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Bovine liver cells in long-term cultivation and their specific properties; albumin production and glycogen storage

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Abstract

The liver cells obtained from a calf have been cultured continuously for 257 days in total at present (May 31, 1967). The primary culture was maintained in rotatory culture for about 2 months with gradual and continuous cell proliferation. The two original strains, LD-BS20 and LD-CS20, have been maintained in static culture since 4th subcultivation. Three substrains, LD-BS10, YLE-BS20 and LD-CS10, were derived from the original strains. Two kinds of appropriate media, in which the cells could be subcultured with trypsin without severe damages and maintained with some characteristic functions of liver cells, were reported. The one consisted of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate and saline D, and the other was added with 0.08 per cent yeast extract to the above mentioned medium. Calf serum examined was not so effective as bovine serum for cell proliferation. Morphologically, the cultured cells resembled parenchymal liver cells quite well. The cells spread wide with abundant pale staining cytoplasm and their large nuclei, oval or round, generally contained one to several nucleoli. The cells as well as the nuclei varied considerably in size, some being two to four times larger than others. Binuclear, trinuclear or polynuclear cells were also observed. No silver impregnated fiber was detected among the epithelial cells. Two attempts to characterize cell types in culture were made. First, the presence of glycogen was tested with PAS reaction and saliva digestion procedure. Secondly, the albumin formation in cultured liver cells was examined with the fluorescent antibody technique. The fact that both albumin and glycogen were observed in the cells suggests strongly that there is a possibility of the continuous cultivation of liver cells by the present method, and by these procedures it seems possible to identify functionally the cultured cells with the parenchymal liver cells.

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**BOVINE LIVER CELLS IN LONG-TERM CULTIVATION
AND THEIR SPECIFIC PROPERTIES ;
ALBUMIN PRODUCTION AND GLYCOGEN STORAGE**

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The establishments of epithelial cell lines from liver tissues of mouse^{1,2}, human^{3,4}, horse⁵, calf⁶, rabbit⁷ and rat^{8,9} have been reported. As the liver tissue consists of various kinds of cells, for instance, parenchymal liver cells, bile duct epithelial cells, endothelial cells including Kupffer's cells, fibroblasts and macrophages, varying outgrowths of different cell types from the original liver tissue explant have been observed. The epithelial-like cells in continuous culture resemble morphologically parenchymal liver cells, but it is an important problem whether or not these cells have originated truly from parenchymal liver cells and whether they can maintain any specific functions of the liver cell in long-term cultivation. The identification as to whether these hepatic cells in long-term cultivation have originated from parenchymal liver cells depends chiefly upon the morphological features, that is to say, epithelial-like or pavement-like growth of the cultured cells. There are only a few reports having identified functionally the liver cells in long-term cultivation with the parenchymal liver cell, i. e. those by EVANS¹ and PIECK⁶ concerning glycogen storage in the liver cells in continuous cultivation. The albumin formation of the rat liver cells in long-term culture and the identification of the cultured cells as parenchymal liver cells were reported in the previous paper¹⁰. The purposes of this study are to explore whether or not the calf liver cells can be cultivated continuously by the present method⁶, and whether the cells can maintain some characteristic functions of the liver cells and to identify functionally the cultured cells with the parenchymal liver cell. With respect to the differential functions of the liver cell, glycogen synthesis and albumin formation of the cultured liver cells were examined in this work.

MATERIALS AND METHODS

Cells and Culture Methods : The cultured cells were derived from the liver tissue of a calf. In the primary culture the liver tissue was minced with knives and the fragments were explanted on the inner surface of roller tubes with no use of clotting materials in various media. The cultures were incubated in a roller drum rotating at 5 rph and maintained in this manner at 37°C. The culture fluid was renewed twice a week. Since the fourth subcultivation all the strains have been cultivated in static culture. Subcultures were made by trypsinizing (0.2 per cent trypsin in phosphate buffered salt solution deficient in Ca⁺⁺ and Mg⁺⁺) at 37°C for 10~20 minutes. The simplified replicate tissue culture method described by KATSUTA¹¹ was employed for the estimation of cell proliferation.

