line in TC100 has been maintained at 17° C for over a yr.

In this paper, I report on the ability of these strains of LdEIta to replicate both AcMNPV and LdMNPV. The comparisons included productivity of occluded virus (OB) by the cells and the relative susceptibility of the cells to infection as determined by the proportion infected at high titer and by endpoint analysis.

MATERIALS AND METHODS

Cell line. The IPLB-LdEIta line was originally developed from *Lymantria dispar* embryos in a 3:1 mixture of IPL-52: IPL-76 media (Goodwin and

Adams, 1980) as LdEI (Lynn et al., 1988). Shortly after establishment, the original line was switched to modified TNM-FH (Hink and Strauss, 1976 = supplemented Grace's, Life Technologies, Grand Island, NY) and TC100 medium (=BML-TC/10, Gardiner and Stockdale, 1975, from Life Technologies but modified as in Lynn, 1989) and its designation changed to LdEIt. This culture had obvious heterogeneity between cell types growing as attached and nonattached forms. The attached cell type was selected by replacing the medium (thereby removing nonattached cells) before subcultivation. This resulted in the LdEIta strain. The strain used in this study was stored frozen in liquid nitrogen but had been subcultured at least 250 times in modified TC-100 medium and is considered the standard in this paper (Ctc strain). Just over a yr ago, some cultures of this strain were subsequently maintained at 17° C (still in modified TC-100 medium; Clt strain) while a third strain has been maintained in serum-free culture medium (ExCell 400®) for over 450 passages (Cex strain).

Virus studies. The two baculoviruses used in this study were a clone of the AcMNPV designated AcMNPV-pxh, which was isolated in my laboratory by plaque purification (unpublished information) and the LdMNPV-a624 clone, which was isolated from the Abington strain of gypsy moth virus (Lynn et al., 1993). Both viruses had been passaged three times in cell cultures, with the inoculum used in these studies having originated from IPLB-TN-R^2 and IPLB-LdEIta, respectively. Virus was stored at −70° C and thawed immediately before use. Titers of the frozen virus stocks were determined by endpoint assay in IPLB-TN-R^2 and IPLB-LdFBc1 as described previously (Lynn, 1992a, 1994).

The sensitivity of the various cell strains to each virus was tested by endpoint assay as previously described (Lynn, 1992a) with the following modifications: before the plates were inoculated with the cells, each strain was centrifuged and resuspended in both ExCell 400 and TC100 media at 10 000 cells/ml, then distributed in 0.2-ml aliquots to each well of a 96-well plate (Falcon®, Franklin Lakes, NJ). Each virus was diluted in TC100 medium, and 10 μl of the 10^{-4} to 10^{-9} dilutions were inoculated into 8 wells of each cell strain/medium combination. The endpoints were scored (positive being the presence of occlusion bodies, OBs) after 2 wk at room temperature and analyzed by Karber's method (Lynn, 1992b).

To test virus productivity in the LdEIta strains, cells of each strain were centrifuged in an IEC tabletop centrifuge (50 × g) and suspended at 7.5 × 10^4 cells/ml in each culture medium. In this paper, the normal maintenance conditions of the cells will be identified by the abbreviations Ctc, Clt, and Cex, whereas the medium used in the tests will be identified by Mtc or Mex for modified TC-100 and ExCell 400, respectively. Thus, for example, CtcMtc are cells normally maintained in TC-100 medium and tested in the same medium, whereas CtcMex are cells normally maintained in TC-100 but tested in ExCell 400. One milliliter of cells was transferred to each well of 24-well tissue culture plates (Costar®, Cambridge, MA) and held at room temperature (approximately 22° C) overnight. The culture plates were inoculated with sufficient volume of the stock virus to result in a multiplicity of infection (moi) of 10 $TCID_{50}$ (50% tissue culture infective dose) per cell. Each cell–medium–virus combination was replicated four times. The plates were incubated at 26° C for 7 d, following which I estimated the number of cells containing OBs and total number of cells in each well by counting cells in a measured area of an ocular on a Leitz inverted microscope. *Note* that at this time period under the conditions used, few of the LdEIta cells are naturally lysed by either virus. Approximately 80% of the medium in each well was carefully removed and replaced with 0.5 ml demineralized water, followed by addition of 50 μl of a 10% (wt/vol) solution of sodium lauryl sulfate (Sigma Chemical Co., St. Louis, MO). The cells were lysed by repeated pipetting and the OBs allowed to settle over a 6-h period, following which they were counted as described above for the cells. The resulting data were used to determine the proportion of cells infected and the total number of OBs produced. The results were analyzed by one-way analysis of variance and Tukey's pairwise multiple comparison test using Sigmastat® (v 2.03).

