Cultivation of V79 Cells in Serum-Free Medium Containing Bovine Serum Albumin and the Use of Gelatin as an Attachment Factor

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V79 cells were cultivated in a serum-free medium. When a serum-free, bovine serum albumin-containing medium was used, removal of trypsin after cell detachment during subcultivation was necessary for routine mass cultivation. For colony formation addition of an attachment factor to the medium was further necessary. Gelatin was proved to serve as a good attachment factor in the colony formation and it could be substituted for fibronectin as an attachment factor for serum-free cultivation of many types of cells. ——— serum-free medium; removal of trypsin; attachment factor; gelatin

Cultivation of animal cells is usually carried out in a basal mixture of amino acids, vitamins, sugars and salts, supplemented with serum. Serum contains various factors which are of critical importance for the maintenance, growth, and/or differentiation of cells in vitro. Although intensive studies have been made to clarify the role of these factors (Temin et al. 1972; Brooks 1975), they have been unsuccessful from the viewpoint of standardizing commercial preparations. For instance, growth-promoting activity of serum is different from batch to batch, and there is no ensured way to obtain serum batches of uniform quality for cell culture. Even more bothering is that a serum batch which is suitable for one type of experiment is not necessarily good for another type of experiment. The use of serum also complicates the testing of chemicals; the effects of which may be influenced by serum components. In such cases, the use of serum-free medium is essential.

Apart from these practical viewpoints, establishment of serum-free, chemically-defined media has been an important research goal for the clarification of physiological and nutritional characteristics of mammalian cells (Phillips and Cristofalo 1981; Walthall and Ham 1981). Sato's group has succeeded in establishing

serum-free, chemically-defined media for some cell lines or strains; and the media contain hormones, growth factors, binding proteins, and attachment factors (Barnes and Sato 1980). Yamane's group has formulated much simpler serum-free media in which bovine serum albumin Fr. V is added (Yamane 1978).

In order to eliminate the problems caused by the use of serum, we tried to use serum-free medium in various experiments. When V79 cells were cultivated in a serum-free medium formulated by Yamane et al. (1976), the medium could not support constant growth of the cells, or colony formation. Therefore, it was necessary to improve the culture conditions or to add some other factors to the medium. In the present communication, we report that the elimination of trypsin at the time of subcultivation and the use of gelatin as an attachment factor improved the growth and colony formation of V79 cells in a serum-free medium.

MATERIALS AND METHODS

Cells. V79 cells were derived from Chinese hamster lung (Chu et al. 1969). Primary cultures of Syrian hamster embryo cells (SHEC) were prepared by trypsinization of 13-day-old embryo carcasses. After cultivation for 2 or 3 days, the cells were processed and stored at -80°C until use. SHEP-T clones were cell lines transformed by treatment of SHEC with N-methyl-N'-nitro-N-nitrosoguanidine as described previously (Umeda and Enaka 1981).

Medium and other chemicals. All cell lines had been maintained in a medium consisting of Eagle's minimum essential medium (MEM) containing 10% fetal bovine serum (FBS). Serum-free medium employed in the present study was based on the formula reported by Yamane et al. (1976) for the cultivation of Yoshida sarcoma. Our preliminary experiments showed that elimination of the heavy metal ions, Zn^{++} and Fe^{++} , from the original formula did not affect the growth of V79 cells. Therefore, the metal compounds were omitted in the present medium (SF-F) which consisted of MEM, Eagle's non-essential amino acids, 0.5% bovine serum albumin Fr. V (BSA), $1\text{ }\mu\text{g/ml}$ thymidine, and $4\text{ }\mu\text{g/ml}$ hypoxanthine.

Gelatin was prepared as a 1% solution in Ca^{++} - and Mg^{++} -free phosphate-buffered saline (CMF-PBS) or in double distilled water, and autoclaved. The solution could be maintained at 4°C or at room temperature without any loss of activity at least for a month.

MEM and Eagle's non-essential amino acids were obtained from GIBCO, Grand Island, NY; BSA, from Armour Pharmaceutical Co., Chicago; insulin, from Shimizu Seiyaku Co., Ltd., Tokyo; gelatin, from Difco Lab., Michigan; Vitrogen 100TM as a collagen source, from Collagen Corp., Calif.; dispase, from Godo Shusei Co., Ltd., Tokyo; and other chemicals, from Wako Pure Chemical Industries, Ltd., Osaka.

