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Abstract

The dispase perfusion technique was used to isolate liver cells from adult rats. The optimum conditions for obtaining many isolated liver cells with high viability were an enzyme concentration of 2000 U/ml, a pH of 7.5 and a perfusion time of 20 min. The population of isolated liver cells prepared with dispase consisted of 43.6% cells with diameters less than 20 micron and 56.4% cells with diameters above 20 micron. The isolated liver cells were cultured in basal culture medium either supplemented with or without dexamethasone (1 X 10(-5)M) and insulin (10 micrograms/ml). The addition of hormones to the culture medium improved the attachment efficiency of the isolated liver cells and delayed the disappearance of mature hepatocytes. Epithelial-like clear cells proliferated early in primary culture even in the presence of hormones. Therefore, functioning mature hepatocytes and proliferating epithelial-like clear cells coexisted well in the hormone-containing medium. Furthermore, the number of cultured cells reached a maximal level earlier in the presence of hormones than in the absence of hormones. The level of TAT activity in primary cultured cells was higher up to 3 days after inoculation in the presence of hormones than in their absence. No difference between G6Pase activity in primary cultured cells in the presence of hormones and that in the absence of hormones was found.

KEYWORDS: dispase-liver-perfusion, sizu distribution, primary liver cell culture, grouwth pattern, liver-specific functions

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PREPARATION AND PRIMARY CULTURE OF LIVER CELLS ISOLATED FROM ADULT RATS BY DISPASE PERFUSION

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Abstract. The dispase perfusion technique was used to isolate liver cells from adult rats. The optimum conditions for obtaining many isolated liver cells with high viability were an enzyme concentration of 2000 U/ml, a pH of 7.5 and a perfusion time of 20 min. The population of isolated liver cells prepared with dispase consisted of 43.6 % cells with diameters less than 20 μm and 56.4 % cells with diameters above 20 μm. The isolated liver cells were cultured in basal culture medium either supplemented with or without dexamethasone (1×10^{-5} M) and insulin (10 μg/ml). The addition of hormones to the culture medium improved the attachment efficiency of the isolated liver cells and delayed the disapperance of mature hepatocytes. Epithelial-like clear cells proliferated early in primary culture even in the presence of hormones. Therefore, functioning mature hepatocytes and proliferating epithelial-like clear cells coexisted well in the hormone-containing medium. Furthermore, the number of cultured cells reached a maximal level earlier in the presence of hormones than in the absence of hormones. The level of TAT activity in primary cultured cells was higher up to 3 days after inoculation in the presence of hormones than in their absence. No difference between G6Pase activity in primary cultured cells in the presence of hormones and that in the absence of hormones was found.

Key words : dispase-liver-perfusion, size distribution, primary liver cell culture, growth pattern, liver-specific functions.

Berry and Friend reported the collagenase perfusion technique to isolate cells from liver (1), and since then trypsin also has been employed in similar procedures (2, 3). Dispase, another proteolytic enzyme which is obtained from Bacillus polymyxa (4, 5), was used in the present experiments to isolate liver cells from adult rats. The optimum conditions, *i.e.*, concentration of the enzyme, pH of the enzyme-containing perfusate and perfusion time were determined for obtaining large numbers of isolated liver cells with high viability.

Biochemical, morphological and growth properties of the isolated liver cells in primary culture are described in this report. Differences in properties between

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liver cells obtained by the dispase perfusion technique and those obtained by collagenase and trypsin perfusion techniques are discussed.

