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Abstract

To obtain a useful rat liver cell line for in vitro carcinogenesis, two rat diploid epithelial cell lines were established from a 7-day-old male rat by the repeated colonial clone method. More than 80% of cells from each cell line have maintained normal diploid karyotype for over 30 months in vitro. The diploid cells were identi. fied as normal diploid karyotype by conventional Giemsa and trypsin. Giemsa techniques. They showed little difference in morphology and growth rate between early and late passages. Without cloning, they tended to be heterogenous in cell morphology, became heteroploid in chromosome and showed increased growth potential with time. Highly heteroploid cells which were derived from one of the lines produced ascites and solid tumors when inoculated into syngeneic rats intraperitoneally. Histologically, the tumors were diagnosed as poorly differentiated hepatocarcinomas. One of these diploid epithelial cell lines in early passage contained some activity of tyrosine transaminase and liver type aldolase and .glycokinase. Therefore, it is suggested that these epithelial cell lines represent liver parenchymal cells.

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LONG-TERM CULTIVATION OF TWO DIPLOID EPITHELIAL CELL LINES DERIVED FROM NORMAL RAT LIVER CELLS

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Abstract: To obtain a useful rat liver cell line for *in vitro* carcinogenesis, two rat diploid epithelial cell lines were established from a 7-day-old male rat by the repeated colonial clone method. More than 80% of cells from each cell line have maintained normal diploid karyotype for over 30 months *in vitro*. The diploid cells were identified as normal diploid karyotype by conventional Giemsa and trypsin-Giemsa techniques. They showed little difference in morphology and growth rate between early and late passages. Without cloning, they tended to be heterogenous in cell morphology, became heteroploid in chromosome and showed increased growth potential with time. Highly heteroploid cells which were derived from one of the lines produced ascites and solid tumors when inoculated into syngeneic rats intraperitoneally. Histologically, the tumors were diagnosed as poorly differentiated hepatocarcinomas. One of these diploid epithelial cell lines in early passage contained some activity of tyrosine transaminase and liver type aldolase and glycokinase. Therefore, it is suggested that these epithelial cell lines represent liver parenchymal cells.

Up to date, in elucidating the mechanism of *in vitro* hepatocarcinogenesis, rat liver epithelial-like cells in culture, especially normal rat liver parenchymal cells, have been used by numbers of workers with various methods (1-10).

In our labolatory, since 1961 primary cultures of rat liver cells have been conducted in the roller tube culture, following the procedures by KATSUTA and TAKAOKA (11, 12). But this method, considered to allow only epithelial cells to grow selectively, requires over two months to obtain the cells. Furthermore the cultures obtained by this method might contain a few fibroblasts, heteroploid in chromosome (13-15), then undergo malignant transformation spontantaneously during long-term cultivation and produce carcinomas and sarcomas in rats by intraperitoneal inoculation of the cultured

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cells (16).

MIYAHARA *et al.* (17) succeeded in inducing malignant transformation *in vitro* using a single cloned rat liver cell line treated with DAB. But in their case, untreated cells, *i. e.* control, were passaged many times serially from primary culture so that they were highly heteroploid in chromosome, thus complicating the problem.

The most ideal materials for *in vitro* carcinogenesis should be derived from a single cell line, having normal diploid karyotype and equipped with function and structure of normal hepatocytes, and further these properties should be stable during *in vitro* cultivation.

This report describes isolation of two rat liver epithelial cell lines from a 7-day-old rat, that have maintained normal diploid karyotype for more than 30 months *in vitro* by repeated colonial cloning, and discusses about evidence that they are progeny of rat liver parenchymal cells.

MATERIALS AND METHODS

Animals: Experimental animals used were Donryu-strain of rats which have been inbred by brother-sister mating in our laboratory since 1965 (18).

Primary culture: The liver tissue was excised from a 7-day-old male rat aseptically, minced with scissors, and then transferred into a 50 ml flask. The minced tissue was added with 20 ml of 0.2% trypsin phosphate buffer solution, stirred gently with pipetting, and incubated at 37°C for 30 min. The supernatant containing mainly blood cells and cell debris was discarded. An equal amount of trypsin solution was added, and the mixture was gently stirred with a magnetic stirring bar for 15 min. After briefly allowing large fragments of tissue to settle, the supernatant was aspirated, poured into a 40 ml conical centrifuge tube containing 15 ml of medium supplemented with 20% bovine serum and mixed with medium gently, and the tube was placed in an ice bath. The liver tissue was trypsinized four times or more, the dissociated cells from each trypsinization were treated as above, and each tube was centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the dissociated cells from each step of trypsinization were collected and resuspended in complete medium, except the first trypsinization containing many blood cells. The cells were seeded into P-2 size glass dishes (3 ml volume) at concention of 10×10^4, 2×10^4, 4×10^3 and 800 per dish. The dishes were placed in a humidified atomosphere of 5% CO_2 and 95% air at 37°C. The culture medium was Eagle's minimum essential medium supplemented with 20% bovine serum, penicilline (100 units/ml) and streptomycin (100μg/ml). The culture medium was renewed once every week.

Isolation of epithelial cells: After two weeks of cultivation, the dishes inoculated with 10×10^4 cells became confluent mixed by fibroblast-like and epithelial-like cells. As inoculum sizes decreased in dishes, the epithelial-like and fibroblast-like cells grew separately from each other and formed their

own colonies. In dishes seeded with 800 cells the epithelial-like cells only grew selectively, while the fibroblast like cells disappeared completely. These epithelial-like colonies were isolated colonially by means of trypsin-paper method and then two isolated clone lines were allowed to proliferate and subcultured under monitoring of morphology and chromosome.