Coating and filming of culture vessels. Coating of plastic dishes with gelatin was done as follows: A few ml of 1% gelatin in CMF-PBS were poured into dishes, usually kept at room temperature approximately for 15 min and then the excess of gelatin was removed. Dishes were used with or without washing twice with Hanks' balanced salt solution. In some experiments dishes were treated with FBS as with gelatin.

In case of preparing gelatin- or collagen-filmed dishes, a few ml of 1% gelatin in water or collagen solution (Vitrogen 100TM) were poured into dishes, kept at room temperature for a few minutes, and then the excess of solutions was removed. After air-drying, the dishes were kept at room temperature until use.

Cultivation. At subcultivation, cells were dissociated with 50 U/ml crystalline trypsin (Trypsilin, Mochida Pharmaceutical Co., Ltd., Tokyo), diluted with Hanks' balanced salt solution, and pelleted by centrifugation. The cell pellet was suspended in the SF-F medium at a density of over 3×10^4 cells/ml. The cells were then inoculated into new culture vessels. The culture was maintained at 37°C in a CO_2 incubator, with medium changes at 2- to 4-day intervals.

For colony formation, 100 cells washed with saline were plated in each dish, cultured for 10 days, fixed with methanol and stained with a Giemsa solution. More than 3 dishes were used for each treatment group.

RESULTS

Cultivation of V79 cells in serum-free medium. When V79 cells were transferred and cultivated in the SF-F medium under proper conditions, they grew steadily and could be split successively. Under improper condition, however, many cells tended to clump, and the cells did not attach and spread on the culture surface.

Our routine method for cell subcultivation in serum-supplemented medium was to trypsinize cells at 37°C for a few minutes, followed by dilution with serum-containing medium and inoculation into new culture vessels. When this method was used for culturing V79 cells in the SF-F medium, however, they did not attach to the culture surface. Tryptic digestion seemed to have a harmful effect on the cells. The use of trypsin at 4°C has been reported to have less harmful effects on cells than at 37°C (McKeehan 1977; Walthall and Ham 1981). When tryptic digestion was performed at 4°C, however, the cells were difficult to detach. Neither EDTA nor dispase addition was helpful for cell detachment. In contrast, when the cells were trypsinized at 37°C and washed once or twice with a sufficient amount of saline, they were subsequently able to attach and grow on the culture surface.

These results suggested that the harmful effects of trypsin at the subcultivation were due to a small amount of trypsin remaining in the SF-F medium. To pursue further this possibility, we tested the effect of trypsin on the cells already spread on the culture surface. Trypsin was added at a final concentration of 2 U/ml in the SF-F medium into a culture containing cells already spread and growing. This concentration of trypsin was chosen as an approximate residual concentration at the usual subcultivation procedure. The cells started to clump and detach after an hour or so. Thus, the harmful effect at the subcultivation was caused by residual trypsin carried over into the SF-F medium.

When the cells were cultivated in the SF-F medium with the above-described attention, mass cultivation of V79 cells continued for more than 10 months with a doubling time of about 20 hr.

Colony formation of V79 cells in SF-F medium. When V79 cells were inoculated at a small inoculum size for colony formation, few attached colonies were formed even though the above-described cautions were exercised. When the dishes were examined microscopically before fixation, floating cell clumps were observed in the medium. Therefore, it seemed necessary to use an attachment factor for colony formation.

The efficacy of various attachment factors added to the SF-F medium was compared by a colony formation technique (Table 1). Serum contains fibronectin, a well-known attachment factor (Yamada and Olden 1978). Dishes pretreated with FBS produced large colonies. Similar numbers of colonies were formed in gelatin-coated and also gelatin-filmed dishes. Colonies formed on collagen-coated

TABLE 1. *Formation of V79 cell colonies in SF-F medium in dishes treated with various substances*

Dish treatment	Plating efficiency	Size of colonies*
FBS-coated	57±12	Large
Gelatin-coated	63± 8	Large
Gelatin-filmed	64± 8	Large
Collagen-coated	64± 5	Small

* Size of colonies is defined as follows: mean diameter of colonies over 2.5 mm, very large; around 2 mm, large; around 1.5 mm, medium; around 1 mm, small; below 0.5 mm, very small.

dishes were similar in number but considerably smaller in size when compared with those in FBS-pretreated dishes. Therefore, we considered gelatin could be substituted for fibronectin as an attachment factor in the SF-F medium.