MATERIALS AND METHODS

Male-Donryu rats, about 200 g in body weight, were used for obtaining isolated liver cells by an enzymatic perfusion technique (1, 3). After perfusing the liver with 100 ml of the 1 st perfusate, which was composed of Ca^{2+} and Mg^{2+}-free Hanks' balanced salt solution (CMF-HBSS, pH 7.5) containing 0.5 mM ethyleneglycol-bis- (β-aminoethylether) -N, N'-tetraacetic acid (EGTA, Dotite, Japan) and 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES, Sigma Chemical Co., USA) (3), the liver was perfused with 100 ml phosphate-buffered saline (PBS) to wash out the residual perfusate, which may inhibit the activity of dispase due to the presence of EGTA (4). The 2 nd perfusate, which was composed of CF-HBSS containing dispase-II (Godo Shusei, Japan) 2 mM $CaCl_2$ and 10 mM HEPES, was circulated through the liver. The concentration of the enzyme, the pH of the 2 nd perfusate and the time of perfusion were determined after repeating a number of experiments. The isolation of the liver cells from the perfused liver tissue was carried out as reported previously (2).

Size measurements were made directly on photomicrographs of the isolated liver cells. More than 1×10^3 cells were scanned to determine the size distribution.

Isolated liver cells were inoculated at a concentration of 3.5×10^5 viable cells/ml into 100 mm Falcon plastic dishes containing 10 ml basal culture medium either supplemented with or without dexamethasone-21-disodium phosphate (dexamethasone, Nippon Merck Banyu Co., Japan, 1×10^{-5} M) and insulin (Sigma Chemical Co., USA, 10 μg/ml). The basal culture medium was Eagle's minimal essential medium (Nissui Seiyaku Co., Ltd., Japan) supplemented with bovine serum (20 %), penicillin (100U/ml), streptomycin (100 μg/ml), Kanamycin (60 μg/ml) and fungizone (1 μg/ml). The isolated liver cells were also inoculated at the same concentration into 35 mm Falcon plastic dishes containing 1.4 ml basal medium either supplemented with or without hormones to determine growth patterns by counting viable cells with trypan blue.

To carry out the enzyme assay, the cultured cells were washed once with PBS before harvesting them by trypsinization, which was stopped by the addition of fresh basal culture medium. These procedures were done to minimize contamination by dead cells.

The preparation of cell homogenates and supernatants, and the assay of tyrosine aminotransferase (L-tyrosine : 2-oxoglutarate aminotransferase, EC 2.6.1.5, TAT) and glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3. 1. 3. 9, G6Pase) activities were carried out as reported previously (2, 6). Protein contents of homogenates and supernatants were determined by the method of Lowry *et al.* (7).

RESULTS

Isolation of the liver cells. The optimum conditions for obtaining large numbers of isolated liver cells with high viability were an enzyme concentration of 2000 U/ml, a pH of the enzyme-containing perfusate of 7.5 and a perfusion time of 20 min. (Tables 1-3).

The isolated liver cells, which were isolated by the dispase perfusion technique under the optimum conditions, were from 6 μm to 34 μm in diameter (Fig. 1). The population of isolated liver cells consisted of 43.6 % cells with diameters less

TABLE 1. EFFECT OF VARIOUS DISPASE CONCENTRATIONS ON TOTAL CELL NUMBER AND VIABILITY OF THE LIVER CELLS ISOLATED BY PERFUSING THE LIVER OF ADULT RATS WITH DISPASE-II-PERFUSATE (pH 7.5) FOR 20 MIN AT 37℃

Dispase concentration (U/ml)	Total cell number ($\times 10^7$ cells)/ 100 g body weight.	Viability (%)
1000	14.3 ± 2.8	86.5 ± 4.5
2000	18.9 ± 1.0	84.2 ± 1.9
3000	12.2 ± 1.9	87.4 ± 0.8
4000	12.2 ± 3.0	79.6 ± 2.7

Results are expressed as the mean ± standard deviation (SD) of 3 different experiments.

TABLE 2. EFFECT OF VARIOUS pH'S ON TOTAL CELL NUMBER AND VIABILITY OF THE LIVER CELLS ISOLATED BY PERFUSING THE LIVER OF ADULT RATS WITH DISPASE-II-PERFUSATE (2000 U/ML) FOR 20 MIN AT 37℃

pH	Total cell number ($\times 10^7$ cells)/ 100 g body weight	Viability (%)
7.0	15.1 ± 2.1	82.8 ± 4.3
7.5	18.9 ± 1.0	84.2 ± 1.9
8.0	14.5 ± 0.5	82.0 ± 1.1
8.5	15.9 ± 4.5	85.2 ± 2.9

Results are expressed as the mean ± SD of 3 different experiments.

