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# A New Serum-Free Medium for Lepidopteran Cell Culture

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A serum-free medium (LM3o) for the culture of lepidopteran cell lines is described. This medium is based on the formulation of the Grace medium, from which calcium chloride, maltose, and β-alanine are omitted. It was supplemented by polyamines and traces of metal to stimulate the cell adherence. High concentrations of vitamins were used to increase cell multiplication. Fetal bovine serum was eliminated and replaced by low concentrations of egg yolk emulsion, a part of which was heat-inactivated at 60°C. The LM3o medium supported the growth of the four established lepidopteran cell lines tested, *Choristoneura fumiferana, Spodoptera frugiperda, S. littoralis,* and *Bombyx mori.* After a period of adaptation to the new medium of about a month, the cell lines grew better in the new medium than in the Grace medium containing 10% fetal bovine serum. The new medium is very low in cost and will enable the rapid culture of many cell lines in large volume to produce insect viruses for molecular studies or for use as biological controls. 

KEY WORDS: Serum-free medium; lepidopteran cell lines; egg yolk.

## INTRODUCTION

Insect pathogenic viruses represent an alternative to conventional pesticides (Khachatourians, 1986). At present, the most commonly employed are produced *in vivo* using insects (Entwhistle, 1983). However, the development of techniques for the growth of insect cells in tissue culture should enable the efficient production of viruses free of microbial contamination by large scale techniques including fermentation (Vaughn, 1976; Weiss et al., 1981).

The most commonly employed media for lepidopteran cell cultivation are based on the formulation of the Grace medium (Grace, 1962). This medium promotes the multiplication of many cell lines, but only if fetal bovine serum is added. A number of scientists have tried to develop chemically defined media, instead of increasing the amount of fetal bovine serum used, in order to reduce costs.

It is well known that it is possible to multiply vertebrate cell lines in chemically defined media lacking fetal bovine serum (Barnes, 1987; Weiss et al., 1980a). Some authors have realized such media using chemical supplements, like Ultroser G (Myara et al., 1986). These media allowed the culture of a number of vertebrate cell lines, but were relatively expensive, owing to the elements added to replace the serum (fibronectin, fibroblast growth factor, etc.).

For insect cells, preliminary results obtained by Wilkie et al. (1980), using a chemically defined medium with the *Spodoptera frugiperda* cell line, or Röder (1982), using the same line but with the addition of 0.5% egg yolk emulsion to the TC10 medium (Gardiner and Stockdale, 1975), indicate a very high specificity of these media. In fact, these two media do not support the growth of either the *S. frugiperda* or the other cell lines we tested.

In this report, we attempted to make a serum-free medium which will support the growth of numerous lepidopteran cell lines at a low cost.

## MATERIAL AND METHODS

Cell cultures. Four established cell lines were used in preliminary experiments, *Choristoneura fumiferana* (gift from Dr. Devauchelle), *Bombyx mori* SPC Bm36 (Quiot, 1982), *S. frugiperda* (IPLB SF21 cl.15), and *S. littoralis* (gift from Dr. Vaughn). They were commonly maintained



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in Grace medium, lacking calcium chloride, maltose (Eurobio), and β-alanine (Léry and Fédière, 1988), supplemented with 10% heat-inactivated fetal bovine serum. To investigate cell behavior during adaptation to the different media tested, the *C. fumiferana* cell line was used.

Cells were grown in 25-$cm^2$ plastic tissue culture flasks (Nunc), containing 4 ml of medium. Cells were routinely counted in a hemocytometer after staining with 0.2% trypan blue. All products were obtained from the Sigma Chemical Co. For subsequent subculturing, confluent monolayers, obtained 5 to 6 days after incubation at 27°–28°C, were detached by gentle agitation and $10^6$ cells were planted in 25-$cm^2$ flasks.

*Cryopreservation of cells.* For freezing and cryopreservation, monolayers 80% confluent were detached and viable cells were adjusted to $5 \times 10^6$/ml with fresh medium containing 10% fetal bovine serum and 10% DMSO. The preparation was dispersed into sterile cryotubes (Nunc) and frozen for 1 hr at −20°C, overnight at −70°C, and in nitrogen for conservation.

