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Letter to the Editor

POLYAMINE AS A GROWTH PROMOTER FOR CULTURED INSECT CELLS

Dear Editor:

The cell line, NIH-SaPe-4, which was derived from embryos of the flesh fly, *Boettcherisca peregrina*, (10) could be cultured for more than 200 passages in a complex, chemically defined medium, MTCM-1520 (6). The cell line has been known to produce an autocrine growth factor (4), and it can continue to multiply without supply of the growth factor if the population density is kept reasonably high. In the course of simplifying the composition of the medium, it was found that at least one polyamine, either putrescine, spermidine, or spermine, is necessary for the proliferation of the flesh fly cells.

The cell line NIH-SaPe-4 had been cultured in Mitsuhashi and Maramorosch's medium (MM) (8) with 3% fetal bovine serum (FBS) up to the 350th passage from its establishment. Then the cells were adapted to the serum-free MM medium, and further adapted to a chemically defined medium, MTCM-1520 (6) at the 430th passage. The cells at the 550 to 600th passages were used for experiments. The cell line was proved free of mycoplasma by the RIKEN Cell Bank. All of the cultures were passed at a split ratio of 1:2, because the cells in the chemically defined medium showed strong density dependency.

For experimental cultures, I formulated various experimental media from the basic medium, MTCM-1520, by removing the constituents singly or as a group. When the cells established confluency in the basic medium, half of the cell suspension was replaced with an experimental medium. When this culture recovered the cell density, half of the cell suspension was replaced with the same experimental medium. The cells were passed for 20 passages in this manner. In the previous study (7), the media which supported the cell growth for 20 passages all supported the unlimited cell passages. Therefore, in the present study if an experimental medium lacking one of the components supported cell growth for more than 20 passages, the component which was omitted from the basic medium was considered nonessential for the growth of these cells. Complete replacement of the medium at one time was avoided, because it caused immediate cell death, probably due to loss of an autocrine growth factor in the medium. In this way, the requirement for each component was determined. All of the cultures were maintained at 25° C and the experiments were run in triplicate.

The basic medium contained 11 fatty acids. Their concentrations (μg per 1000 ml) were oleic acid (75), palmitic acid (75), palmitoleic acid (75), myristic acid (75), stearic acid (75), linoleic acid (75), linolenic acid (75), arachidonic acid (15), docosahexaenoic acid (25), eicosapentaenoic acid (25), and octadecatetraenoic acid (25). These fatty acids did not affect cell growth when removed from the basic medium one at a time. Also, cells could be continuously cultured when all of these fatty acids were removed altogether (data not shown).

The basic medium contained four diglycerides and seven triglycerides. Their concentrations (μg per 1000 ml) were 1,3 dimyristin (50), diolein (50), dipalmitin (50), 1,3 distearin (50), trilaurin (50), trilinolein (50), trilinolenin (50), trimyristin (50), triolein (50), tripalmitin (50), and tristearin (50). These glycerides did not affect cell growth when removed from the basic medium singly or altogether (data not shown).

The basic medium contained three sterols and one phospholipid. Their concentrations (μg per 1000 ml) were cholesterol (550), β-sitosterol (550), stigmasterol (550) and L-α-phosphatidylcholine (150). These lipids could be removed from the basic medium singly or altogether without detrimental effects on cell growth (data not shown).

The basic medium contained four lipid-soluble vitamins. Their concentrations (μg per 1000 ml) were calciferol (150), menadione (150), retinol acetate (174) and α-tocopherol acetate (6). These vitamins could be removed singly or altogether from the basic medium without detrimental effects on cell growth (data not shown).

From the above results, it became evident that NIH-SaPe-4 cells did not require any lipid or lipid soluble vitamins. The cells could be cultured continuously in the basic medium from which all the lipids and lipid-soluble vitamins were removed (data not shown). Therefore, experiments were conducted thereafter with the lipid-free and lipid-soluble vitamin-free MTCM-1520 medium (MTCM-1520 LF) as the basic medium.