TABLE 3. EFFECT OF VARIOUS PERFUSION TIMES ON TOTAL CELL NUMBER AND VIABILITY OF THE LIVER CELLS ISOLATED BY PERFUSING THE LIVER WITH DISPASE-II-PERFUSATE (2000 U/ML, pH 7.5) AT 37℃.

Perfusion time (min)	Total cell number ($\times 10^7$ cells)/ 100 g body weight	Viability (%)
10	10.2 ± 0.2	88.8 ± 3.1
20	18.9 ± 1.0	84.2 ± 1.9
30	11.5 ± 2.1	82.9 ± 0.1

Results are expressed as the mean ± SD of 3 different experiments.

than 20 μm and 56.4 % cells with diameters greater than 20 μm (Fig. 1).

Primary culture of isolated liver cells. Growth patterns of isolated liver cells in primary culture are illustrated in Fig. 2. The attachment efficiency of the isolated liver cells 1 day after inoculation was 34 %, and the cell number markedly decreased during 4 days of a lag period, falling to 19 % of the initial cell number 4 days after inoculation. After the lag period, the number of cultured cells increased rapidly until a maximum (82×10^4 cells/dish) was reached 17 days after inoculation. Addition of hormones to the basal culture medium improved the attachment efficiency (74 %). In the presence of hormones, the culture showed

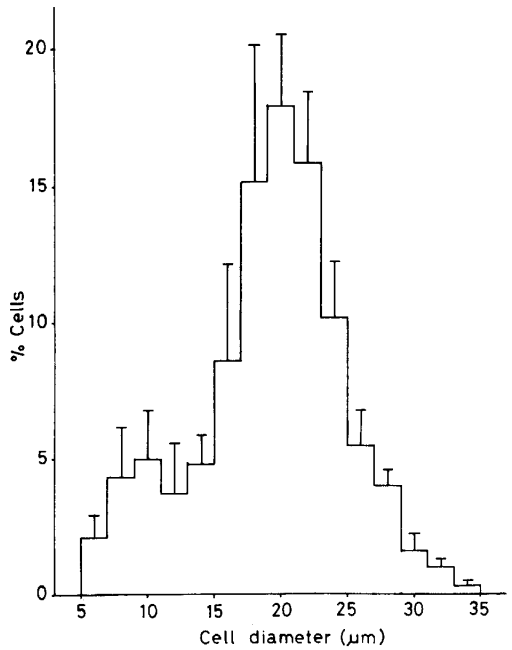

Fig. 1. Size distribution of the liver cells isolated from adult rats by the dispase perfusion technique. Results are expressed as the mean of 4 different experiments. The vertical liens indicate the standard deviations.

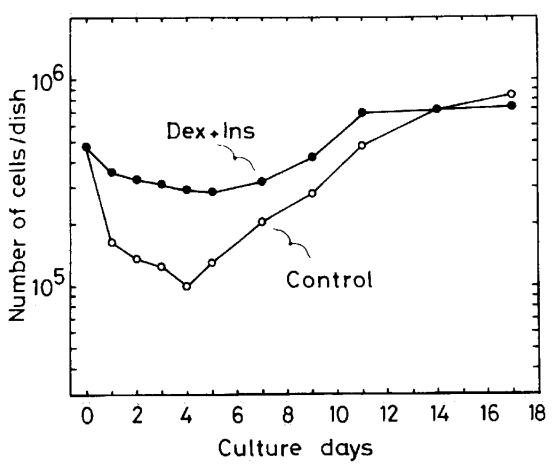

Fig. 2. Growth patterns in primary culture of the liver cells isolated from adult rats by the dispase perfusion technique. The isolated liver cells were cultured in the basal culture medium either supplemented with (●——●) or without (○——○) dexamethasone (1×10^{-5} M) and insulin (10 μg/ml). The numbers of viable cells are expressed as the mean of 3 different dishes.

