**77. α -Cyclodextrin, a Novel Substitute for Bovine
Albumin in Serum-Free Culture of
Mammalian Cells**

By Isao YAMANE, Mikio KAN, Yoshiki MINAMOTO,
and Yasuo AMATSUJI

The Research Institute for TB and Cancer,
Tohoku University, Sendai 980

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So far most mammalian cells have been cultivated with the use of serum-containing media. These several years G. Sato *et al.*¹⁾ succeeded in cultivating many kinds of cells in chemically defined media supplemented with various growth factors, but the most cells employed by them are limited to established cell lines. Also Walthall and Ham³⁾ recently reported successful culture of human diploid fibroblasts with a chemically defined medium supplemented with growth factors and lipids but their serial cultures were restricted only to three or four passage. We have so far employed bovine serum albumin (BSA) as a main serum substitute for the culture of various established and diploid mammalian cells.⁴⁾⁻⁷⁾ Further we have tried to search a substitute for BSA which can be a unsaturated fatty acids vehicle and recently discovered α -cyclodextrin included with unsaturated fatty acids can be at least feasible for such function. This paper briefly deals with the availability of lipids-included α -cyclodextrin as a BSA substitute.

Materials and methods. *Cells:* A human lymphoblast cell line named UMCL was employed which was transformed from cord blood by EB virus in authors laboratory. This cell line has been so far transferred in RPMI 1640 supplemented with fetal calf serum for about a year. Human diploid fibroblasts derived from embryo lungs (HEL) were maintained in serum-containing MEM in authors laboratory. These two kinds of cells were cultured as indicated in the previous reports.^{5),7)} *Basal Test Media:* In order to examine the growth promoting activity of various test compounds for UMCL cells, we employed RITC 56-1 medium supplemented with synthetic lecithin (2.5 mg/l), cholesterol (1 mg/l) galactose (500 mg/l) and mannose (500 mg/l).⁷⁾ For the culture of HEL we employed a serum-free chemically defined medium named RITC 80-7 as described previously.⁵⁾ For each experiment with HEL, the cells of confluent layer were harvested with cold trypsinization and plated in diameter

35 mm plastic Petri dish (Lux Co.) in the concentration of 2×10^4 cells/dish. The cultures were incubated in O₂-CO₂ incubator (Tabai Co., Osaka, Japan) at 37°C in 5% CO₂ and 7% O₂. *Tested Chemicals:* Various cyclodextrins were purchased from Wako Chemicals Co. Ltd., Osaka, Japan. Lipid samples were obtained from following sources: oleic and linoleic acids: Sigma Chemical Co. (St. Louis, Mo, USA), cholesterol: Eastman Kodak Co. (Rochester N.Y., USA), synthetic lecithin: Serdary Research Lab. (Ontario, Canada). *Preparation of fatty acids included with α -cyclodextrin:* After 1 g of α -cyclodextrin (α -CD) was dissolved in 7 ml of distilled water, 7 ml of ethanol dissolved with 10 mg oleic and linoleic acids was added to the above α -CD solution bubbled with nitrogen gas and stored with tight stopper. This was warmed in water bath at 75°C until the solution became opalescent, and left for 2 hr at room temperature and further for 20 hr at 4°C. Then after it was centrifuged at 3,000 rpm, the supernatant was discarded, while the precipitate lightly washed with ethanol and dried *in vacuo*. This was further washed two times with petroleum-ether and dried *in vacuo*. The above preparation method was made, referring Szejtli and Bánky-Elöd's paper.²⁾

Results. 1) *Cytotoxicity test of cyclodextrin (CD) and actual amount of fatty acids included in CD complex.* In order to examine the cytotoxicity of CDs, we employed UMCL cells grown in serum-supplemented RPMI 1640. 5×10^5 UMCL cells/ml were inoculated to RITC 55-9 medium containing various amounts of α - and β -CDs and incubated at 37°C for 4 days. α -CD did not show any cytotoxic effect for the above cultures to the amount of 2 g/l medium, whereas β -CD showed definite cytotoxic effect in the amount of more than 0.25 g/l as indicated in Fig. 1. γ -CD did not show any cytotoxic effect similarly to α -CD, but γ -CD is very expensive and further experiments were carried out with α -CD alone. The inclusion complex of α -CD with both oleic and linoleic acids was extracted with petroleum-ether and about half an amount of the added fatty acids was proved to be actually included (unpublished data).

