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LEPIDOPTERAN CELL LINES AFTER LONG-TERM CULTURE IN ALTERNATIVE MEDIA: COMPARISON OF GROWTH RATES AND BACULOVIRUS REPLICATION

DWIGHT E. LYNN[1]

Insect Biocontrol Laboratory, USDA/ARS, Beltsville, Maryland 20705-2350

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Summary

Three insect cell lines, IPLB-LdFB and IPLB-LdEIta from gypsy moth fat body and embryos and UFL-AG-286 from velvetbean caterpillar embryos, have been concurrently maintained for 1 to 12 yr on two media formulations, modified TC-100 containing 9% fetal bovine serum and Ex-cell 400, a commercial serum-free medium (SFM). Cells grown in each medium were tested for susceptibility to and productivity of various multiply embedded nucleopolyhedroviruses. The three lines chosen for these experiments fall into three categories of relative growth in SFM versus TC-100: LdFB cells grew similarly in each medium, LdEIta grew better in Ex-Cell than in TC-100, and AG-286 grew better in TC-100 than in Ex-Cell. The susceptibility of cells to infection also varies, although without any apparent correlation to which medium was best for supporting growth. Endpoint assays suggested that LdFB cells grown in serum-containing medium are more susceptible to virus infection than their SFM counterparts, while the opposite is true for LdEIta cells. Production of virus, based on numbers of occlusion bodies, showed fewer differences with only AcMNPV production with AG-286 in TC-100 being statistically higher than production of the same virus in Ex-cell 400. These studies suggest that long-term passage in alternative media may impact the ability of cells to support virus infection and replication, but the effects on each cell line and virus system need to be determined.

Key words: nucleopolyhedrovirus; insect cell lines; serum-free medium; virus productivity.

Introduction

Well over 500 insect cell lines have been reported in the literature, with about half of these lines having been developed from 56 species of Lepidoptera (Lynn, in press). These lines represent a substantial amount of material available for uses in research and biotechnology. With the development of new cell lines, new media formulations have followed. The first cells developed from insects, the moth cell lines by Grace (1962), were grown in a formulation Grace described in the same paper, and this medium is still in use today with a variety of supplements, but so are a number of other media described later. In particular, several manufacturers of cell culture media began marketing serum-free medium (SFM) formulations for the growth of lepidopteran cells in the 1980s to capture the potentially lucrative market of using insect cell lines for production of proteins by the baculovirus expression vector system (Summers and Smith, 1987). The first of these was Ex-cell® 400 produced by JR Scientific (now JRH Biosciences). In an effort to simplify maintenance of multiple cell lines in my laboratory, I tested Ex-Cell 400 for support of growth of over 20 insect cell lines. Of those tested, several grew poorly or not at all on the SFM (such as TN-368 and IPLB-TN-R2 from the cabbage looper), while others grew much better than on their "normal" medium (such as IPLB-Tcon and IPLB-Tex2 from *Trichogramma* wasps and IPLB-Ld652Y, IPLB-LdEp, and IPLB-LdEIta from the gypsy moth). In general, when cell growth was as good or better on SFM, I subsequently maintained those cells on Ex-Cell 400, while if growth was poorer, the cell line maintenance was continued on their original medium. In a few cases, cell lines were maintained on both SFM and serum-supplemented modified TC-100 (which is a modification of Grace's medium), and these form the foundation for the present study.

The wide availability of the cell lines along with a diversity of media can create problems of reproducibility between laboratories ostensibly using the same cell lines. For example, laboratory A may get substantially different results from laboratory B if A grows a particular cell line in medium X while B uses medium Y. The situation can become even more complicated if laboratory B provides the cell line to laboratory C, which returns to using medium X or starts using medium Z. Every change of medium or other maintenance protocols creates a selection pressure on the cells. Based on the numbers of publications from different researchers over the past few years, insect cell lines are being maintained in scores, if not hundreds, of facilities around the world.

Since the widest use of lepidopteran cell lines is in applications with insect viruses, the availability of cells that have been maintained for extended periods on alternative media can provide an opportunity to examine the effects such activity may have on virus replication—to see, in effect, if the different media results in a selection pressure resulting in cells that are less useful in insect virus research—and this is the purpose of this current study. While the experiments described here will directly reveal the media ef-

[1] USDA/ARS, Insect Biocontrol Lab, BARC West, Building 011A, Room 214, 10300 Baltimore Boulevard, Beltsville, Maryland 20705-2350. E-mail: lynnd@ba.ars.usda.gov

fects, the results could also answer broader questions on the effects of different cell line maintenance procedures.