The MTCM-1520 LF medium contained some nucleic acid-related compounds. Their concentrations (μg per 1000 ml) were adenine (5000), adenylic acid (100), 2-deoxyribose (250), guanine hydrochloride (150), hypoxanthine (20 000), *d*-ribose (250), thymine (150), uracil (150) xanthine (150), adenosine 5′-triphosphate disodium salt (5), and adenosine 5′-monophosphoric acid (0.1). They could be removed from the MTCM-1520 LF medium singly or altogether without deleterious effects on cell growth (data not shown).

The MTCM-1520 LF medium contained three polyamines. Their concentrations (μg per 1000 ml) were putrescine (2), spermidine (2), and spermine (2). When these polyamines were removed from the MTCM-1520 LF medium singly, the cells could be passed continuously (data not shown). However, when all of the polyamines were removed altogether, the cells could not be passed more than nine passages (Fig. 1). When only one polyamine was put back into the polyamine-deficient MTCM-1520 LF medium, any one of these three polyamines could support the continuous passages of the cells (Fig. 1). Among these three polyamines, spermine supported cell passages most strongly.

The MTCM-1520 LF medium contains several compounds which are not regular components of many insect cell culture media. These substances and their concentration (mg/1000 ml) were L-hydroxyproline (19.5), cysteine hydrochloride (0.1), DL-α-aminobutyric acid (3.98), *o*-phosphorylethanolamine (4), taurine (2), L-ascorbic acid (0.43), cyanocobalamine (2), glutathione (0.0025), and iron (III) nitrate enneahydrate (0.05). These substances could be removed from

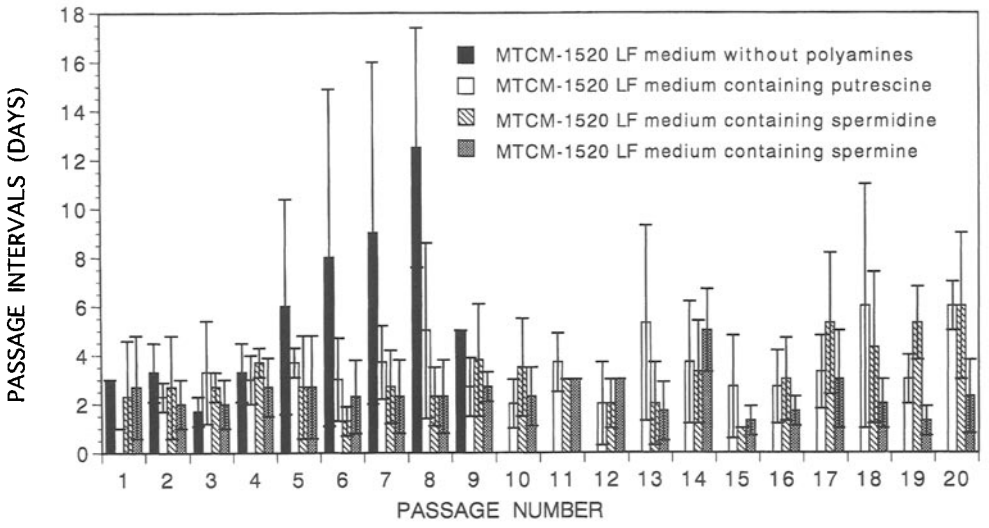

FIG. 1. Passage intervals up to the 20th passage when the NIH-SaPe-4 cell line was cultured in the medium lacking all the polyamines or in the medium containing one polyamine. The *vertical lines* at the *top* of the *bars* indicate standard deviation for experiments run in triplicate.

TABLE 1

THE COMPOSITION OF MTCM-1583 MEDIUM[1]