Culture Media : Various kinds of media were prepared. The medium for both of the mother cultivation and the control culture of the experiments consisted of 20 per cent bovine serum or 20 per cent calf serum, 0.4 per cent lactalbumin hydrolysate (NBC) and saline (mixture D)¹². For the purpose of examining the effect of the serum concentration on the proliferation of liver cells, the media containing bovine or calf serum at the final concentration of 10 per cent or 20 per cent were prepared. For another purpose of examining the influence of other kinds of sera on liver cells, chicken, rabbit, rat and human sera were also prepared. One of the mother cultures which had been maintained in the control medium containing 20 per cent bovine serum has been removed to the medium added with 0.08 per cent yeast extract (Difco) to the control medium. The lot of bovine and calf sera, respectively, was collected from several animals.

Fluorescent Antibody Technique : Crystallized bovine serum albumin (BSA, Armour) was used as antigen. Alum-precipitated albumin was injected subcutaneously and intramuscularly into rabbits. After 2 months, the precipitation titer of the sensitized rabbits' sera was about 16, and subsequently 20 mg of BSA dissolved in physiological saline were injected intramuscularly 3 times at the intervals of 3 to 5 days. The procedure increased the precipitation titer up to 128. Total blood was collected from carotic artery 5 days after the last injection. The properties of the antigen (BSA) and the antibody were examined according to the microimmunoelectrophoresis. The labelling of the antiserum with fluorescein isothiocyanate (FITC) was as follows; The fraction of gamma-globulin was precipitated from the pooled serum by 33.3 per cent saturated ammonium sulfate and labelled with fluorescein isothiocyanate at FITC : γ G ratios of 1 : 100 at pH 9.4 for 10 hr at 4°C. After removing free FITC from the conjugate by passage through a Sephadex G-50 column using as eluent 0.1M NaCl in 0.01M phosphate buffer, the labelled serum was eluted through diethylaminoethyl

cellulose (0.80 meq) column with the same eluent. The final molecular ratio of FITC to protein was about 2.

Cells cultured on coverslips were fixed with 1 per cent acetic acid alcohol for 30 min according to HAMASHIMA¹³ and rinsed for 10 min in phosphate buffered saline (PBS, 0.01M phosphate buffer, 0.15M NaCl, pH 7.4) with three changes of solution. A few drops of the conjugate, which had been previously absorbed with human liver homogenates, were applied to the specimen in a moist chamber for 30 min at room temperature. The slide was then rinsed three times in PBS during a period of 10 min and mounted with 10 per cent glycerol PBS. The specimen was observed by darkfield using the microscope illuminated with high pressure mercury lamp (Nikkon Co.). UV filter was employed through the observations and for the photomicrography.

Demonstration of Glycogen : Small coverslip was inserted into a small square bottle prior to the cultivation. When a full sheet of cells was observed on the coverslip, the cells were fixed with absolute methanol and stained with periodic acid-Schiff reaction (PAS) by the method of LILLIE¹⁴. After the PAS staining the nuclei were stained with Harris' hematoxylin for 24 hr. For the proof of glycogen, fixed liver cells were washed with PBS and treated with saliva at 37°C for 60 min before the reaction.

Preparation of Specimens : Small coverslips inserted into a small square bottle were fixed with methanol or neutral formol and stained with Giemsa, Sudan III or Gomori's silver method.

RESULTS

1. Primary Cultures Arising from Original Explant

The cultivation of liver cells from a calf was started on September 16, 1966. After one week, the proliferation of cells from explanted tissues was observed. Very gradual and steady growth of the cells continued within 2 months after the planting of the tissues. The general cell type observed in this period was characteristic large flattened cells and these sheet-like cells had clear cytoplasm containing granules of varying size. The spheroidal, well-defined nuclei were one to three in number and small as compared with the cytoplasm. These cells, which appeared to have originated from hepatic parenchymal tissue, were subcultured with trypsin to the second transfer generation. Some of the cells were subcultured in the bottle containing a small coverslip and fixed with methanol and stained with Giemsa's solution.