*Medium assays.* Cells ($2 \times 10^6$) were planted in 25-$cm^2$ flasks to permit the subculture of cells because of the possible mortality found with the new media due to cell adaptation. The cells were maintained in the new media for a minimum of five passages.

*Growth kinetics and adhesion.* Kinetics were performed in 35-mm Petri dishes. Cells ($4 \times 10^5$) were planted with 2 ml of growth medium. Cells from the supernatant were cut off and fresh medium was added. Adherent cells were scraped, the two populations were counted, and their viabilities were determined.

*Assays with cholesterol and albumin.* Cholesterol was prepared at a concentration of 2 mg/liter with water and ethanol (37.5:62.5%). Cholesterol and albumin were tested at different concentrations, corresponding to those employed in different media (Morgan et al., 1950; Wilkie et al., 1980) or to those observed in the egg yolk emulsion used by other authors (Röder,1982; Yunker et al., 1967). Cholesterol (1, 10, and 100 mg/liter) and albumin (5, 50, and 500 mg/liter) were tested separately, or in combination, with the Grace medium, with or without fetal bovine serum. Two assays were attempted, without treatment and after heat inactivation of both products at 60°C during 30 min in a water bath.

*Preparation of egg yolk.* After being washed in alcohol, egg yolk was extracted from a chicken egg using a sterile syringe through an incision in the shell. The yolk was diluted to a quarter in PBS (NaCl 0.15 M; KCl 5 mM; glucose 5 mM; Na $HCO_3$ 4 mM; penicillin, streptomycin 100 U/ml) and centrifuged at 28,000*g* for 20 min. The supernatant was centrifuged twice under the same conditions. The clear supernatant was diluted to the final concentration of 1/10 and filtered on millex 0.22 μm. One fraction was used without treatment and another employed after heat inactivation during 30 min at 60°C in bath water.

## RESULTS

*Serum-free medium.* With concentrations of fetal bovine serum in the Grace medium of 5%, cells became detached and burst. Cells grew very slowly and died. A semi defined medium (LM) in which cells do not survive alone, but which supports cell growth when 2% fetal bovine serum is added, was performed (Table 1). Earlier results obtained with the same line indicated that it was possible to increase the cell adhesion by increasing vitamin concentration (Léry and Fédière, in press) and eliminating β-alanine from the Grace medium (Léry and Fédière, 1988). The composition of LM medium was also based on results obtained by Weiss et al. (1980a) using traces of the metals Zn and Al, which stimulate cell attachment and increase cell yield significantly. Using polyamines (Table 1), the percentage of fetal bovine serum decreased to 1% (LM3). No improvement was ob-

TABLE 1
MEDIUM COMPOSITION

| | LM medium[a] | | |
|---|---|---|---|
| Inorganic acids (Grace) | | Organic acids (Grace) | |
| $CaCl_2$ | 1,000 | Malic acid | 670 |
| KCl | 2,240 | Ketoglutaric acid | 370 |
| $MgSO_4$ | 2,780 | Succinic acid | 60 |
| $NaHCO_3$ | 350 | Fumaric acid | 55 |
| $NaH_2PO_4$ | 1,013 | | |
| $MgCl_2$ | 2,280 | Antibiotics (Grace) | |
| | | Penicillin | 100 U |
| Specific inorganic salts | | Streptomycin | 100 U |
| $FeSO_4$ | 1.0 | | |
| $ZnCl_2$ | 0.2 | Amino acids (Grace) | |
| $CuSO_4$ | 0.2 | Alanine | 225 |
| $MnCl_2$ | 0.1 | Arginine | 700 |
| | | Aspartic acid | 350 |
| Sugars | | Asparagine | 350 |
| Glucose | 700 | Cystine | 220 |
| Sucrose | 26,680 | Glutamic acid | 600 |
| Maltose | 750 | Glutamine | 600 |
| | | Glycine | 650 |
| Vitamins | | Histidine | 2,500 |
| Choline chloride | 20.0 | Isoleucine | 50 |
| Folic acid | 0.5 | Leucine | 75 |
| Thiamine–HCl | 0.1 | Lysine | 625 |
| Biotin | 0.1 | Methionine | 50 |
| Pantothenic acid | 0.1 | Phenylalanine | 150 |
| myo-Inositol | 0.5 | Proline | 350 |
| Niacin | 0.2 | Serine | 1,100 |
| Riboflavine | 0.1 | Threonine | 175 |
| Pyridoxine–HCl | 0.5 | Tryptophan | 100 |
| *p*-Aminobenzoic acid | 0.4 | Tyrosine | 50 |
| | | Valine | 100 |
| | | β-Alanine | Omitted |
| | LM3 medium[a] | | |
| LM + polyamines | | | |
| Taurine | 1 | | |
| Putrescine | 1 | | |
| Spermine | 1 | | |
| Spermidine | 1 | | |

