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Effect of Different Amino Acids and Vitamins on Lepidopteran Cell Cultures

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The composition of the medium is an important factor in the regulation of cell growth and adherence. We have attempted to stimulate those properties by adding some amino acids or vitamins to the culture medium. The results obtained on the *Choristoneura fumerana* (Lepidoptera, Tortricidae) cell line show that the augmentation of the concentration of folic acid (×10) increases the cell adherence (only 0.15×10^6 supernatant cells) and stimulates their multiplication (more than 20%). The same results are obtained with a mixture containing high concentrations of all the Grace' vitamins. Choline chloride does not modify cell behavior. An amino acid mixture only stimulates adherence. The stimulation of the production of adhesion factors represents a first stage in the elimination of fetal bovine serum from media, which ordinarily supply those factors. The mass production of cells at a cheaper cost and the multiplication of insect viruses could permit the replacement of chemical agents for biological control.

KEY WORDS: *Choristoneura fumiferana;* folic acid; cell growth; cell adherence.

INTRODUCTION

During culture, cells are normally able to adhere to the flask walls. This faculty is an important factor in infecting cells with viruses. The factors which affect adhesion have been poorly understood. In the last few years, studies of vertebrate cell lines have shown the importance of certain factors such as fibronectin (Barnes, 1987; Guichard-Balestrini, 1987). The composition of the medium can also affect cell growth and adhesion (Priston, 1985; Summers and Smith, 1987).

In this study, we have tried to improve the adhesion characteristics of the medium by varying the concentration of several components. Some earlier studies made on a Drosophila cell line (unpublished) showed that the growth rate and adhesion of these cultures could be increased by adding a number of vitamins. We have tested the effect of this mixture on a lepidopteran cell line to determine which were the most important vitamins. Other factors were also tested.

MATERIALS AND METHODS

Cell Cultures

The continuous cell line CF131 (gift from Dr. Devauchelle, St. Christol-les-Alès) used in these studies was derived from the ovarioles of *Choristoneura fumiferana* (Lepidoptera: Tortricidae).

Cells in more than 100th passage were cultured in 25-cm^2 polystyrene tissue culture flasks (Nunc) containing 4 ml of growth medium. Monolayers attained full confluency after incubation at 28°C for 5 days. At the sixth day, cells often displayed degeneration. Cells become detached from flask walls and die rapidly.

Confluent monolayers were detached with gentle agitation and cell viability was determined in a hemocytometer after staining with 0.2% trypan blue.

For subsequent subculturing, 2×10^6 cells were planted in 25-cm^2 flasks containing 4 ml of growth medium.

Cryopreservation of Cells

For freezing and cryopreservation, the monolayers, 80% confluent, were detached and viable cells were adjusted to 5×10^6 cells/ml with fresh medium containing 10% fetal bovine serum and 10% dimethyl sulfoxide. The preparation was dispersed into sterile cryotubes (Nunc) and frozen for 1 hr

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at −20°C, overnight at −70°C, and in nitrogen for conservation.

Medium

Antheraea Grace medium (Grace, 1962) containing 10% fetal bovine serum was used for this study according to formulation of EUROBIO catalog, except that β-alanine was eliminated from the medium (in preparation).

Growth Kinetics and Adhesion

Kinetics were performed in 35-mm Petri dishes. Cells (5×10^5) were planted with 2 ml of new growth medium. Cells from supernatant were removed and added to fresh medium. Adherent cells were scraped and the two populations were counted and their viabilities determined. Cells were maintained for three passages in the different media to see if any subsequent adaptation could be detected.