Materials and Methods

Cells and media. The IPLB-LdFB cells were originally established from *Lymantria dispar* larval fat bodies, and IPLB-LdEIta were from embryos of the same species (Lynn et al., 1988). Both of these were established originally in a mixture of Goodwin's IPL-52B and IPL-76 (Goodwin and Adams, 1980) but were switched to TC-100 at early passages. The UFL-AG-286 line is from *Anticarsa gemmatalis* embryos (Sieburth and Maruniak, 1988) and was originally grown in modified Grace's medium.. All three lines had been maintained in modified TC-100 (= BML-TC/10 [Gardiner and Stockdale, 1975], prepared from powder obtained from GIBCO, Grand Island, NY, product no. 11600-53[2] and modified as described in Lynn, 1989) for over 12 yr of continuous, weekly subcultures. The cells were concurrently maintained in Ex-cell® 400 (JRH Biosciences, Lenexa, KS[3]) for 3 (LdFB), 12 (LdEIta), and 1.5 yr (AG-286), respectively. The cell lines were authenticated annually using an isozyme electrophoresis method (Innovative Chemistry, Marshfield, MA).

Virus. Clonal isolates of three nucleopolyhedroviruses (NPVs) were used in these studies. Each clone was isolated in my laboratory as previously described: AcMNPV-pxh (Lynn, 1999), LdMNPV-a624 (Lynn et al., 1993), and AgMNPV-ag3 (Lynn, 2003), and the inocula used were cell culture–derived budded virus (BV) stocks.

The relative susceptibility of each line on each media was determined using an endpoint assay as previously described (Lynn, 2002). The cell strain being maintained on each medium was used to initiate 96-well tissue culture plates (Corning, Cambridge, MA) with 5000 cells in 0.2 ml of the medium (supplemented with 0.1 mg/ml gentamicin sulfate; Sigma Chemical Co., St. Louis, MO) per well. The BV samples of the appropriate virus (LdMNPV for LdFB and LdEIta, AcMNPV for LdEIta and AG-286,[4] and AgMNPV for AG-286) were serially diluted in the same medium, and 10μl samples were inoculated into 12 wells for each dilution. Plates were sealed with masking tape and incubated at 22° C for up to 4 wk. The wells were scored positive when infected cells were present (determined by the presence of viral occlusion bodies [OBs]) and the endpoint values determined by Karber's method (Lynn, 1992).

Optimal OB production was determined by initiating 24-well tissue culture plates (Corning) with each cell line at four cell densities (1×10^4, 3×10^4, 1×10^5, and 3×10^5 per well in 0.5 ml of the respective medium supplemented with 0.1 mg/ml gentamicin sulfate, Sigma). The wells were then inoculated with one of the virus samples at a multiplicity of infection of 0.01, 0.1, or 1.0 $TCID_{50}$ units (using the $TCID_{50}$ values obtained for each cell line in the susceptibility studies described previously). Plates were incubated at 22° C for 2 wk, after which the culture medium was replaced with distilled H_2O, and then sodium lauryl sulfate was added to a final volume of 1% (w/v). The cells were disrupted by repeatedly passing four to six times through a Pasteur pipette. The plates were left on the bench at room temperature overnight to allow the OBs to settle, and the number per well was estimated by counting the number in a measured area of an ocular on a Nikon TMS Inverted Microscope. Each cell, medium, and virus combination was tested three times, and the mean number of OBs produced at each virus and cell concentration was determined. The maximum mean was used as the optimum productivity of that virus in the cells with each medium. These optimal OB numbers were analyzed by Student's paired *t*-test to determine differences in virus productivity between the media for each cell line.

[2] Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

[3] JRH Biosciences is now called SAFC Biosciences. Ex-cell 400 is no longer listed in their catalog; however, new formulae, Ex-cell 405 and Ex-cell 420, seem to have similar growth-supporting properties for each of these cell lines.

[4] The IPLB-LdFB cell line is only semipermissive to AcMNPV virus with no production of progeny virus (Guzo et al., 1991). Thus, this virus was not used on this cell line in these studies.