2. Tissue Culture History, Morphology and Cell Proliferation Rate of Two Original Strains, LD-BS20 and LD-CS20

The strain, LD-BS20, has been maintained in the medium composed of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate and saline D and the other strain, LD-CS20, has been cultured in the medium containing 20 per cent calf serum instead of bovine serum. Both strains had been incubated in a roller drum until the third subcultivation, that is to say, the cells were removed from the static culture to the rotatory within 24~48 hours after they were subcultured. A steady and slow rate of cell proliferation has been maintained since the first subcultivation. As will be understood from the column of "Duration" in Tables 1 and 2, it took about a month to two months to subculture cells. The proliferation rates of two strains, LD-BS20 at 5th subcultivation on 209th culture day and LD-CS20 at 5th subcultivation on 171st day, were about 3 and 1.6-fold in cell population during a week, as illustrated in Fig. 2. The LD-BS20 and the LD-CS20 have attained the 7th and the 6th subcultivation respectively and 257th culture day in total at present (May 31, 1967). The history of each strain is summarized in Fig. 1.

On histological examination, no distinguishable difference was detected between two strains. Flat sheets of epithelial-like cells were always observed. The cells spread largely with abundant pale staining cytoplasm and their large

Table 1 Tissue culture history of the original strain LD-BS 20 maintained in the medium 20% bovine serum, 0.4% lactalbumin hydrolysate and saline D

Subculture	Duration (days)	Date	Total days in culture	Culture method
Primary	70	Sept. 16, '66	0	Rotatory
1	22	Nov. 25	70	"
2	38	Dec. 17	92	"
3	44	Jan. 24, '67	130	"
4	35	Mar. 9	174	Stationary
5	22	Apr. 13	209	"
6		May 5	231	"

Table 2 Tissue culture history of the original strain LD-CS 20 maintained in the medium 20% calf serum, 0.4% lactalbumin hydrolysate and saline D

Subculture	Duration (days)	Date	Total days in culture	Culture method
Primary	71	Sept. 16, '66	0	Rotatory
1	18	Nov. 26	71	"
2	63	Dec. 14	89	"
3	64	Feb. 15, '67	152	"
4	27	Apr. 20	216	Stationary
5		May 17	243	"

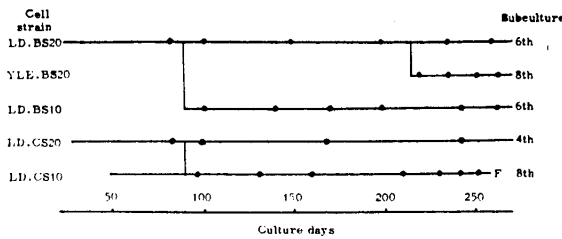


Fig. 1 Culture history of liver cells from calf
 —•— : Subculturing, F : Stored being frozen

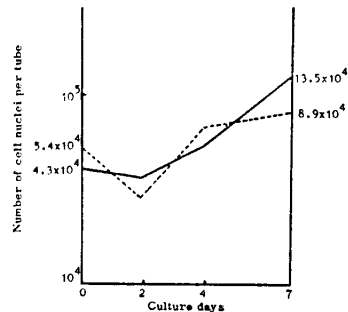


Fig. 2 Cell proliferation rate of two original strains, LD-BS 20 and LD-CS 20
 — : LD-BS 20, 5th subculture, 209th culture day, : LD-CS 20, 5th subculture, 171st culture day

nuclei, oval or round, generally contained one to several nucleoli. The cells as well as the nuclei varied considerably in size, some being two to four times larger than others. Binuclear, trinuclear or multinuclear cells were also sometimes observed. These cells appeared to be typical hepatic parenchymal cells (Photo 1). The cells were disposed to aggregate together in clumps or to make branches when the culture was maintained for a long period (Photo 2).

3. Tissue Culture History, Morphology and Cell Proliferation Rate of the Substrains, YLE-BS20, LD-BS10 and LD-CS10

The substrains, YLE-BS20 and LD-BS10, were derived from the strain LD-BS20 and the substrain, LD-CS10, was from the strain LD-CS20. The YLE-BS20 has been cultivated in the medium containing 20 per cent bovine serum, 0.08 per cent yeast extract, 0.4 per cent lactalbumin hydrolysate and Earle's balanced salt solution. The LD-BS10 and LD-CS10 have been maintained in the medium composed of 10 per cent bovine serum and 10 per cent calf serum respectively, 0.4 per cent lactalbumin hydrolysate and saline D. The historical records of each substrain are illustrated in Fig. 1.