Results and Discussion

Comparison of the sensitivity of the various cell strains through the use of an endpoint technique shows that variability was very slight in endpoints with AcMNPV, with only CtcMex showing reduced susceptibility (Fig. 1). The variation was greater in the LdMNPV tests in which Cex showed lower infectivity in both media (Figs. 1 and 2).

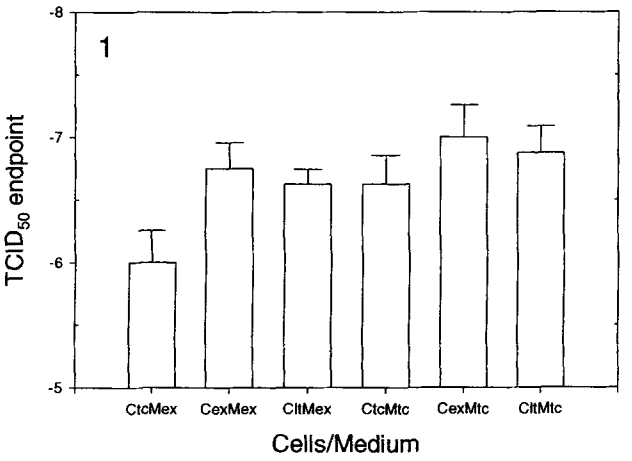

FIG. 1. Susceptibility of IPLB-LdEIta strains to infection by AcMNPV. $TCID_{50}$ endpoint titers were measured by inoculating the same number of cells of each strain with the same virus suspension. The difference in resulting titer (recorded as the log of the endpoint titer; *error bars* are one standard error as calculated by Karber's method) is thus a measure of the sensitivity of the cell population to the virus with the smaller log indicating greater susceptibility. The *x*-axis legends indicate the cell strains (Ctc = cells maintained in TC100; Cex = cells in ExCell 400; and Clt = cells in TC100 maintained at 17° C) and the medium used in the endpoint assay (Mex = ExCell 400 and Mtc = TC100).

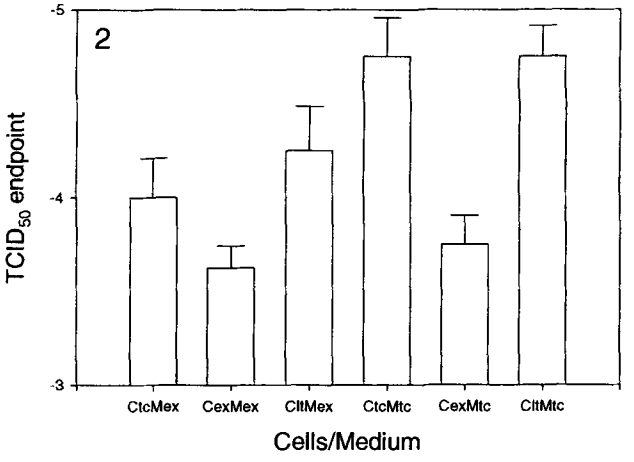

FIG. 2. Susceptibility of IPLB-LdEIta strains to infection by LdMNPV. The results are presented as described in Fig. 1.