[a] All values expressed in mg/liter.

served when the concentration of lipids was modified. On the contrary, an increase provoked a decrease in cell growth.

Different supplements were tested as replacements for fetal bovine serum (coconut milk, latex serum, and egg yolk). Only the egg yolk emulsion resulted in an improvement. Contrary to the results obtained by Röder (1982), the Grace medium, with the addition of 0.5% egg yolk, did not permit the survival of cells during many passages.

With LM3 medium, the egg yolk was lethal to cells at a concentration of 0.1%. The best results were obtained with 0.01% egg yolk nontreated, with the addition of 0.02% egg yolk heat-inactivated. This mixture was used as a supplement to our serum-free medium (LM3o).

Egg yolk contains cholesterol and albumin in significant amounts. The results obtained after adding different concentrations of these two elements, either separately or

combined to the Grace medium containing 10% fetal bovine serum, are given in Table 2. Without heat treatment, cholesterol induced a decrease in the cell growth at all concentrations. At both 10 and 100 mg/liter, cells died rapidly after a few passages. At lower concentrations, about 1 mg/liter, cells multiplied slowly and the cultures could be maintained. There was a decrease of 26% in the number of cells after 5 days of culture and a slight increase in doubling time. The cell adhesivity was not affected and the percentage of nonadherent cells decreased at the 1 mg/liter concentration. Cholesterol appears to inhibit cell growth.

With heat-treated cholesterol, cell growth was affected little. A small increase in the number of cells was noted at the 1 mg/liter concentration (10.5%). Cell adherence however was affected, with a significant increase of the percentage of nonadherent cells (×3).

The presence of albumin produced similar responses. At the 5 mg/liter concentration, cell growth was significantly reduced. Heat-treated albumin induced the same response as did denatured cholesterol. There was an increase in the number of nonadherent cells correlated with the increase of the concentration of albumin even though the cell growth was not affected.

The presence of both cholesterol (1.6 mg/liter) and albumin (5 mg/liter), equivalent to 0.01% egg yolk, in Grace medium lacking fetal bovine serum did not promote the same degree of cell multiplication as occurred in the presence of fetal bovine serum. The results with LM3 medium were worse than those obtained with the egg yolk mixture.

*Cell behavior and adaptation to the new medium LM3o.* The passage of cells in the new LM3o medium provoked a cellular adaptation during the first subcultures (Fig. 1). Many cells died during the first two passages and small rounded cells could be observed (Figs. 1a and 1b). With the third passage, only the duration of the culture was affected (Fig. 1c), while from the fourth passage, cells grew normally (Fig. 1d).

Analysis of the growth kinetics (Fig. 2) indicates that, during the first passage in LM3o, 8 days were required to obtain a yield (in comparison with the 5 days necessary in Grace medium containing 10% fetal bovine serum) and a 20% decrease in the number of viable cells was observed. At the second passage, 12 days were required, but only if the medium is replaced after 7 days. During these two passages, the growth kinetics showed two phases. Initially, the cell growth was very slow (doubling time 5 days). From the fourth day, the culture developed normally with a doubling time of 48 hr, but still slower than in Grace medium containing 10% fetal bovine serum. If 0.5%