Assays with Amino Acids and Vitamins

A mixture of six amino acids was tested, which contained hydroxyproline and isoleucine (500 mg/liter), methionine and phenylalanine (1000 mg/liter), and tyrosine and valine (1500 mg/liter). A mixture of 10 vitamins, including biotin, pantothenate, riboflavin, and thiamine (0.1 mg/liter), niacin (0.2 mg/liter), paraaminobenzoate (0.4 mg/liter), folic acid, myoinositol, and pyridoxine (0.5 mg/liter), and choline chloride (20 mg/liter) was also tested. The effect of individual vitamins was tested using folic acid (0.02, 0.2, and 1 mg/liter) and choline chloride (0.2 and 20 mg/liter).

RESULTS

Effect of Amino Acid and Vitamin Mixtures

Influence on cell growth. As seen in Figure 1, differences occur by the second day of culture. The multiplication of the adherent cells was approximately the same with or without the addition of the amino acid mixture. The doubling time was also approximately the same (30 hr). As the number of supernatant cells was greater without the amino acid mixture, the total number of viable cells, after 5 days of culture, also increased (Table 1: 2.97×10^6 instead of 2.60×10^6).

With the addition of the vitamin mixture

TABLE 1
EFFECT ON CELL GROWTH OF DIFFERENT AMINO ACIDS AND VITAMINS[a,b]

Days of culture	Grace (G)	G + amino acids mixture (a)	G + vitamins mixture (b)	G + folic acid 0.2 mg/liter	G + folic acid (1 mg/liter)	G + choline chloride (20 mg/liter)
2	0.55	0.53	0.70	0.76	0.67	0.60
3	1.15	0.95	1.30	1.57	1.10	1.07
5	2.97	2.60	3.25	3.25	2.75	2.90
6	2.49	1.52	2.34	2.93	2.35	2.39

[a] All values are expressed in 10^6 cells.

[b] G—Grace medium EUROBIO without β-alanine and containing asparagine (350 mg/liter), isoleucine and methionine (50 mg/liter), valine (100 mg/liter), phenylalanine (150 mg/liter), no hydroxyproline. All vitamins were at the concentration of 0.02 mg/liter, except biotin (0.1 mg/liter) and choline chloride (0.2 mg/liter).

a—hydroxyproline and isoleucine (500 mg/liter), methionine and phenylalanine (1000 mg/liter), and asparagine and valine (1500 mg/liter).

b—biotin, pantothenate, riboflavin, and thiamine (0.1 mg/liter), niacin (0.2 mg/liter), paraaminobenzoate (0.4 mg/liter), folic acid, myoinositol, and pyridoxine (0.5 mg/liter), and choline chloride (20 mg/liter).

ulated (more than 20%) and the number of total viable cells increases to 3.25×10^6. In this case the doubling time is about 27 hr.

Influence on cell adhesion. After 2 days of culture, some significant differences appeared in the adhesion of cells. The proportion of the supernatant cells, which represented 42% if cells were grown in the Grace medium (Table 2), decreases in the two mixtures tested to approximately 20 or 25% of the total viable cells (1.5×10^5 cells instead of 2.3×10^5).

The differences were more important after the third day. The proportion of the supernatant cells decreased to 7%, representing 2×10^5 cells (less than half those obtained under normal conditions). During the culture time, the number of the supernatant cells remained between 1.5 and 2×10^5 cells. This number increased proportionately in the Grace medium.

Effect of Folic Acid

Influence on cell adhesion. The effects obtained using folic acid depended upon the concentration used (Table 1). At concentrations below 0.1 mg/liter, the results obtained were the same as those found for the vitamin mixture. Above this concentration, the number of supernatant cells increased more than 20%, representing 6×10^5 cells.

Influence on cell growth. From Figure 2, it is clear that the concentration of folic acid influenced cell growth in the same way as was found for cell adhesion. Differences were observed from the second day of culture. Using 0.2 mg/liter, the number of cells was the same as that recorded for the vitamin mixture (3.25×10^6). At concentrations less than 1 mg/liter, cell multiplication rates were comparable to those found using the Grace medium (growth increased by 10%). The differences were greater for the adherent cells (2.15×10^6 instead of 2.5×10^6), representing a reduction of 15% and an augmentation of the doubling time to 34 hr.

