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Abstract

In the experiments with cultured liver cells it is very important to know whether or not the cells in vitro have the same properties and functions as in vivo. The purposes of this work were to investigate the functions of the cultured liver cells and to identify functionally the liver cells cultured by our present method with the parenchymal liver cells. At first, the albumin production of the cultured liver cells, one of the well known functions of the liver cells, was examined by the immunological methods, especially, the fluorescent antibody technique and the complement fixation test. Culture methods which could display the functions of the liver cells as much as possible were explored simultaneously. The results were as follows: 1. Albumin production was detected in the strain RLN·10 liver cells established from the liver tissues of a Donryu rat with immunofluorescent method and complement fixation test. This confirms that the cultured liver cells maintain the function to produce albumin and these cells have originated from the parenchymal liver cells. 2. Hepatoma strains (AH 66-TC-l, AH 7974-TC-l) also showed the albumin production but the extent of its production was less than that of the strain RLN-10. 3. In the short-term cultured liver cells, the albumin production was testified only slight in one month and was exhibited in a small amount in three months. 4. Every culture method examined exhibited no appreciable difference in the albumin production in the cultured liver cells.

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FUNCTION OF THE LIVER CELLS IN THE SHORT-TERM AND THE LONG-TERM CULTURES
I. ALBUMIM PRODUCTION OF THE LIVER CELLS *IN VITRO*

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When we culture the rat liver tissues by the present method[1], we can obtain the liver cells which morphologically belong to the parenchymal liver cells. Now, there arise the questions what functions the cultured liver cells maintain *in vitro* and whether or not these cells are only proliferating *in vitro* without characteristic functions of the liver cells. These problems are very important in the planning of some experiments using the cultured liver cells. The purposes of this experiment were to investigate the function of the cultured liver cells and to identify functionally the cultured liver cells with parenchymal liver cells.

It is generally recognized that albumin can be produced only in the liver cells *in vivo* or in the fragments of the liver tissue *in vitro*[2,3,4]. But no work concerning albumin production in the cultured liver cells, especially in the long-term culture, has been reported. The present work explored the albumin production in the long- and short-term cultured liver cells by the method of immunofluorescence and complement fixation test. At the same time, every culture method, by which the cultured liver cells may display better functions, was examined.

MATERIALS AND METHODS

Cells and Culture Methods : Five strains established in our laboratory were utilized in the present experiment. They were designated as strains RLN-10, RLD-10, AH 66-TC-1, AH 7974-TC-1 and JTC-11, as shown in Table 1. The strain RLN-10 was derived from liver tissues of Donryu 14-day-old rats which had been inbred in our laboratory. The strain RLD-10 was from the same rat from which the strain RLN-10 cells were obtained and induced to proliferate by the use of 1γ per ml DAB for the initial 4 days of cultivation[5]. AH 66-TC-1 and AH 7974-TC-1, which were generously supplied by Dr.

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TABUCHI of our laboratory, were derived from the ascites hepatoma cells of the rats fed the azo dyes and the strain JTC-11 was from Ehrlich ascites carcinoma cells[6]. The four strains except JTC-11 were subcultured with 0.2% trypsin but JTC-11 was done with pipetting. The cultivation of primary culture of liver cells was used as the short-term culture. The culture medium consisted of 20% inactivated bovine serum, 0.4% lactalbumin hydrolysate (NBC) and buffered saline, mixture D.

Table 1 Cell Strains used in the Present Work

Cell strain	Origin of tissues	Age (days) of culture
RLN-10	Donryu rat liver	1447
RLD-10	Donryu rat liver	1430
AH 66-TC-1	rat hepatoma	378
AH 7974-TC-1	rat hepatoma	414
JTC-11	Ehrlich	2011

Preparation of labelled antiserum : Rat serum albumin as antigen was purified according to the method of ADAIR and ROBINSON[7]. This albumin fraction was checked by the electrophoresis (Photo 1). Fifty mg rat albumin were injected intracutaneously and intramuscularly into each rabbit in form of Freund's incomplete adjuvant. Animals were bled after 4 months. The precipitation titer was ×64. The fraction of gamma-globulin was precipitated from the pooled serum by 33.3% saturated ammonium sulfate and labelled with fluorescein isothiocyanate (BBL) at FITC : γG ratios of 1 : 100 at pH 9.4 for 10 hr at 4°C. After free FITC was removed 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 (0.80meg) column with the same eluent. The final molecular ratio of FITC to protein was about 2.

Staining Procedures : Cells cultured on cover slips were fixed with 1% acetic acid alcohol for 30 min 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 mouse and bovine liver homogenates, respectively, was 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% glycerol PBS. The specimen was observed by darkfield using the microscope illuminated with high pressure mercury lump (Nikkon Co.). UV filter was employed through the experiments and for the photomicrography.