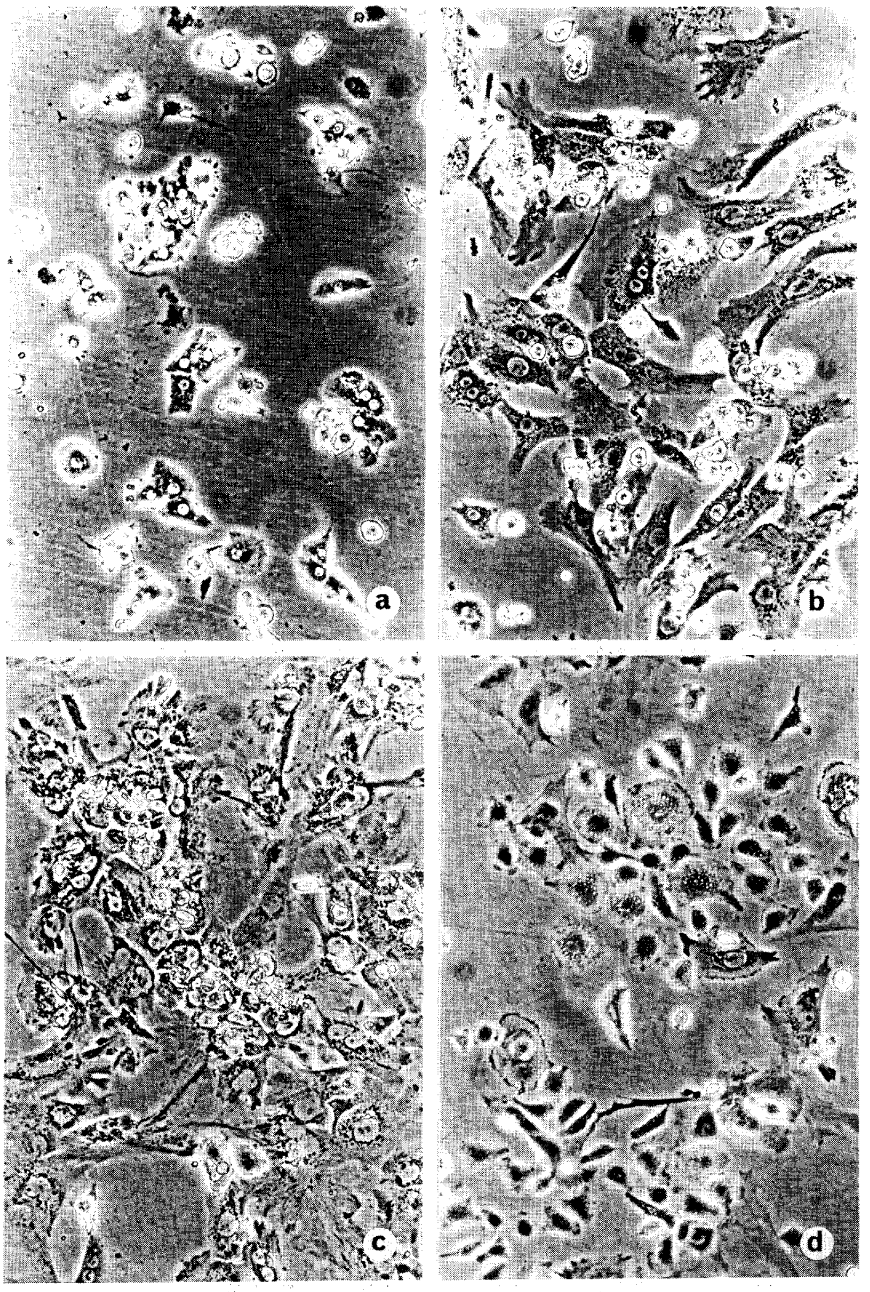

Fig. 3. Morphology of primary cultured liver cells isolated from adult rats by the dispase perfusion technique. The isolated liver cells were cultured in the basal culture medium. The culture ages of a, b, c and d were 1, 3, 5 and 9 days postinoculation, respectively. The cultured cells were observed under a phase contrast microscope at a magnification of 100 ×.