Effect of Choline Chloride

No effects, on either cell growth or cell adherence, was found when the concentration of the choline chloride, which was high in Grace medium, was multiplied ×100.

Effect of Passages

With all media tested, cell growth and adherence showed no change after the first passage.

DISCUSSION AND CONCLUSIONS

Cell adhesion results can be compared with those obtained in another study on the

TABLE 2
EFFECT ON CELL ADHESION OF DIFFERENT AMINO ACIDS AND VITAMINS[a]

Days of culture	Grace (G)	G + amino acids mixture (a)	G + vitamins mixture (b)	G + folic acid 0.2 mg/liter	G + folic acid 1 mg/liter	G + choline chloride (20 mg/liter)
2	42	25	24	21	37	43
3	25	15	15	11	36	25
5	15	8	7	5	22	19
6	45	32	11	9	55	36

[a] All values expressed as nonadherent cells as percentage of total viable cells.

stimulation of growth of adherent cells by the elimination of the supernatant cells during the first hour of the culture (unpublished). These results show that, during culture, under normal conditions, 4% of adherent cells float in the supernatant, which represents 1.5×10^5 cells. Twenty percent of these cells died in the medium in the last few hours and the number of cells remains at this stage. If these cells are not eliminated, they multiply slowly, and the number of supernatant cells increases during culture.

In this study, we obtained similar results without removal of the supernatant cells by adding the two mixtures tested to the medium or by increasing the concentration of folic acid to 0.2 mg/liter.

Cell multiplication can also be stimulated by increasing the growth rate of adherent cells, but only by increasing the level of certain vitamins. Other vitamins, such as choline chloride, did not affect the culture. Pantothenic acid was found to decrease the cell multiplication (manuscript in preparation).

These results show that it is possible to increase the degree of cell adhesion by modifying the culture medium. In vertebrate cell culture, it is well known that some factors such as fibronectin stimulate this adhesion (Yamada and Olden, 1978). Fibronectin occurs in fetal bovine serum, at 0.2 to 0.3 μg/ml (Guichard-Balestrini, 1987), and is also synthesized by cells (Yamada and Olden, 1978). So, it suggests that the production of such adhesion factors could be stimulated in insect cell cultures.

The stimulation of production of adhesion factors is the first stage in the elimination of fetal bovine serum from culture media. This should, in turn, reduce the mass

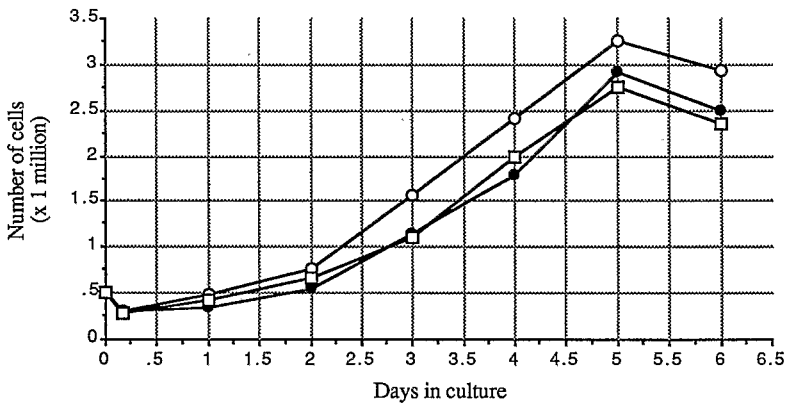

FIG. 2. Effect on cell growth of the augmentation in the concentration of folic acid ●, folic acid (0.02 mg/liter); ○, folic acid (0.2 mg/liter); □, folic acid (1 mg/liter).

production costs necessary in the multiplication of insect viruses for biological control. Decreasing in the cost of production of such viruses means they could provide a real alternative to chemical controls.

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