Ingredient	mg/100 ml	Ingredient	mg/100 ml
NaCl	700	L-Tryptophan	10
NaH_2PO_4	20	L-Tyrosine	10
$NaHCO_3$	12	L-Valine	6
KCl	20	Glucose	300
$CaCl_2 \cdot 2H_2O$	20	Fructose	20
$MgCl_2 \cdot 6H_2O$	10	Sucrose	496
L-Arginine-HCl	33	Malic acid	30
L-α-Alanine	15	α-Ketoglutaric acid	15
L-Asparagine	20	Succinic acid	3
L-Aspartic acid	70	Fumaric acid	3
L-Cystine	20	Sodium acetate	3
L-Glutamic acid	50	Spermine	0.6
L-Glutamine	30	Thiamine-HCl	0.016
Glycine	15	Riboflavin	0.016
L-Histidine	25	Pyridoxine-HCl	0.016
L-Isoleucine	30	Niacin	0.016
L-Leucine	30	Calcium pantothenate	0.016
L-Lysine	30	Inositol	0.016
L-Methionine	15	*p*-Aminobenzoic acid	0.016
L-Phenylalanine	8	Choline chloride	0.016
L-Proline	15	Biotin	0.0016
L-Serine	20	Folic acid	0.016
L-Threonine	10		

[1]pH: 6.3 with KOH.

the MTCM-1520 LF medium singly or altogether without deleterious effects on cell growth (data not shown).

Then, based on the MTCM-1520 medium from which all of the above components except spermine were omitted, and whose inorganic salts were replaced with a simpler salt solution (1), a simplified chemically defined medium (MTCM-1583) was formulated (Table 1). In this medium the NIH-SaPe-4 cells could be passed continuously. The population doubling time of the cells in this medium was 2.22 d at 25° C.

From the above experiments, it became evident that no lipid and lipid-soluble substances were essential for the growth of the NIH-SaPe-4 cells. In mammalian cell cultures, a lipid- and protein-free chemically defined medium has been developed (5), whereas in insect cell cultures, most media contain some natural substances, and essentialness of lipid and lipid-soluble substances has been obscure. Also, some nucleic acid-related substances were found unnecessary.

A polyamine, either putrescine, spermidine or spermine, was found essential for the continuous multiplication of the NIH-SaPe-4 cells. Polyamines have been used as a component of culture media for mammalian cells (3,12). In insect cell culture media, only the medium of Wilkie et al. (11) contains three polyamines, putrescine, spermidine and spermine. In mammalian cell cultures, only putrescine has been used. Putrescine is said to be toxic to the cells at relatively high concentration but increase the plating efficiency of mouse L cells (3) if the concentration is adequate. Yamane (12) successfully developed a primary culture medium for mammalian cells by fortifying Eagle's MEM with a supplement containing putrescine. Although the mechanism of putrescine action is unknown, spermidine and spermine have been known as growth factors for microorganisms (9). In insect cell cultures, effects of adding polyamines to culture media has not been shown. In the present study, putrescine, spermidine, and spermine were all effective in supporting continuous cell growth, spermine being the most effective. Because putrescine and spermidine are the precursors of spermine, spermine may be the real effective substance, and insect cells may have the ability to synthesize spermine from putrescine via spermidine. In Chinese hamster cell cultures, Ham (2) reported that putrescine, spermidine, and spermine were all equally active in promoting the cell growth in the absence of fetuin.

The essentialness of a polyamine was also confirmed by omitting it from the MTCM-1520 LF medium. Without a polyamine the NIH-SaPe-4 cells could not be passed continuously. Because an autocrine growth factor is present in the medium of the NIH-SaPe-4 cell cultures (4), a polyamine may be necessary for the action of the growth factor. In the MTCM-1583 medium, population doubling time of the flesh fly cell line at 25° C was 2.2 d, which is comparable to that in the MTCM-1520 medium (2.17 d) as well as that in MM medium (2.27 d) (6). Lipid, lipid-soluble vitamins, nucleic acid-related substances, and some other substances were successfully eliminated

from MTCM-1520 medium, but not one of polyamines. Ham (2) reported that putrescine can replace fetuin. Because most insect cells need FBS and fetuin is a protein of FBS, a polyamine may be used in lieu of FBS for insect cell culture media.

The MTCM-1583 medium has relatively simple composition and can be prepared easily. It is a protein-free, lipid-free, chemically defined medium used for the first time for insect cell culture media. It is useful for biochemical studies of insect cells. Although only the flesh fly cell line, NIH-SaPe-4, has been adapted to this medium at present, other insect cell lines will adapt to it or a modification of it. The cell line, NIH-SaPe-4, is available from the RIKEN Cell Bank (Koyadai, Tsukuba City, Ibaraki 305, Japan) upon request.

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