We exploited the optimum method of gelatin application. The cells suspended in gelatin-containing SF-F medium formed as large colonies as the cells seeded in gelatin-coated dishes. Gelatin had less effect when it was added after cell seeding. Dishes filmed with gelatin supported good colony formation. Fig. 1 showed a dose-dependency of the plating efficiency on gelatin concentration.

Fig. 1. Effect of gelatin concentration on V79 cell colony formation. The data are mean values $\pm$ s.d. from 4 dishes.

Growth of various other cells in SF-F medium. Although the SF-F medium used for V79 cells did not seem to be a universal formula for serum-free cultivation of many kinds of cells, the capacity of gelatin to support the growth of cells in the medium was examined (Table 2). When the cells did not grow in the SF-F medium supplemented with gelatin, 1% FBS was added to the culture medium.

Tertiary-cultured Syrian hamster embryo cells (SHEC) could not grow in the gelatin-containing SF-F medium. When 1% FBS was added to the medium, SHEC grew well in the gelatin-containing medium, but less in the medium without gelatin.

TABLE 2. *Growth capacity of various cells in SF-F medium with or without gelatin (mass cultivation)*

Additives to SF-F medium	V79	SHEC	SHEP-T		
			1 cl	4 cl's	4 cl's
Gelatin	##	—	+	±	—
None	+	—	±	—	—
1% FBS+gelatin	NT	+	+	±	+
1% FBS	NT	+	+	±~—	±~—

—: Cells were dead or detached from the culture surface.

±: Cells were maintained in the culture with little evidence of cell growth.

+: Slight cell growth was observed.

+: Fairly well cell growth was observed.

##: Abundant cell growth was observed.

NT: Not tested.

Several transformed cell lines (SHEP-T) from SHEC were also tested. All of these cell lines formed colonies in soft agar and tumors when inoculated into nude mice (unpublished data). One cell line could be maintained in the SF-F medium without gelatin, but it grew better in the medium with gelatin. Four cell lines could be maintained in the medium with gelatin but not without it. The other 4 cell lines could not grow in the SF-F medium even when gelatin was added. When 1% FBS was added to the medium, these cells grew a little, but the addition of gelatin provided little improvement in these cases.

DISCUSSION

Serum has been known to contain an anti-tryptic factor(s) and, therefore, dilution with serum-supplemented medium at the time of subcultivation leads to the inactivation of trypsin. In contrast, the BSA in our serum-free medium (SF-F) had no anti-tryptic action and the residual trypsin brought into the new culture medium exerted a harmful effect on V79 cells. Thus, inactivation or removal of trypsin was necessary during the subcultivation procedure. When trypsin was removed by washing before cell inoculation, we were successful in obtaining good culture conditions for V79 cells in the SF-F medium.

It has been reported that BSA has an inhibitory action on cell spreading (Grinnell 1976) and that cells could attach and spread on a culture surface which had already been coated with fibronectin produced by cells even when such culture surface was later treated with BSA (Grinnell and Feld 1979). It has been reported that fibronectin is produced not only by normal cells but also by malignant ones (Yamada and Olden 1978; Zardi et al. 1979). Therefore, in the case of mass cultivation of V79 cells in the SF-F medium, sufficient amounts of fibronectin which block the anti-attachment action of BSA may be produced by the cells. Indeed, our separate paper (Kan et al. 1982) shows that cells which produce fibronectin can grow in a serum-free medium, whereas cells which do not produce it cannot grow under such conditions.

Although V79 cells in mass culture could grow well in the SF-F medium in proper culture vessels, few attached colonies were observed when a small number of the cells were inoculated for colony formation. Actually, cell clumps were floating in the medium in these cultures. This fact suggested that addition of some attachment factor might help cell attachment and allow colony formation, because there is a possibility that the production of fibronectin by the small number of cells inoculated for colony formation was too little to overcome the anti-attachment effect of BSA.