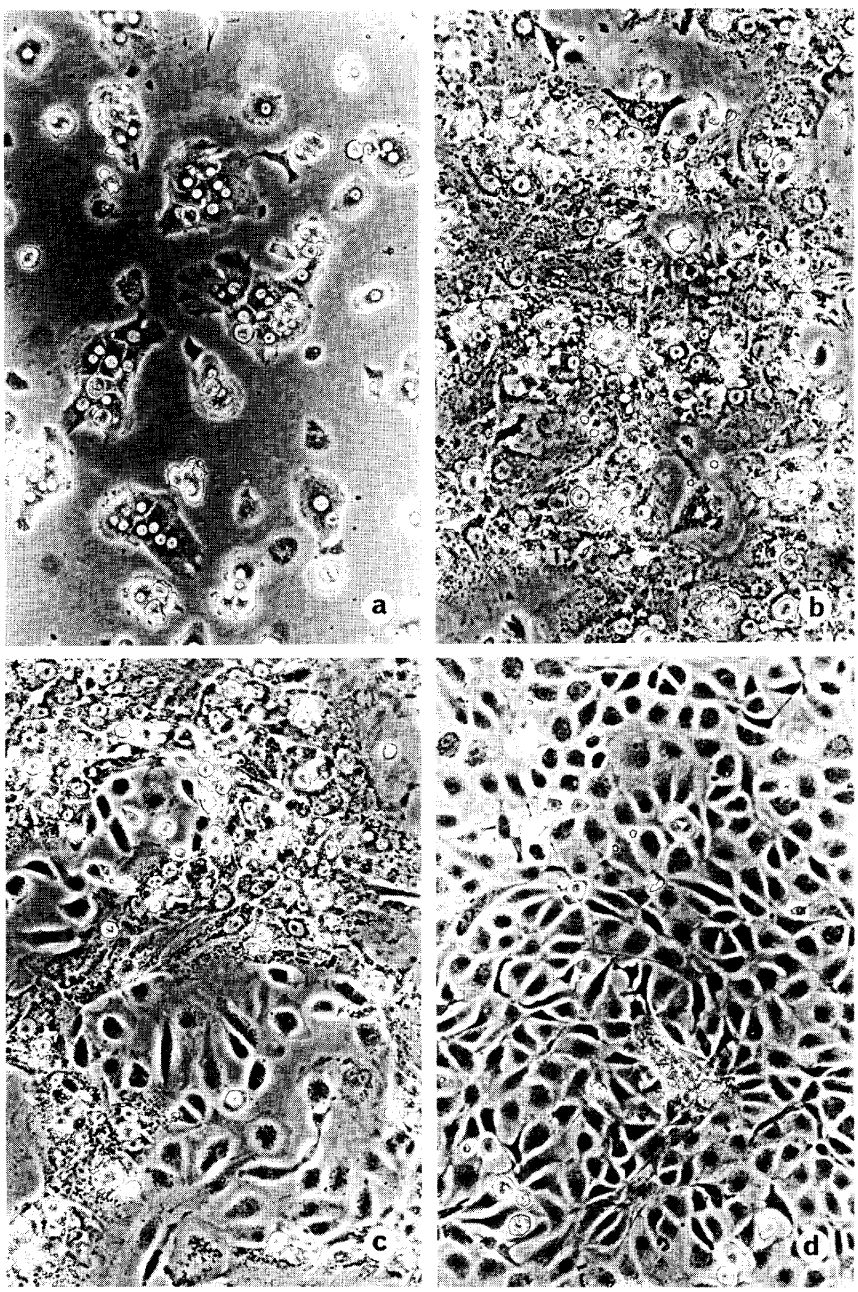

Fig. 4. Morphology of primary cultured liver cells isolated from adult rats by the dispase perfusion technique. The isolated liver cells were cultured in the basal culture medium supplemented with dexamethasone (1×10^{-5} M) and insulin (10 μg/ml). The culture ages of a, b, c and d were 1, 3, 5 and 9 days postinoculation, respectively. The cultured cells were observed as in Fig. 3.

almost the same lag period as that in the absence of hormones. However, the cell number did not decrease markedly during the lag period, and only dropped to 60 % of the initial cell number. The number of cultured cells in the hormone-containing medium reached a maximal number (75×10^4 cells/dish) 11 days after inoculation, which was 6 days earlier than in the hormone-free medium.