TABLE 2

NUMBER OF CELLS AFTER A CULTURE OF 5 DAYS IN GRACE MEDIUM CONTAINING OR NOT CONTAINING CHOLESTEROL AND ALBUMIN

| Cells | Grace medium (G) | G + cholesterol (1 mg/liter) | | G + cholesterol (10 mg/liter) | | G + albumin (5 mg/liter) | | G + albumin (50 mg/liter) | | G + albumin (500 mg/liter) | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Normal | Treated[a] | Normal | Treated | Normal | Treated | Normal | Treated | Normal | Treated |
| Total cells[b] | 2.85 | 1.82 | 3.05 | 1.45 | 2.80 | 1.78 | 2.94 | 1.60 | 2.80 | 1.05 | 3.00 |
| Adherent cells[b] | 2.50 | 1.72 | 2.55 | 1.26 | 1.50 | 1.58 | 2.57 | 1.35 | 2.30 | 0.95 | 1.75 |
| % of nonadherent cells | 12.3 | 5.5 | 16.4 | 13.1 | 46.4 | 11.2 | 12.6 | 15.6 | 17.9 | 9.5 | 41.7 |
| Doubling time[c] | 34 | 36 | 30 | 40 | 34 | 36 | 34 | 38 | 36 | 44 | 30 |

[a] Heat inactivation in water bath during 30 min at 60°C.
[b] Values expressed in million cells.
[c] Values expressed in hours.