As attachment factors in monolayer cultures, fibronectin (Yamada and Olden 1978), fetuin (Fisher et al. 1957), polylysine (McKeenhan and Ham 1976), collagen (Hauschka and Konigsberg 1966), and gelatin (Forkman et al. 1979) are known. Fibronectin has been used efficiently in most serum-free cultures (Hook et al. 1977; Orly and Sato 1979; Rizzino and Crowley 1980; Rockwell et al. 1980), but it is known to be unstable and very expensive. The effectiveness of polylysine does not seem universal. Collagen has also been used in various kinds of cell cultures as a substrate for cell attachment (Hauschka and Konigsberg 1966; Klebe 1974; Pearlstein 1976; Grinnell and Minter 1978; Wu et al. 1980). Gelatin was introduced by Yaffe (1973) as a substitute for collagen in the culture of rat muscle cells. Among these attachment factors, gelatin is the most inexpensive, stable, and easy to handle.

When FBS, gelatin and collagen were compared for their capacity to promote colony formation, gelatin was demonstrated to be as good an attachment factor as FBS. Gelatin is known to have a special affinity for fibronectin as well as collagen (Engvall et al. 1978). When gelatin is sufficiently present on the surface of culture vessels, the fibronectin on the cell membrane, even in small amounts, may be able to bind easily to the gelatin, thus allowing cells to attach and spread on the surface. This binding capacity would allow colony formation when the small number of cells were inoculated. In this connection, the use of gelatin-filmed dishes is practically very convenient.

Although we examined only one formula of serum-free medium, it is suggested that the removal of trypsin at subcultivation and the use of gelatin as an attachment factor are important procedures for improving cultivation of cells in other serum-free media.

References

- 1) Barnes, D. & Sato, G. (1980) Serum-free cell culture: a unifying approach. *Cell*, **22**, 649-655.
- 2) Brooks, R.F. (1975) Growth regulation *in vitro* and the role of serum. In: *Structure and Function of Plasma Protein*, edited by A.C. Allison, Plenum, New York, pp. 1-112.
- 3) Chu, E.H.Y., Brimer, P., Jacobson, K.B. & Merrian, V. (1969) Mammalian cell genetics. I. Selection and characterization of mutations auxotrophic for l-glutamine or resistant to 8-azaguanine in Chinese hamster cells *in vitro*. *Genetics*, **62**, 359-377.
- 4) Engvall, E., Rvoslanti, E. & Miller, E.J. (1978) Affinity of fibronectin to collagens of different genetic types and to fibrinogen. *J. exp. Med.*, **147**, 1584-1595.
- 5) Fisher, H.W., Puck, T.T. & Sato, G. (1957) Molecular growth requirements of