RESULTS

1. Assay of Nonspecific Staining of the Labelled Antiserum

When the fluorescent antibody technique is used, the problem of nonspecific staining always comes up. For that reason, at first, the specificity of the labelled serum was examined. The sections of mouse and bovine liver and rat kidney were stained with the conjugate. No specific fluorescence was admitted in these specimens. The liver cells having albumin showed specific fluorescence, when the section of a rat liver was stained with the conjugate. Besides, both the blocking and the absorption tests of the liver specimens were negative. The labelled antiserum was highly specific from the results and utilized in the following works.

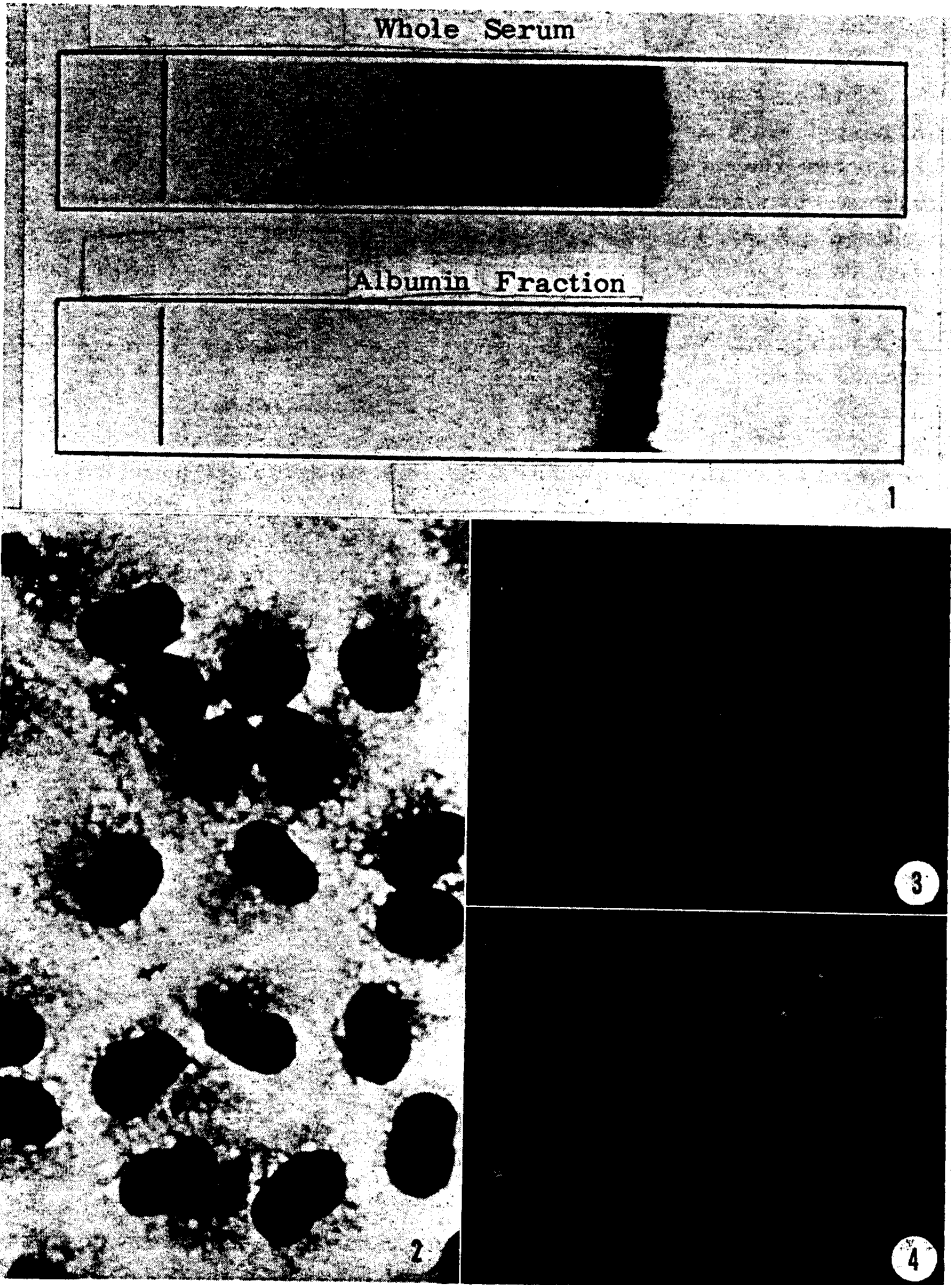
2. Albumin Production in the Cultured Cells

Strain cells cultured on cover slips for 4 to 6 days after the subculture were stained with the labelled antiserum. Fine fluorescent granules were observed in the cytoplasm of RLN-10 cells. The granules were abundant near the nucleus and its localization in the cell was consistent with the microsomes (Photo 3). The strains, RLD-10, AH 66-TC-1 and AH 7974-TC-1 exhibited the identical findings of fluorescence but the number of cells having fluorescent particles and the distribution of luminous microsomes were generally less than those of RLN-10. The immunofluorescent findings of the RLD-10 cells are shown in Photo 4.