A large number of inoculated cells did not attach to the surface of dishes 1 day after inoculation. Furthermore, the attached cells looked round, not flat. An elongated, flat morphology of the cultured cells was observed 3-4 days after inoculation. The cultured cells did not arrange themselves in cords and clusters. Polygonal cells with granular cytoplasm (mature hepatocytes) formed interrupted cords 5-6 days after inoculation, and then gradually decreased in number. The epithelial-like cells with clear cytoplasm (epithelial-like clear cells) were first observed 5-6 days after inoculation and proliferated to the point where they became dominant 10-11 days after inoculation (Fig. 3). The addition of dexamethasone and insulin to the basal culture medium markedly improved the attachment of the inoculated cells to the surface of the dishes. The mature hepatocytes already looked flat 1 day after inoculation and formed interrupted cords 3-4 days after inoculation. The addition of hormones to the basal culture medium also delayed the disappearance of mature hepatocytes and accelerated the appearance of the epithelial-like clear cells. The epithelial-like clear cells proliferated vigorously in hormone-containing medium and became dominant 8-9 days after inoculation (Fig. 4).

The time courses of G6Pase and TAT activities in primary cultured liver cells isolated from adult rats by the dispase perfusion technique are listed in Table 4. One hundred % of the G6Pase activity in rat liver homogenate (53 ± 5 mU/mg protein) (3) was observed in primary cultured liver cells 1 day after inoculation. However, the activities rapidly decreased with time in culture. Addition of hormones to the basal culture medium did not increase the level of G6Pase activity

TABLE 4. G6PASE AND TAT ACTIVITIES IN PRIMARY CULTURED ADULT RAT LIVER CELLS ISOLATED BY PERFUSING THE LIVER WITH DISPASE-II-PERFUSATE (2000 U/ML, pH 7.5) FOR 20 MIN AT 37℃.

Culture day	G6Pase activity (mU/mg protein)		TAT activity (mU/mg protein)	
	Basal medium	DI medium	Basal medium	DI medium
1	53.0 ± 1.2	50.1 ± 6.0	6.4 ± 1.2	161.5 ± 25.8
2	23.1 ± 4.3	23.5 ± 0.4	15.4 ± 4.4	53.3 ± 4.1
3	21.3 ± 5.8	18.6 ± 2.9	18.6 ± 2.5	28.2 ± 5.5
4	6.9 ± 0.6	8.9 ± 2.4	14.4 ± 5.4	18.2 ± 4.6
5	5.6 ± 1.9	5.0 ± 0.5	12.3 ± 3.6	14.2 ± 3.4

Results are expressed as the mean ± SD of 3 different experiments.
DI = Dexamethasone and Insulin.

in the cultured cells. TAT activity in primary cultured liver cells 1 day after inoculation was 15 % of that in liver supernatant (44 ± 7 mU/mg protein) (3). However, the TAT activity showed a 2.4-fold increase 2 days after inoculation, and maintained this level for 2-5 days following inoculation. Addition of hormones to the basal culture medium markedly increased the level of TAT activity. TAT activity in cultured cells 1 day after inoculation in hormone-containing medium was 25-fold higher than that in hormone-free medium. However, the activities markedly decreased thereafter. The level of TAT activity in primary cultured liver cells 5 days after inoculation in the presence of hormones was nearly the same as that in the absence of hormones.