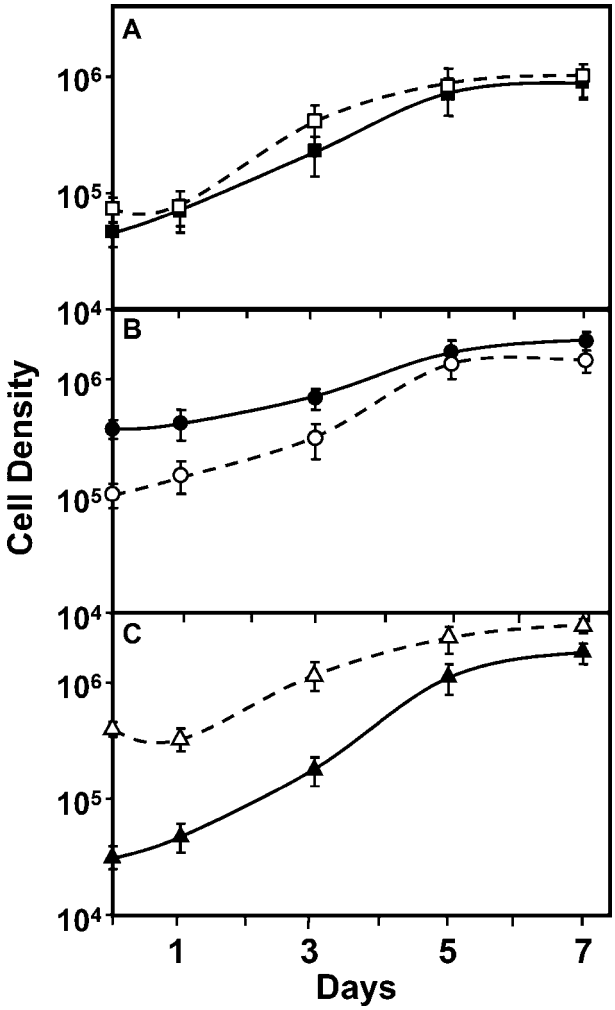

Fig. 1. Growth curves for cell lines in TC100 (solid points/solid line) and Ex-cell 400 (open points/dashed line). (*A*) IPLB-LdFB cells; (*B*) IPLB-LdEIta cells; (*C*) UFL-AG-286 cells. Each point is the mean of cell estimates for three 25-cm² tissue culture flasks; error bars are one standard deviation.

Results

Growth rates. The three cell lines used in these studies represent three categories of growth response to the two media as shown in Figure 1. The LdFB cells grew similarly in each medium (Fig. 1*A*), while the LdEIta cells grew better in Ex-Cell than in TC-100 (Fig. 1*B*). The initial cell density routinely used with these cells was approximately four times greater in the medium with FBS than in SFM so as to reach similar final densities after 7 d of growth. Alternatively, the AG-286 cells grew better in TC-100 than in Ex-Cell (Fig. 1*C*), requiring about 12 times as many initial cells to reach the same density at 7 d.

Susceptibility to virus. As with growth rates, the cells varied in their relative susceptibility to virus in the alternate media (Fig. 2). The LdFB cells were 7.5-fold more susceptible to LdMNPV BV infection in Ex-cell 400 compared to TC-100. Conversely, the LdEIta cells were about 20-fold more susceptible to the same virus in TC-100 and more than 50-fold more susceptible to AcMNPV in this medium relative to Ex-cell 400. While the susceptibility of LdEIta to these two viruses was statistically different between the

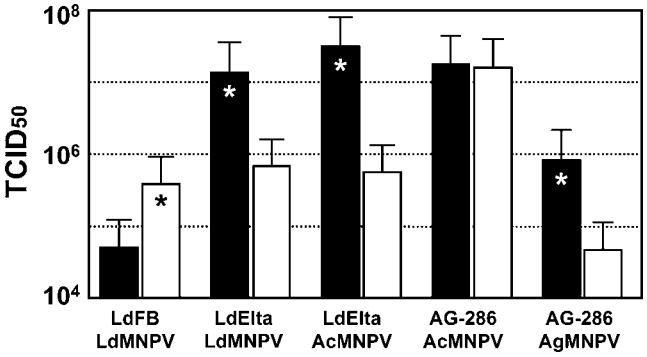

FIG. 2. Relative susceptibility of cell lines grown in TC-100 (black bars) or Ex-cell 400 (white bars). Each bar represents the estimate of viral titers in budded virus samples of the nucleopolyhedroviruses in the cells as indicated on the *x* axis. Each bar is the mean of three replicate test, and the error bars are one standard deviation. Titers marked with an asterisk are statistically different from the corresponding titer for the same cell line/virus combination in the alternative medium as determined by Student's *t*-test at $P < 0.05$.

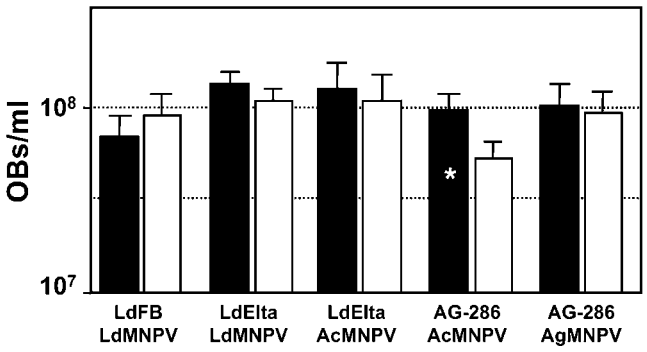

FIG. 3. Production of occlusion bodies of the indicated virus in cells with each medium. Black bars are cells grown in TC-100; white bars are cells grown in Ex-cell 400. Each bar is the mean of three replicate test, and the error bars are one standard deviation. The AcMNPV production in AG-286 in TC-100 medium (the bar marked with an asterisk) was statistically different from the same virus and cells in Ex-cell as determined by Student's *t*-test at $P < 0.05$.

two media ($P < 0.05$ by Student's *t*-test), the relative differences between the two viruses were not statistically significant. The AG-286 cells showed no difference between the two media to AcMNPV but were 18-fold more susceptible to the AgMNPV BV in TC-100.