The cell proliferation rates of the substrains, YLE-BS20 and LD-BS10 were compared with the original strain LD-BS20 from 187th culture day to 216th, as shown in Fig. 3. The proliferation rate of the LD-BS10 substrain was lowest and that of the LD-BS20 strain highest. The substrain, YLE-BS20 showed gradual and steady proliferation and was not affected so much by the damage of subculturing with trypsin. The cell proliferation rate of the LD-CS10 was very low and the same cells were severely injured by the subculture (Fig. 4).

Histologically, the two substrains, YLE-BS20 and LD-BS10, showed practically the same appearance as the original strains, but the cells of the substrain LD-CS10 were a little smaller than those of original strains. As the cells

appeared a little fibroblastic by the observation of phase contrast microscopy, the silver staining was performed but no argentaffine fiber was detected.

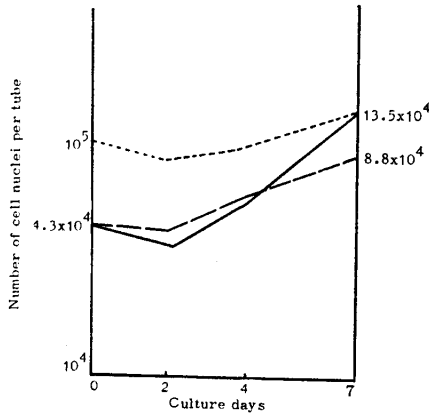


Fig. 3 The comparison of cell proliferation of substrains, YLE-BS 20 and LD-BS 10 with that of the original strain LD-BS 20

— : LD-BS 20, 6th subculture, 209th culture day. : YLE-BS 20, 7th subculture, 209th culture day. — · — : LD-BS 10, 5th subculture, 187th culture day

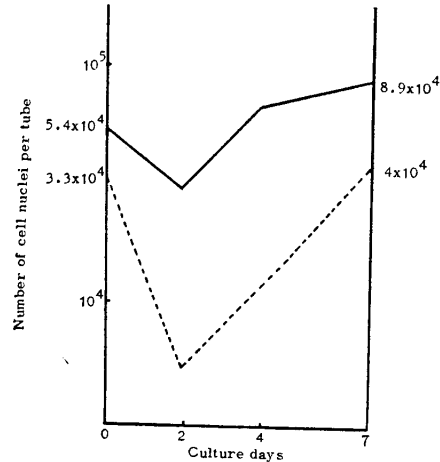


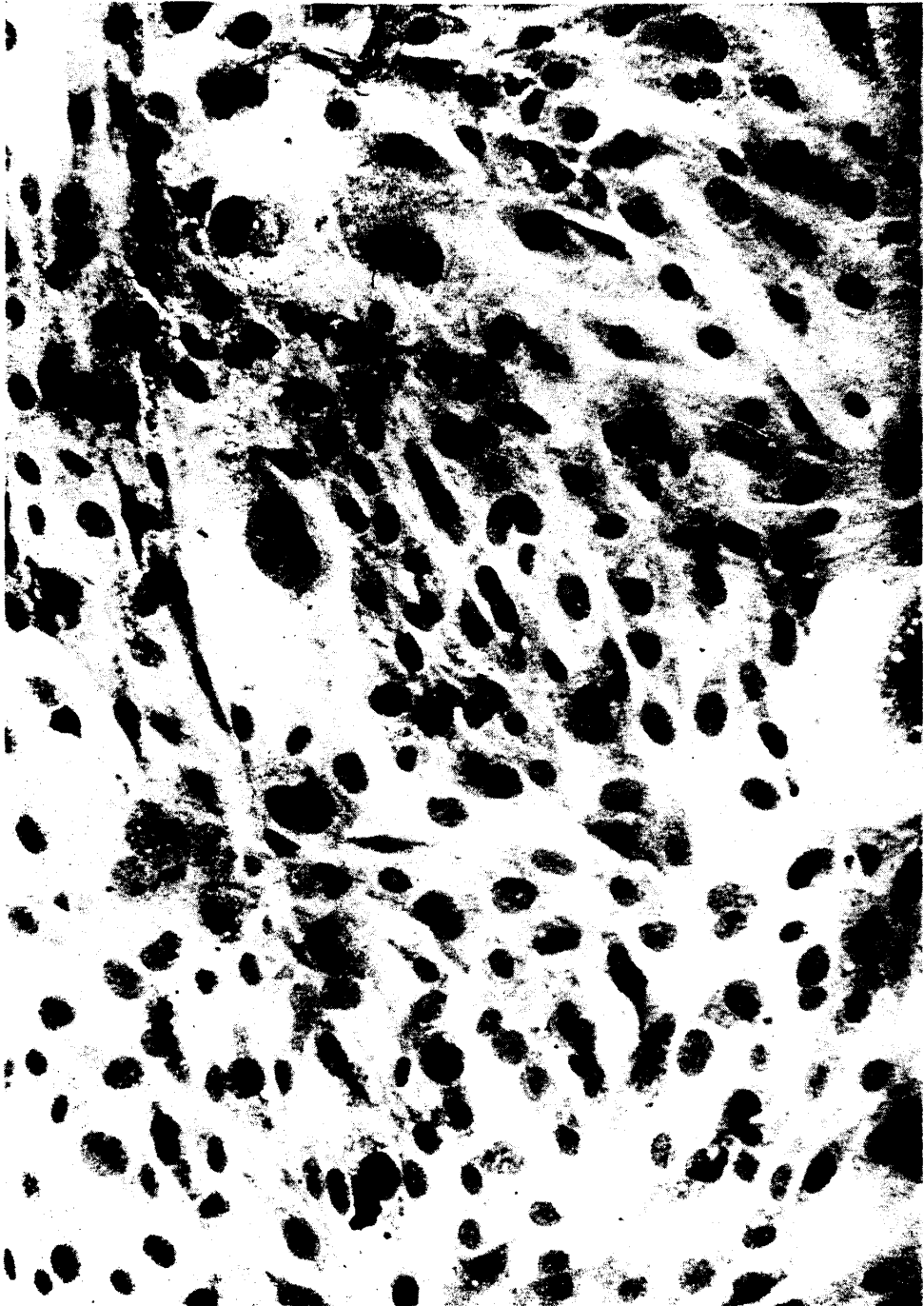
Fig. 4 The comparison of cell proliferation of the substrain LD-CS 10 with that of the original strain LD-CS 20