DISCUSSION

The population of liver cells isolated by dispase, trypsin and collagenase perfusion techniques consisted of 43.6, 43.1 and 32.2 % cells with diameters less than 20 μm and 56.4, 56.9 and 67.8 % cells with diameters above 20 μm, respectively (3). The dispase and trypsin perfusion technique yielded much larger numbers of smaller cells than the collagenase perfusion technique (3). The proliferating epithelial-like clear cells were small in size. Therefore, the richness of smaller isolated liver cells seems to cause earlier proliferation of epithelial-like clear cells in primary cultures of liver cells isolated by the dispase and trypsin perfusion techniques than in cultures of cells isolated by the collagenase perfusion technique (3). These results are interesting in that the epithelial-like clear cells may originate from stem cells or partially differentiated hepatic precursor cells (3, 8, 9, 10). The dispase and trypsin perfusion techniques can be used to prepare much larger numbers of these cells which give rise to proliferating epithelial-like clear cells than the collagenase perfusion technique. Dispase may be useful in preparing epithelial-like clear cells for various studies, *e.g.*, those concerned with regulation of cell proliferation (11), cell differentiation (9, 10) and the acquisition of transformation markers induced by chemical carcinogens *in vitro* (12-14).

Epithelial-like clear cells did not appear earlier in the presence of hormones than in their absence in the primary cultures of liver cells isolated by trypsin and collagenase perfusion techniques (3). On the other hand, in the primary cultures of liver cells isolated by the dispase perfusion technique epithelial-like clear cells appeared earlier in the presence of hormones than in the absence of hormones. Furthermore, the number of cultured cells reached a maximal level earlier in the presence of hormones than in their absence. The differences in these results may be due to the different conditions of the cell-cords formed in culture. Mature hepatocytes obtained by the trypsin and collagenase perfusion techniques arranged themselves in uninterrupted cords and formed big cell-islands in the presence of hormones (3, 15). On the other hand, mature hepatocytes obtained by the dispase perfusion technique arranged themselves in interrupted cords even in the presence of hormones, and the cell-islands were small and separated from each

other. It has already been reported that the presence of less than 20 U/ml of dispase influenced neither the cell attachment to the substrate nor the rate of cell proliferation (4). However, the difficulty of forming cell-cords by liver cells prepared with dispase might be due to active dispase remaining on cell membrane, because the activity of dispase is not inhibited by addition of serum (5). Furthermore, the condition of cell membranes of liver cells prepared with dispase might be different from those prepared with trypsin or collagenase. In primary culture of liver cells isolated by the dispase perfusion technique, the rapid proliferation of epithelial-like clear cells in the presence of hormones may be due to the presence of appropriate places around cell-islands for epithelial-like clear cells to proliferate. This characteristic might be valuable if rapid proliferation of epithelial-like clear cells were desired when functioning mature hepatocytes are maintained in culture. In other words, the dispase perfusion technique which can provide both functioning mature hepatocytes and proliferating epithelial-like clear cells might be suitable for studying the effect of substance(s) metabolized or synthesized by functioning mature hepatocytes in primary culture on the proliferating epithelial-like clear cells in "mass" culture of liver cells (11, 16, 17).

Addition of dexamethasone and insulin to the basal culture medium improved attachment of mature hepatocytes, which were observed morphologically in primary culture. This effect of hormones can be observed regardless of the variety of proteolytic enzymes which were used for liver cell isolation (3, 15). However, the best attachment of mature hepatocytes was observed in the primary culture of liver cells isolated by the collagenase perfusion technique (3). The addition of hormones to the basal culture medium also maintained the mature hapatocytes for a much longer time in primary culture. Therefore, the hormones may influence the liver specific enzyme activities of cultured cells. However, addition of hormones to the basal culture medium influenced the TAT activity only, and no hormonal effect was found on G6Pase activity. These results were the same regardless of which proteolytic enzyme was used for liver cell isolation (3 ,15). These results are probably due to the biochemical difference between G6Pase and TAT rather than the conditions of liver cell isolation and culture.