2) *Growth promoting activity of included α -CD for UMCL cells.* As shown in Fig. 2, both α -CD included with oleic acid and that with linoleic acid, expressed growth enhancing effect in accordance with their supplemented amount up to the concentration of 100 mg/l medium, but the supplementation of the more amount displayed a conspicuous toxic effect. However, when 100 mg of α -CD included with both fatty acids and 1000 mg of free α -CD were supplemented to one liter of the medium, rather stable growth promoting effect of the inclusion complex was observed with high reproducibility. Fig. 3a shows the growth curve of UMCL cells cultured in the medium sup-

Fig. 1

Fig. 2

Figs. 1-2. 1: Cytotoxic effect of free α - and β -cyclodextrins for the growth of UMCL cells. The cells were inoculated in the density of 2×10^5 cells/ml in the presence of the figure plotted concentration of each cyclodextrin and incubated for 5 days at 37°C . 2: Growth response of UMCL cells for various amounts of α -CD included with oleic acid and linoleic acids in the serum-free medium. Each α -CD complex was included with oleic acid and linoleic acid respectively in the ratio of 1 g CD to 10 mg fatty acid. The cells were inoculated in the size of 5×10^5 and incubated for 4 days at 37°C .

Fig. 3. The growth curve of UMCL cells in the serum-free, α -CD complex containing medium (a) and the result of their serial transfer culture in the same medium (b).

plemented with both the inclusion complex and free α -CD, comparing that with serum-containing RPMI 1640. Both growth curves run roughly parallel, although that with the latter in medium is slightly more abundant than that with the former medium. Fig. 3b shows the result on the serial transfer culture of UMCL cells with the present serum-free medium and this medium is proved to support the stable long-term culture of UMCL cells.

3) *Growth promoting activity of α -CD inclusion complex for human diploid fibroblasts culture.* As made in the case of UMCL cell

culture, various amounts of α -CD included with the fatty acids were added to RITC 80-7 basal medium and its growth promoting activity was examined. 10 mg of the inclusion complex/l medium was found to be optimal in the case of HEL at a inoculum concentration of 2×10^4 cells/dish. But the growth in this serum-free medium was inferior to that in RITC 80-7 supplemented with 5 g/l of bovine serum albumin (BSA) in which the cells can grow just like in serum-containing MEM. However when the cells were inoculated to the medium supplemented with 10 mg/l α -CD complex and further added to this medium with the α -CD complex every other day in the final concentration of 10–20 mg/l, the growth was similar to that with the above BSA supplemented medium, as shown in Fig. 4.

Fig. 4. The growth curves of human diploid fibroblasts in the different serum-free media. The cells were cultured with RITC 80-7 (▲), BSA supplemented RITC 80-7 (●) and α -CD complex supplemented RITC 80-7 (○). Arrows indicate the addition of concentrated α -CD complex in the final concentration of 10 mg/l.

Discussion. So far we have developed serum-free media for the cells of both suspension and monolayer growth for their primary culture and their transfer.^{4),5),7)} But these serum-free media contain a pretty large amount of BSA. BSA is, needless to say, a serum protein which is often discrepant in its cell growth promoting action of each commercial preparation. Therefore we sought the effective compounds replacing to BSA. Cyclodextrins have been known to include various hydrophobic compounds and let them be water-soluble as their inclusion complex.²⁾ We applied such technique of the inclusion complex to the preparation of oleic and linoleic acids which are contained in the commercial BSA as contaminants and were found to be essential for the growth of various mammalian cells. The removal of BSA from our serum-free media has been anyway achieved, utilizing the technique of this inclusion complex preparation, although the stable establishment of conclusive serum-free media still remains.

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