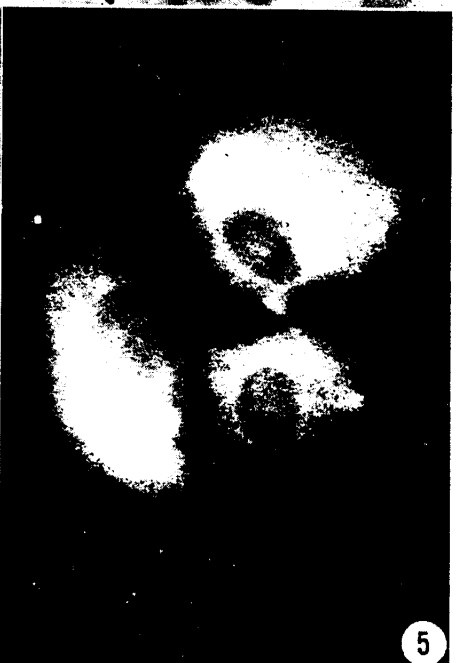
— : LD-CS 20, 5th subculture, 171st culture day. : LD-CS 10, 8th subculture, 233rd culture day

4. The Demonstration of Glycogen in Cultured Liver Cells

The metabolic passway from glucose to glycogen is generally admitted in liver cells *in vivo*. In this experiment it was examined whether or not glycogen existed in the cultured liver cells in order to identify the cells with parenchymal liver cells. The two original strains and the substrain YLE-BS20 reacted positively with PAS reagent and a new substrain, YLE-HuS20, which was derived from the substrain YLE-BS20 and maintained in the YLE medium supplemented with 20 per cent human serum, gave the same positive results. This last new substrain was mainly used in the following experiment to prove the albumin production of the cultured liver cells. The PAS positive granules observed in the cytoplasm varied in size but, generally speaking, most cells produced PAS positive fine granules (Photo 3). The reaction of PAS reagent with these gran-

Photo 1 Flat sheet of epithelial-like cells is observed. The cells spread widely with abundant pale staining cytoplasm and their large nuclei, oval or round, generally contain one to several nucleoli. The cells as well as the nuclei vary considerably in size, some being two to four times larger than others. Giemsa, $\times 100$





ules was completely prevented after the treatment with saliva.