REFERENCES

1. Berry, M.N. and Friend, D.S.: High-yield preparation of isolated rat liver parenchymal cells. *J. Cell Biol.* **43**, 506-520, 1969.
2. Miyazaki, M.: Primary culture of adult rat liver cells. I. Preparation of isolated cells from trypsin-perfused liver of adult rat. *Acta Med. Okayama* **31**, 351-360, 1977.
3. Miyazaki, M., Tsunashima, M., Wahid, S., Miyano, K. and Sato, J.: Comparison of cytologic and biochemical properties between liver cells isolated from adult rats by trypsin-perfusion and those isolated by collagenase-perfusion. *Res. Exp. Med. (Berl)*, 1984 (in press).
4. Matsumura, T., Yamanaka, T., Hashizume, S., Irie, Y. and Nitta, K.: Tissue dispersion, cell harvest and fluid suspension culture by the use of bacterial neutral protease. *Jpn. J. Exp. Med.*

45, 377-382, 1975.

5. Matsumura, T., Nitta, K., Yoshikawa, M., Takaoka, T. and Katsuta, H.: Action of bacterial neutral protease on the dispersion of mammalian cells in tissue culture. *Jpn. J. Exp. Med.* **45**, 383-392, 1975.
6. Miyazaki, M.: Primary culture of adult rat liver cells. II. Cytological and biochemical properties of primary cultured cells. *Acta. Med. Okayama* **32**, 11-22, 1978.
7. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J.: Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275, 1951.
8. Grisham, J.W., Thal, S.B. and Nagel, A.: Cellular derivation of continuously cultured epithelial cells from normal rat liver. In *Gene Expression and Carcinogenesis in Cultured Liver.* ed. L.E. Gerschenson and E.B. Thompson, Academic Press, Inc., *New York*, pp. 1-23, 1975.
9. Malan-Shibley, L. and Iype, P.T.: The influence of culture conditions on cell morphology and tyrosine aminotransferase levels in rat liver epithelial cell lines. *Exp. Cell Res.* **131**, 363-371, 1981.
10. Iype, P.T., Issaq, H.J. and Shaikh, B.: Synthesis of primary bile acids by rat liver epithelial cell lines. *J. Steroid Biochem.* **16**, 333-337, 1982.
11. McMahon, J.B., Farrelly, J.G. and Iype, P.T.: Purification and properties of a rat liver protein that specifically inhibits the proliferation of nonmalignant epithelial cells from rat liver. *Proc. Natl. Acad. Sci. USA* **79**, 456-460, 1982.
12. Iype, P.T. and Pontzer, C.H.: Identification of transformed liver cell colonies using concanavalin A attached to agarose beads. *Cancer Res.* **40**, 4663-4668, 1980.
13. Iype, P.T., Currens, M.J., Herring, A., Kelley, S.P., McMahon, J.B., Ray-Chaudhuri, R, and Malan-Shibley, L.: Sequential changes in diploid rat liver epithelial cells treated with afiatoxin B_2 in culture (abst.). *Proc. Am. Assoc. Cancer Res.* **23**, 74, 1982.
14. Miyazaki, M., Wahid, S., Miyano, K., Yabe, T. and Sato, J.: Expression of gammaglutamyltranspeptidase in cultures of spontaneously and chemically transformed rat liver cells. *Int. J. Cancer* **32**, 373-377, 1983.
15. Miyazaki, M., Watanabe, A., Shiraishi, M., Hoshika, T., Miyano, K. and Sato, J.: Primary culture of adult rat liver cells. III. Hormonal effects on cytological and biochemical properties of primary cultured cells. *Acta Med. Okayama* **32**, 85-96, 1978.
16. Wahid, S.: *In vitro* transformation of adult rat liver cells by 3'-methyl-4-dimethylaminoazobenzene. *Acta Med. Okayama* **37**, 31-44, 1983.
17. Miyazaki, M., Wahid, S., Matsuura, K., Tsunashima, M., Nakabayashi, H., Miyano, K. and Sato, J.: Effect of 3'-methyl-4-dimethylaminoazobenzene on liver cells from adult rat in primary culture. *Res. Exp. Med. (Berl)* **181**, 27-38, 1982.