Virus productivity. Fewer differences in maximum viral OB production resulted from infection of cells grown in the two media (Fig. 3). The only statistically different productivities were with the AG-286 cells grown in TC-100; these produced more AcMNPV than the same line in Ex-cell 400. Remember that these optimal values were obtained by inoculating four cell densities with three different multiplicities of infection of the budded virus. The range of production in individual virus/cell/media tests varied substantially in some cases. For example, AG-286 cells in Ex-cell 400 medium infected with AgMNPV ranged from a minimum of 2.06×10^4 to a maximum of 5.65×10^7 OBs/ml (more than a 4000-fold difference), and the same cells and medium had a 500-fold difference with AcMNPV. However, the range was much smaller in most tests (8- to 20-fold difference with infections in TC-100 and 10- to 100-fold differences in Ex-cell 400; data not shown).

DISCUSSION

The variability in growth rates between the two media in the three cell lines can be considered somewhat surprise considering the similarity in the lines. Both LdFB and LdEIta were developed from gypsy moth tissues, and the primary cultures and early passages were in the same media (initially a 3:1 mixture of IPL-52B and IPL-76 [Goodwin and Adams, 1980] but with each switched to TC-100 during early passages), yet LdFB grew similarly in each medium, while LdEIta grew much better in Ex-cell 400. While still a lepidopteran cell line, the third line examined, AG-286, is from a different species (the velvetbean caterpillar) but has the tissue source (embryos) in common with LdEIta and was initiated in TC-100, yet it grew much slower in Ex-cell 400 compared with TC-100. Thus, the three cell lines examined here fall into three categories with regard to how they respond to being grown in the serum-free medium.

Changing the media composition in cell cultures almost certainly results in selection for different cell types in the cultures. None of these cell lines was cloned prior to the change in media, so the cultures could have been quite heterologous. However, normal maintenance procedures will select for cells that can grow fastest, and since all three lines had been maintained in culture at least a couple of years before a separate strain was developed by growth in the serum-free medium, they were already well adapted to growth in the serum-containing TC-100 medium. Switching to SFM would probably result in a further selection of a subset of the original cell population.

I would expect this selection to affect some of the properties of the cultures, including how they respond to virus infection. I have used comparative endpoint assays between different cell lines or strains in several previous studies (Lynn, 2002, 2003), and, in my experience, this is one of the most sensitive and consistent measure of differences in virus replication between cells. This appeared to be the case in the current study as well. As shown in Figure 2, all three cell lines showed significantly different levels of susceptibility to at least one of the viruses tested on each. In fact, the only cell/virus combination that did not show a difference in these assays was AG-286 with AcMNPV. The other virus used with this line, AgMNPV, was 18-fold more infectious to cells in serum-containing medium, and a similar result was observed with both viruses tested on LdEIta cells in which LdMNPV was 20 times more infectious and AcMNPV 56 times more infectious to cells grown in the serum-containing medium. These results are consistent with the idea that transferring cells to the serum-free medium resulted in a selection of a subset of the cells and that cells with greater susceptibility to the cells were lost during this selection. Curiously, the LdFB line had an opposite effect since the cells adapted to growth in SFM were 7.5 times more susceptible to LdMNPV, suggesting that, in some systems, the selection in SFM can result in higher levels of infection.

Very few differences occurred in the optimal production of OBs results. The only cell line with a significant difference in OBs between the two media was AG-286 with the AcMNPV virus. This was the only cell/virus combination that showed no difference in susceptibility. The seemingly contradictory nature of the results on susceptibility versus productivity may simply indicate that, while it takes greater amounts of input virus to initiate an infection in two of the lines grown in the serum-containing medium, once a cell is

infected it appears to produce as many or more (in the case of AG-286 cells with AcMNPV) viral OBs.

The virus susceptibility and productivity results are consistent with my earlier study (Lynn, 1999) in which I compared virus replication in long- and short-term passage of the LdEIta cell line. One conclusion that can be made from the results in this study is that the growth rates of cells in various media are not a predictor of the cell's utility for virus production. As long as the cell density and virus inocula are adjusted at the time of infection, similar yields can be obtained even if cell growth is not optimal.

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