The results of infection of LdEIta cells with AcMNPV are shown in Figs. 3–4 and with LdMNPV in Figs. 5–6. Although there was a significant difference in the proportion of cells infected with AcMNPV between two of the treatments (CtcMex and CexMex) as compared with the rest of the treatments, these treatments resulted in only about 10% fewer cells containing OBs (Fig. 5). The variability in proportion of cells infected with LdMNPV was greater (ranging from 54% to 90%, Fig. 6) although statistically, only the low value (CexMtc) was significantly different from values from the other treatments.

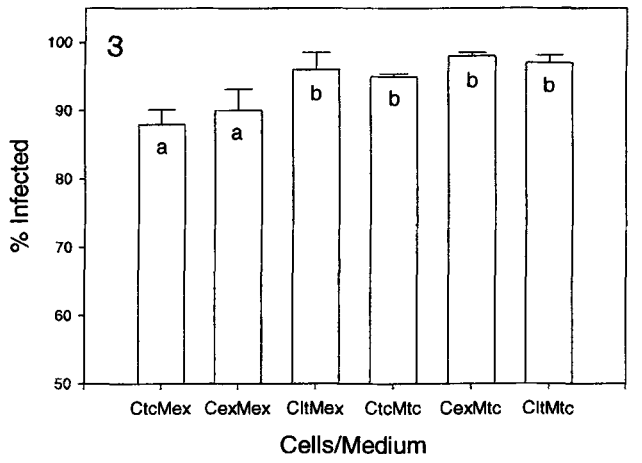

FIG. 3. Percent infection of IPLB-LdElta strains 1 wk after inoculation with AcMNPV at an moi of 10 $TCID_{50}$/cell. Cells were scored as positive if occlusion bodies were present in the nuclei. Each *bar* is the mean of four replicate wells in a 24-well plate; *error bars* represent one standard deviation. *Bars* with the same letter are not statistically different at $P < 0.05$ based on Tukey's multiple comparison test. The *x*-axis labels indicate the cell strain and medium used in each test as described for Fig. 1.

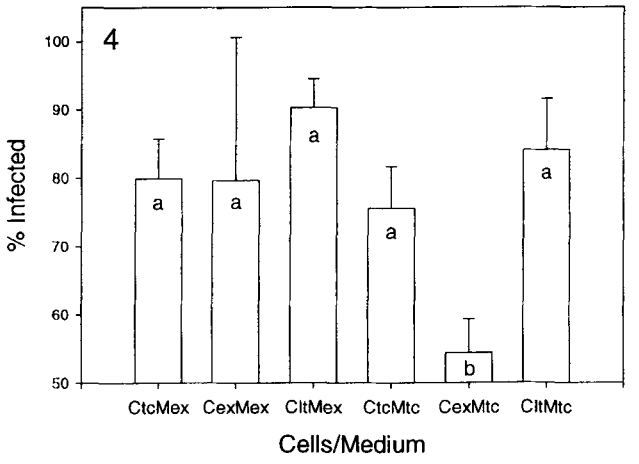

FIG. 4. Percent infection of IPLB-LdElta strains 1 wk after inoculation with LdMNPV at an moi of 10 $TCID_{50}$/cell. The results are presented as described in Fig. 3.

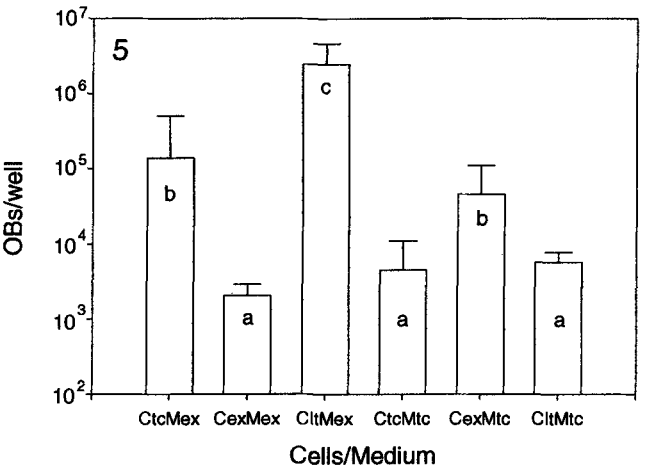

FIG. 5. Occlusion body production in IPLB-LdElta strains 1 wk after inoculation with AcMNPV at an moi of 10 $TCID_{50}$/cell. Each *bar* is the mean of counts in four replicate wells for each treatment in a 24-well plate; *error bars* represent one standard deviation. *Bars* with the same letter are not statistically different at $P < 0.05$ based on Tukey's multiple comparison test. The *x*-axis labels indicate the cell strain and medium used in each test as described for Fig. 1.