- single mammalian cells: The action of fetuin in promoting cell attachment to glass. *Proc. nat. Acad. Sci. USA*, **44**, 4–10.
- 6) Forkman, J., Haudenschild, C.C. & Zetter, B. (1979) Long-term culture of capillary endothelial cells. *Proc. nat. Acad. Sci. USA*, **76**, 5217–5221.
 - 7) Grinnell, F. (1976) Cell spreading factor: Occurrence and specificity of action. *Exp. Cell Res.*, **102**, 51–62.
 - 8) Grinnell, F. & Feld, M.K. (1979) Initial adhesion of human fibroblasts in serum-free medium: Possible role of secreted fibronectin. *Cell*, **17**, 117–129.
 - 9) Grinnell, F. & Minter, D. (1978) Attachment and spreading of baby hamster kidney cells to collagen substrata: Effects of cold-insoluble globulin. *Proc. nat. Acad. Sci. USA*, **75**, 4408–4412.
 - 10) Hauschka, B.D. & Konigsberg, I.R. (1966) The influence of collagen on the development of muscle clones. *Proc. nat. Acad. Sci. USA*, **55**, 119–126.
 - 11) Hook, M., Rubin, K., Oldberg, A., Obrink, B. & Vaheri, A. (1977) Cold-insoluble globulin mediates the adhesion of rat liver cells to plastic petri dishes. *Biochem. biophys. Res. Commun.*, **79**, 727–733.
 - 12) Kan, M., Minamoto, Y., Sunami, S., Yamane, I. & Umeda, M. (1982) A role of fibronectin and gelatin for cell adhesion in serum-free, BSA-containing medium. *Cell Struct. Funct.*, **7**, 245–252.
 - 13) Klebe, R. (1974) Isolation of a collagen dependent cell attachment factor. *Nature*, **250**, 248–251.
 - 14) McKeehan, W.L. (1977) The effect of temperature during trypsin treatment on viability and multiplication potential of single normal human and chicken fibroblast. *Cell Biol. int. Rep.*, **1**, 335–343.
 - 15) McKeehan, W.L. & Ham, R.G. (1976) Stimulation of clonal growth of normal fibroblasts with substrata coated with basic polymers. *J. Cell Biol.*, **71**, 727–734.
 - 16) Orly, J. & Sato, G. (1979) Fibronectin mediates cytokinesis and growth of the follicular cells in serum-free medium. *Cell*, **17**, 295–305.
 - 17) Pearlstein, E. (1976) Plasma membrane glycoprotein which mediates adhesion of fibroblasts to collagen. *Nature*, **262**, 497–499.
 - 18) Phillips, P.D. & Cristofalo, V.J. (1981) Growth regulation of WI38 cells in a serum-free medium. *Exp. Cell Res.*, **134**, 297–302.
 - 19) Rizzino, A. & Crowley, C. (1980) Growth and differentiation of embryonal carcinoma cell line F9 in defined media. *Proc. nat. Acad. Sci. USA*, **77**, 457–461.
 - 20) Rockwell, G.A., McClure, D. & Sato, G.H. (1980) The growth requirements of SV40 virus-transformed Balb/c-3T3 cells in serum-free monolayer culture. *J. cell. Physiol.*, **103**, 323–331.
 - 21) Temin, H.M., Pierson, R.W. & Dulak, N.C. (1972) The role of serum in the control of multiplication of avian and mammalian cells in culture. In: *Nutrition and Metabolism of Cells in Culture*, Vol. 1, edited by G. Rothblat & V.J. Cristofalo, Academic Press, New York, pp. 50–81.
 - 22) Umeda, M. & Enaka, K. (1981) Some aspects of *in vitro* carcinogenesis using Syrian hamster cells. In: *Gann Monograph on Cancer Research*. No. 27. *Mutation, Promotion and Transformation in Vitro*, edited by N. Inui, T. Kuroki, M. Yamada & C. Heidelberger, Japan Scientific Societies Press, Tokyo, pp. 183–194.
 - 23) Walthall, B.J. & Ham, R.D. (1981) Multiplication of human diploid fibroblasts in a synthetic medium supplemented with EGF, insulin, and dexamethasone. *Exp. Cell Res.*, **134**, 303–311.
 - 24) Wu, T., Smith, D. & Chang, L. (1980) Requirements of collagen-coated substratum for the growth of rat tracheal epithelial cells in culture. *J. supramol. Struct.*, Suppl. **4**, 179.
 - 25) Yaffe, D. (1973) Rat skeletal muscle cells. In: *Tissue Culture: Methods and Applications*, edited by P.F. Kruse, Jr. & M.K. Patterson, Jr., Academic Press, New York, pp. 106–114.

- 26) Yamada, K.M. & Olden, K. (1978) Fibronectin-adhesive glycoproteins of cell surface and blood. *Nature*, **275**, 179-184.
 - 27) Yamane, I. (1978) Development and application of serum-free culture medium for primary culture. In: *Nutritional Requirements of Cultured Cells*, edited by H. Katsuta, Scientific Societies Press, Tokyo, pp. 1-21.
 - 28) Yamane, I., Murakami, O. & Kato, M. (1976) Serum-free culture of various mammalian cells and the role of bovine albumin. *Cell Struct. Funct.*, **1**, 279-284.
 - 29) Zardi, L., Cecconi, C., Barbieri, O., Carnemolla, B., Picca, M. & Santi, L. (1979) Concentration of fibronectin in plasma of tumor-bearing mice and synthesis by Ehrlich ascites tumor cells. *Cancer Res.*, **39**, 3774-3779.
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