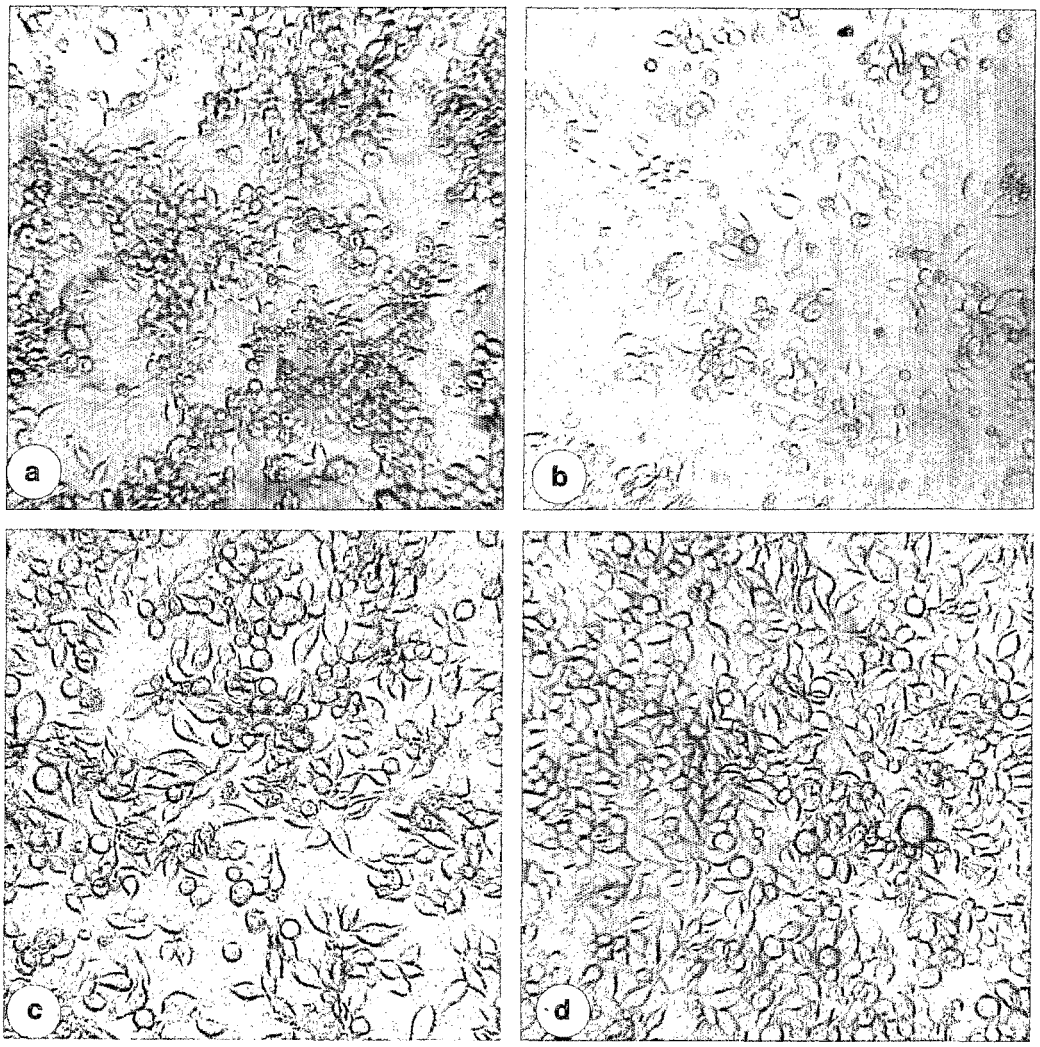


FIG. 1. *Choristoneura fumiferana* cell line after a culture of 5 days in LM3o medium. Cellular adaptation to the new medium. (a) Cells during the first passage (×141). (b) Cells during the second passage (×141). (c) Cells during the third passage (×141). (d) Cells during the fourth passage (×141).

fetal bovine serum was added at this point, the culture did not show any adaptation.

On the third passage, the growth kinetics showed a reversed pattern compared with those of the first two. During the first 3 days, the doubling time was about 34 hr as also found with Grace medium. From the fourth day, there was a decrease in cell growth and the doubling time increased to 65 hr.

From the fourth passage, the growth kinetics became identical to those observed with Grace medium containing 10% fetal bovine serum. The exponential multiplication of cells began after 24 hr. By the end of the culture (5 days) $3.35 \times 10^6$ cells were counted in the Petri dishes, of which less than 1% were floating in the medium. The doubling time was about 34 hr.

After passages in the LM3o medium, the four established cell lines performed as well as in Grace medium containing 10% fetal bovine serum while the adherence was increased by about 20%.