No fluorescent granule was observed in short-term cultured liver cells within one month and faint fluorescence was displayed in three months in the outgrowth area of the explanted liver tissues. The results are illustrated in Table 2.

Table 2 Immunofluorescent Results of Albumin Production in Short-term Cultured Liver Cells

Expt. No.	Tissue	Total days	Subculture	Albumin production
1	rat liver	19	primary	—
		36	primary	±
2	rat liver	24	1st	±
		26	1st	±
		36	2nd	±
3	rat liver	81	3rd	+
		97	3rd	+
4	rat spleen	8	primary	—


Whole Serum
Albumin Fraction
1
2
3
4

3. The Demonstration of Albumin in the Culture Fluid of RLN-10 Cells with Other Immunoreactions, Precipitation Test, Complement Fixation Test, Ouchterlony's Method and Reversed Passive Cutaneous Anaphylaxis

The strain RLN-10 cells were inoculated in TD 40 bottle with about 10 ml medium and the cultured media were respectively collected on 6th, 8th and 24th culture day. The number of cells was about one million at the start and increased up to about 10 millions after 24 days of the culture. Each medium collected was checked with the rat albumin antiserum. No albumin production was noticed by the use of precipitation test and Ouchterlony's method. But the test of the reversed passive cutaneous anaphlaxis was positive. The method of R-PCA was as follows. The culture fluid was injected intradermally at various sites in shaved dorsal skin of an albino guinea pig. After a latent period of 20 minutes, the animal was challenged intravenously with 0.5 ml of the rat albumin antiserum mixed with 0.5 ml of 1% Evans blue solution. Reactions became visible as blue spots within a few minutes after the challenge.

Complement fixation test was utilized to examine the albumin in the culture medium. As shown in Table 3, the reaction began on 8th culture day and was admitted clearly on 24th culture day. From the results, the existence of the albumin in the culture fluid was testified quantitatively.

Table 3 Albumin in the Culture Fluid Incubated with the RLN-10 Cells Detected with Complement Fixation Test, Control Medium was the One which was not Incubated with the Cells.

Culture days \ Dilution of med.	×1	×2	×4	×8	×16
4	−	−	−	−	−
6	±	−	−	−	−
8	+	±	−	−	−
24	+	+	±	−	−
Control med.	−	−	−	−	−

Photo 1 Electrophoretic patterns of whole rat serum and rat serum albumin isolated with ammonium sulfate. The albumin fraction was utilized as antigen.

Photo 2 The cells of the strain RLN-10 established from the Donryu rats. Cytoplasm containing oval nuclei spread thinly on the glass surface and each nucleus has several nucleoli. The morphology suggests the cells to have originated from parenchymal liver tissues (stained specimen with Giemsa). (10×20)

Photo 3 Immunofluorescent finding of the strain RLN-10 cell. Fine granules in the cytoplasm show the existence of albumin and its localization in the cells is consistent with microsomes. (10×40)

Photo 4 Immunofluorescent findings of the strain RLD-10 cells (10×20)

4. The Effect of the Concentration of Bovine Serum on the Albumin Production

The function of cells is generally considered to be in the inverse proportion to the cell growth. If the growth of cells can be inhibited, a greater function may be expected. The strain RLN-10 cells were used in this experiment and bovine serum per cent in the medium was reduced down to 5 per cent. No significant difference between 20% and 5% group, however, was found out during 2 or 4 days of the subculture.

5. The Effect of Rotatory and the Stationary Culture on the Albumin Production

If more oxygen can be supplied to the cultured cells, it may be expected for the cells to display better functions. The rotatory culture can give more oxygen to the cultured cells than the stationary culture, because the cells can more readily touch oxygen. From this viewpoint, the difference between the rotatory and the stationary cultures about the albumin production in RLN-10 cells was examined, but no difference was observed with immunofluorescent technique.

6. The Effect of Oxygen and Nitrogen Gas on the Albumin Production

This experiment was intended to supply as much oxygen as possible to the cultured liver cells. The albumin production of RLN-10 cells was observed by the immunoflucrescent method 2 days after the air in the culture test tubes was displaced by oxygen gas or nitrogen gas. No difference between them was noticed.