On the other hand, other substrains, LD-BS10 and LD-CS10, were not stained with PAS reagent. The cultured liver cells from a rat were also stained with PAS reagent but no positive results were obtained.

5. Albumin Production in Cultured Liver Cells

One important problem rises in this experiment. That is, how much bovine serum albumin is incorporated in the cells cultured in the medium containing bovine serum. Rat liver cells cultured in the medium supplemented with 20 per cent bovine serum reacted with rabbit anti-bovine serum albumin globulin labelled with fluorescent isothiocyanate. Cytoplasm of all the cells observed was very luminous, but nuclei were not so. From this viewpoint, it is concluded that all the cells cultured in the medium containing bovine serum have incorporated appreciably bovine serum albumin in their cytoplasm (Photo 4). Consequently, it had to be investigated with immunofluorescent technique how long bovine serum albumin existed in the cells after the bovine serum was replaced by other kinds of sera. Bovine albumin disappeared completely from the rat liver cells in long-term culture 3 days after the replacement of the bovine serum. This fact was identical with the case of JTC-11 cell line, which was established from Ehrlich ascites carcinoma by SATO and HAMASAKI¹⁵.

When bovine serum in the culture medium was changed to other sera, namely, rabbit, chicken and rat sera, rough and relatively large granules appeared in the cytoplasm of the cultured cells. Most of these granules were positive with Sudan III staining. As rabbit, chicken and rat sera were not suitable for the observations of morphology and proliferation, these sera were not used in this experiment.

Calf liver cells had been cultured in the medium containing human serum in place of bovine serum 2 to 4 weeks before this experiment was started. After that period, the cells were subcultured with trypsin and inoculated in small square bottles containing small coverslips. The cells were harvested when they grew fully sheet-like on the coverslip.

Albumin produced in the cultured liver cells was observed to be granular

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- Photo 2 The cultured cells are disposed to aggregate together in clumps while the culture is maintained for a long period. Harris hematoxylin, $\times 400$
- Photo 3 The PAS positive granules are observed in the cytoplasm as black spots. PAS and Harris hematoxylin, $\times 400$
- Photo 4 Rat liver cells cultured in the medium supplemented with bovine serum react with rabbit anti-bovine serum globulin labelled with fluorescent isothiocyanate. Cytoplasm of all the cells is very luminous, but nuclei are not so. $\times 200$
- Photo 5 Albumin produced in the bovine liver cells cultured in the human serum supplemented medium is observed in the cytoplasm with the immunofluorescent antibody technique. $\times 400$

or homogenous in the cytoplasm of the liver cells with the immunofluorescent antibody technique. The albumin was situated in the cytoplasm mainly near the nucleus (Photo 5). The cytoplasm of cultured liver cells spread so thinly and widely that a small amount of albumin in the peripheral region might have escaped the detection.

DISCUSSION

The successes in the continuous cultivation of epithelial cells from liver tissues by the present tissue culture methods have been reported by several authors¹⁻⁹, but there are few attempts to identify functionally the cultured cells with the parenchymal origin. When the method of the identification is discovered, it will enable us to investigate more effectively how to culture liver cells which hold more prominent characteristic liver functions. The success will afford many advantages for many *in vitro* researches of carcinogenesis, virus, especially hepatitis virus, metabolism and diseases of liver cells.

EVANS succeeded to cultivate the epithelial cells from the liver tissue of a newborn mouse and she attempted to examine the estradiol inactivating capacity of the cultured cells but she found no inactivation¹. Although EVANS and PIECK demonstrated glycogen in the epithelial cells established from liver tissues, such a proof of glycogen does not necessarily indicate the cells to have originated from parenchymal liver cells, because glycogen has been detected also in other cells *in vitro* (L line cells)¹, even though the parenchymal cells of liver are the major storage site of glycogen *in vivo*. With exception of EVANS and PIECK, all other attempts to identify functionally the epithelial cells obtained from liver tissues with parenchymal liver cells have failed up to date.

Most liver cell lines except those of PIECK⁶, CHANG³ and SYVERTON⁴ were cultivated continuously in the medium containing the heterogeneous serum to the cells in culture. In the present experiment calf liver cells were cultured in the medium containing calf serum and bovine serum respectively. No appreciable difference was observable concerning the morphology and glycogen synthesis in the culture medium supplemented with 20 per cent calf or bovine serum, but proliferation rate was low in the calf serum containing medium and the cells subcultured with trypsin were severely damaged in the same medium. Therefore, bovine serum may be recommended for the culture of the calf liver cells. Homogeneous serum to the culture cells may be able to maintain some functions of the cells in a long-term cultivation.