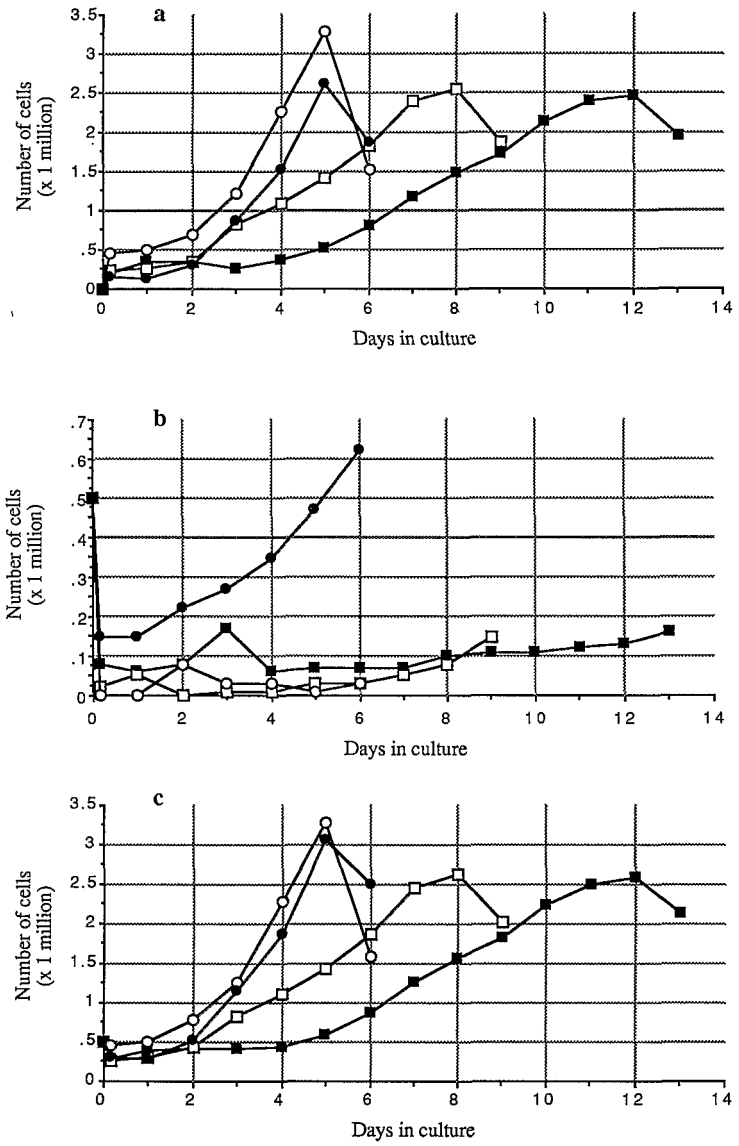


FIG. 2. Comparison of the growth kinetics of the *Choristoneura fumiferana* cell line in LM3o medium at different passages and in Grace medium. (a) Adherent cells. (b) Nonadherent cells. (c) Total viable cells. (○) During the first passage in LM3o medium. (●) During the second passage in LM3o medium. (□) During the fourth passage in LM3o medium. (■) In Grace medium.

## DISCUSSION

Results obtained using the new fetal bovine serum-free medium (LM3o), show that it is possible to multiply a number of lepidopteran cell lines in the same medium. The modification of various components in the Grace medium, as described above, resulted in a significant improvement in the efficacy of the medium, particularly in the increase of the cell adherence to the flask walls.

The fetal bovine serum contains 0.2 to 0.3 μg/ml of fibronectin (Guichard-Balestrini, 1987). In vertebrate cell cultures, fibronectin is synthesized by cells under nor-

mal conditions (Yamada and Olden, 1978). The elimination of fetal bovine serum results in the removal of the supply of fibronectin. It induces poor synthesis of this molecule by the cells, with cell deformation and detachment from the flask walls (Bornstein et al., 1978), probably due to the abscence of components necessary for the synthesis of fibronectin. The presence of $Mg^{2+}$, $Fe^{2+}$, and $Cu^{2+}$ ions in the chemically defined medium increased the cell adherence. Similarly, the addition of polyamines, which act on the crosslink cell wall components and replace $Mg^{2+}$ in certain reactions, also stimulates cell adhesivity (Cohen and McCormick, 1979). Polyamines represent viral components such as Densonucleosis viruses. Thus virus multiplication could be stimulated (Cohen and McCormick, 1979).

Furthermore, the absence of fetal bovine serum eliminates the supply of cholesterol, necessary for making cell walls (Gachelin, 1980), and albumin. These substances represent the main components of the serum. The results obtained after the addition, either separately or in combination of these elements, indicate that very low concentrations of them are essential for cell growth and adherence. Indeed, a toxic effect was noted if the concentration of which exceeded 5 mg/liter. Thus, the quantity of egg yolk added to the medium needs to be carefully calculated to avoid such toxic effects. To avoid toxicity due to high concentrations of cholesterol, only 0.01 to 0.05% egg yolk must be used. The results obtained by Röder (1982) with 0.5 to 1% egg yolk, representing a concentration of 100 mg/liter cholesterol and 48 mg/liter albumin, do not agree with this result.

Other essential elements are certainly present in the egg yolk, such as hormones, growth factors, and vitamins. This explains why the cells did not grow as well in the LM3 medium supplemented with the cholesterol and albumin mixture as they did with the total fraction of egg yolk.

The cell adaptation to the new medium observed during the first four passages is typical of cell behavior when stressed. Only half of the cells adhered to the flask walls when they were subcultured, representing $2 \times 10^5$ cells for C35 Petri dishes. This result is probably correlated to the absence of the fibronectin from the medium. Only those cells which adhered could multiply and their doubling time was greater than that under normal conditions. In fact, the adaptation of cells and the synthesis are altered.

The time required to obtain a yield which was 12 days at the second passage decreased regular thereafter, until the fifth passage, when the cells had stabilized. This stabilization was achieved after 1 month. Whatever the line used, the adaptation occurred in the same time period. All the lines tested grew in our serum-free medium (LM3o).

The presence of very low concentrations of egg yolk decreases the price of the medium considerably. This medium makes the rapid, large scale culture of these cell lines possible. These techniques which are now being developed by different laboratories (Weiss et al., 1981) will be served by a such medium. Its wide range activity and its low cost greatly assist in the production of insect viruses. For example, the Densonucleosis virus of *Casphalia extranea,* which is multiply in our laboratory on the *B. mori* cell line (in press), will be produced in large quantities for molecular studies or for use in biological control.

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