7. Biological Aspects of the Strain RLN-10 Liver Cells

The proliferation rate of the strain cells was about 3-5-fold in 7 days using the medium consisting of 20% bovine serum, 0.4% lactalbumin hydrolysate and buffered saline. The liver cells cultured on a coverslip are illustrated in Photo 2. The cytoplasm is thinly spread on the glass surface and oval nuclei containing a few of nucleoli are observed. These morphological findings of the cells are epithelial-like and suggest that the cells originated from the parenchymal liver tissues. The modal chromosome number of the cells existed near the triploidy region (Fig. 1). The specimens were prepared by the squash method.

DISCUSSION

1. Albumin was detected as granules in the cytoplasm of the cultured liver cells by the immunofluorescent technique and the localization of albumin in the cell probably was consistent with the microsomal particles. It was not deter-

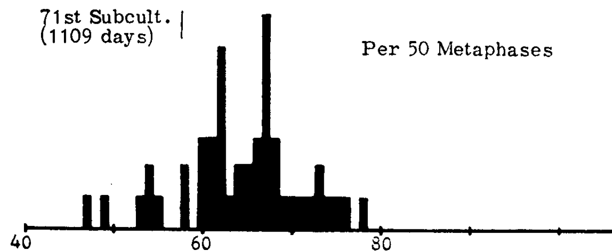


Fig. 1 Distribution of Chromosome Number of the Strain RLN-10

mined, however, whether the granular particles of albumin might be produced at the microsomes or merely contained in the microsomal sites before its excretion after they had been produced at other sites. These problems will be resolved in near future.

2. The number of cells producing albumin *in vitro* increased day by day after the subculture in the immunofluorescent observation. This was in accord with the result using the complement fixation test. CF test will be recommended to the quantitative estimation of the albumin production in culture fluid but far many cells must be prepared to detect the albumin. On the other hand, the existence of albumin in the cells cannot be recognized by CF test.

3. AH 66-TC-1 and AH 7974-TC-1, hepatoma cell strains in culture, exhibited slight albumin production. The results indicate hepatoma cells to maintain some functions of liver cells.

4. In the short-term culture, the liver cells did not show albumin production in one month, but slightly exhibited the production in three months. It seems probable that cultured liver cells adapt themselves to the new culture conditions and begin to display gradually some functions of the liver cell. At any rate, a certain period may be necessary before the cultured cells adapt to the *in vitro* environments.

5. The results 5 and 6 failed to give any singificant meanings. This fact suggests that oxygen might not be necessary to the albumin production. But it cannot be concluded that the albumin production is independent of the supply of oxygen, because the oxygen dissolved in the culture medium could not be excluded by the methods of these experiments. There is no denying the fact that oxygen must be necessary for the cultured liver cells to exhibit some functions.

6. It is very difficult to maintain the diploid number of chromosomes in the long-term cultured cells. The chromosome distribution of RLN-10 is near the triploidy region, as illustrated in Fig. 1. The relation between the albumin production and the chromosome number could not be ascertained, but, when the clonal culture of the liver cells is established, the relation may be elucidated.

The work along this line is now in progress.

7. It is of a great interest to know whether or not the cultured liver cells have functions other than that of albumin production. At the present time, the function of bilirubin metabolism in the cultured liver cells is being investigated. In the series to follow further results will be reported.

8. It is generally believed that the cultured cells lose their specific function or character and dedifferentiation ensues, and that the more the cells proliferate, the less their functions are retained. It has been very difficult to ascertain the relation between the cell proliferation and the albumin production throughout the experiments. But it is very important to prove whether the proliferating liver cells have the function of the albumin production. Though it is not decisive yet whether the proliferating liver cells showed the albumin formation or they lost the function, it seems probable that the proliferating liver cells produce albumin. As far as the albumin production and the proliferation are concerned, the relation between them is inferred from the results not to be so dependent on each other.

SUMMARY

In the experiments with cultured liver cells it is very important to know whether or not the cells *in vitro* have the same properties and functions as *in vivo*. The purposes of this work were to investigate the functions of the cultured liver cells and to identify functionally the liver cells cultured by our present method with the parenchymal liver cells. At first, the albumin production of the cultured liver cells, one of the well known functions of the liver cells, was examined by the immunological methods, especially, the fluorescent antibody technique and the complement fixation test. Culture methods which could display the functions of the liver cells as much as possible were explored simultaneously. The results were as follows:

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4. Every culture method examined exhibited no appreciable difference in

the albumin production in the cultured liver cells.

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