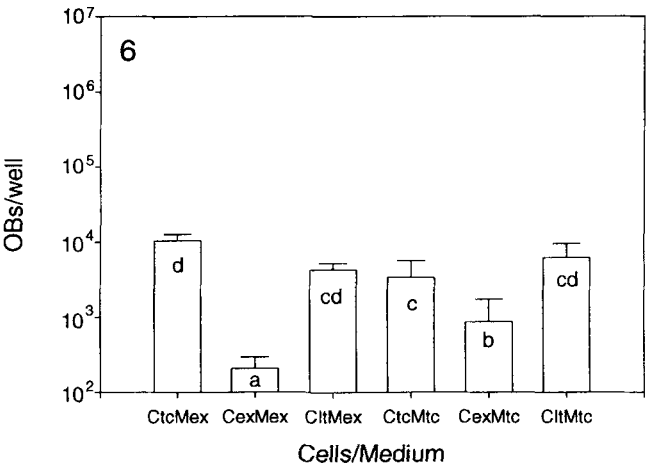

FIG. 6. Occlusion body production in IPLB-LdElta strains 1 wk after inoculation with LdMNPV at an moi of 10 $TCID_{50}$/cell. The results are presented as described in Fig. 5.

The variability in OB production in the various cell–medium combinations was much greater than the proportion of cells showing infection and the results differed between the two viruses. For AcMNPV-infected cells, no correlation seemed to exist between either the medium used in the test or the source of the cells. For example, whereas the cells maintained in TC100 (both Ctc and Clt) produced significantly greater numbers of OBs than cells maintained in and tested in ExCell medium, Cex produced more OBs in the TC100 medium than the cells normally maintained in TC100 (again with both Ctc and Clt strains) (Fig. 4). This suggests that for AcMNPV, switching media at the time of virus infection resulted in greater virus production and that perhaps each is lacking in some component(s) necessary for optimal OB production.

The effect of the maintenance medium was more clear-cut with LdMNPV. Cells maintained on TC100 medium (both Ctc and Clt) produced more OBs than the Cex cells, regardless of which medium was used during the infection (Fig. 6). Even so, the pattern of improved productivity in cells maintained in one medium but infected in the other was duplicated in the LdMNPV virus tests (although to a lesser extent as compared with what was observed with AcMNPV). Cex cells produced significantly more OBs in TC100 than the same cells in ExCell, whereas Ctc cells produced more virus in ExCell 400 than in TC100.

The use of low temperature for maintenance of cells does not appear to be detrimental to the productivity of either virus (Figs. 5 and

6). With both viruses, the numbers of OBs produced were either not significantly different from those of cells maintained at 26° C or were better (in the case of AcMNPV replicated in ExCell medium). On the basis of these results, I believe that for laboratories that only periodically need cells for virus production, maintaining cells at low temperature on a 3-wk schedule would be a viable alternative to the more labor-intensive maintenance of cells on a weekly schedule at 26° C or using liquid nitrogen freezing.

Overall, my results suggest that the long-term maintenance of cells in serum-free medium may cause a slight decline in virus productivity. However, returning the cells for even a brief time to serum-containing medium (in the case of this study, during just the virus replication) is sufficient to increase production, suggesting that the serum-free effect is not irreversible. Another suggestion from my results is that changing to a different culture medium at the time of inoculation may stimulate OB production. This occurred with both viruses when Cex cells were changed to TC100 or Ctc cells changed to ExCell 400, and only Clt cells did not respond this way with both viruses.

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