The albumin formation is well known as one of the specific functions of liver cells *in vivo*, but it is very difficult to detect the produced albumin in the liver cells cultured in the medium containing bovine serum, because, as men-

tioned in the results, bovine serum albumin is contained invariably in the medium, hence in all cells in culture. For that reason, bovine serum in the medium had to be replaced by rabbit, chicken, rat or human serum for the purpose of investigating the albumin production. When rabbit, rat or chicken serum was used, many lipid particles were observed microscopically in the cells. The appearance of the lipid granules has usually been ascribed to crowding, aging, degeneration or reduction of extracellular pH of the culture. However, these factors just mentioned may not be applicable to the abundance of lipid particles in the present experiment.

It is reported that albumin fraction enhances the cell growth in cultures^{16,17}. The beneficial effects of albumin on the cells in culture may stem from a double action as described by DUBIN¹⁷: 1) from the provision of essential nutrient elements deficient in the serum, such as fatty acids, and 2) from the removal of inhibitory substances in the serum through binding with albumin. In the present experiment the bovine serum albumin was observed to be contained remarkably in all the cells cultured in the medium containing bovine serum by the immunofluorescent antibody technique. Though the role of the albumin incorporated in the cell is still unknown, some albumin in the medium enters cells and may be utilized as growth promoting nutrients.

Albumin formation and glycogen synthesis have been observed in the liver cells from a calf up to now, but the fact these characteristics are not always observable, especially at the early stage after the subculture, suggests that the cells require some period before they display these specific functions. This fact might also depend on the cell proliferation, namely, proliferative cells cannot show the functions appreciably. It is interesting to note how long these characteristic functions of liver cells will be maintained *in vitro*. The possibility of the recovery of the specific functions will be pursued by using various kinds of culture methods, when these characteristics are lost in culture.

The aggregated cells showed more distinct glycogen storage in the cells. It is important whether or not aggregation or organisation of cells *in vitro* is related to exhibition of cell functions and this problem will be resolved by the utilisation of the gyratory culture method¹⁸. When the serum concentration in the culture medium was reduced down to 10 per cent, no glycogen storage was detected. This may suggest the medium containing 10 per cent serum to be lacking some essential factors to synthesize glycogen from glucose.

The genetic problems, what kind of cell is producing albumin or glycogen, whether or not the same cell forms albumin and glycogen simultaneously, and if the albumin formation or glycogen depends on the stage of cell generation, will be explored in near future by cloning method.

SUMMARY

The liver cells obtained from a calf have been cultured continuously for 257 days in total at present (May 31, 1967). The primary culture was maintained in rotatory culture for about 2 months with gradual and continuous cell proliferation. The two original strains, LD-BS20 and LD-CS20, have been maintained in static culture since 4th subcultivation. Three substrains, LD-BS 10, YLE-BS20 and LD-CS10, were derived from the original strains. Two kinds of appropriate media, in which the cells could be subcultured with trypsin without severe damages and maintained with some characteristic functions of liver cells, were reported. The one consisted of 20 per cent bovine serum, 0.4 per cent lactalbumin hydrolysate and saline D, and the other was added with 0.08 per cent yeast extract to the above mentioned medium. Calf serum examined was not so effective as bovine serum for cell proliferation.

Morphologically, the cultured cells resembled parenchymal liver cells quite well. The cells spread wide with abundant pale staining cytoplasm and their large nuclei, oval or round, generally contained one to several nucleoli. The cells as well as the nuclei varied considerably in size, some being two to four times larger than others. Binuclear, trinuclear or polynuclear cells were also observed. No silver impregnated fiber was detected among the epithelial cells.

Two attempts to characterize cell types in culture were made. First, the presence of glycogen was tested with PAS reaction and saliva digestion procedure. Secondly, the albumin formation in cultured liver cells was examined with the fluorescent antibody technique. The fact that both albumin and glycogen were observed in the cells suggests strongly that there is a possibility of the continuous cultivation of liver cells by the present method, and by these procedures it seems possible to identify functionally the cultured cells with the parenchymal liver cells.

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