Isolation of normal diploid cells: When the culture began to deviate from the normal pattern in karyology and morphology during serial passages, the cells were plated again in decresing inoculum size, and the colonies consisting of uniform epithelial-like cells were isolated by the same procedures as in the primary culture, and checked for chromosomes. Thus, only the clone in which the majority of cells showed normal diploid pattern was allowed to proliferate and subcultured. Thereafter, to maintain diploid cells *in vitro*, the procedures mentioned above were repeated.

Chromosome analysis: For chromosome study, the culture was treated with colchicine (final concentration 1 μg/ml) 4 hrs before harvest. Then, they were treated with a hypotonic solution of 1% sodium citrate at 37°C for 15 min and fixed in Carnoy's mixture (1 : 1, absolute ethanol-glacial acetic acid), which was changed several times until complete dehydration was achieved. A small droplet of the suspension containing fixed cells was carefully dropped on clean and chilled glass slides and allowed to dry at room temperature. After drying, the cells were stained with 3% Giemsa-PBS solution (pH 6.8) for 4 hrs. In addition to a conventional Giemsa staining, the G-banding procedure (19) was used to identify individual chromosomes more precisely, according to a slightly modified Seabright's method (20). Instead of Leishman's solution Giemsa solution was used, and trypsin (Difco) was used in several different concentrations and treating times.

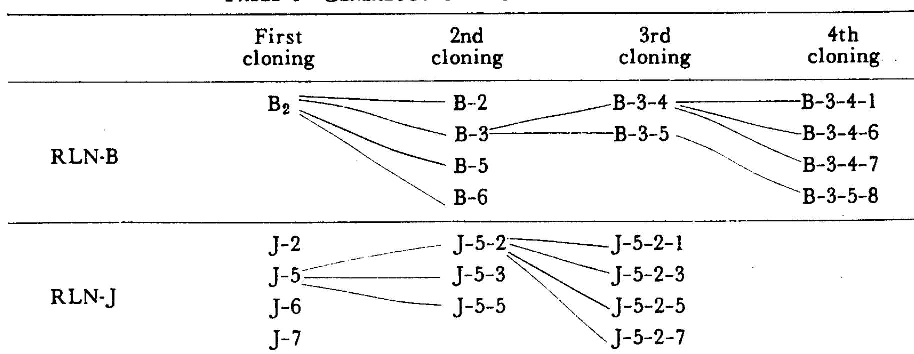
RESULTS

1. *Establishment of rat epithelial cell lines*

Two rat liver epithelial cell lines were established from a 7-day-old male rat respectively. RLN-B line was derived from a colonial clone of primary culture, and thereafter cloned at day 260 (19th subculture), day 520 (36th subculture) and day 760 (51st subculture). B-3-4-7 line was cloned four times up to now. RLN-J line grew and subcultured in mass-culture from primary culture until the first cloning at day 330 (41st subculture), and thereafter the cells were cloned at day 660 (67th subculture) and day 780 (75th subculture). J 5-2-1 line, therefore, was cloned three times up to now (Table 1).

Table 2 shows the cell lines which were maintained in long-term cultivation *in vitro* and their percent diploid cells at about day 900 *in vitro*. In RLN-B line, the cells of B-3-4-7 line have 82% diploid at day 922 *in vitro*, while the stem cell of B-3-5-8 line have pseudodiploid karyotype with one medium-size subtelocentric marker chromosome and have none of cells with

TABLE 1 GENEALOGY OF TWO DIPLOID CELL LINES



Two rat liver epithelial-like cell lines were established from a 7-day-old male rat respectively. RLN-B_2 line was derived from a colonial clone of primary culture, and thereafter has been cloned four times up to now. RLN-J line has been subcultured in mass-culture from primary culture before the first coloning at day 330, and then cloned three times up to now.

TABLE 2 CHROMOSOME ANALYSIS OF THE REPEATED CLONE LINES DERIVED FROM NORMAL RAT LIVER CELLS AT ABOUT 900 DAYS IN VITRO

Cell line	Total culture days	% of diploid cells	Abnormal karyotype
B-3-4-1	873	56	
B-3-4-6	887	64	
B-3-4-7	886	94	
	922	82	
B-3-5-8	739	0	*m*ST marker
J-5-2	780	88	
	854	84	
	915	88	
	976	30	*l*M marker
J-5-3	727	86	
	833	74	
	915	68	
J-5-5	730	84	
J-5-2-1	854	84	
	914	82	
J-5-2-3	863	74	
	915	36	
	949	0	*l*M marker
J-5-2-5	920	90	
J-5-2-7	877	0	*l*M marker

*m*ST marker : medium-size subtelocentric marker chromosome
*l*M marker : large size metacentric marker chromosome

When the cells were cloned based on the morphological characteristics, the clone lines obtained varied in the degree, from the lines with high rate of diploid (B-3-4-7, J-5-2-1) to those with none of diploid (B-3-5-8, J-5-2-7). After cloning, the diploid cells tended to decrease invariably in number with lapse of times, while the cells with abnormal karyotype increased, and eventually the latter overgrew the former in every diploid lines such as in J-5-2 and J-5-2-3.

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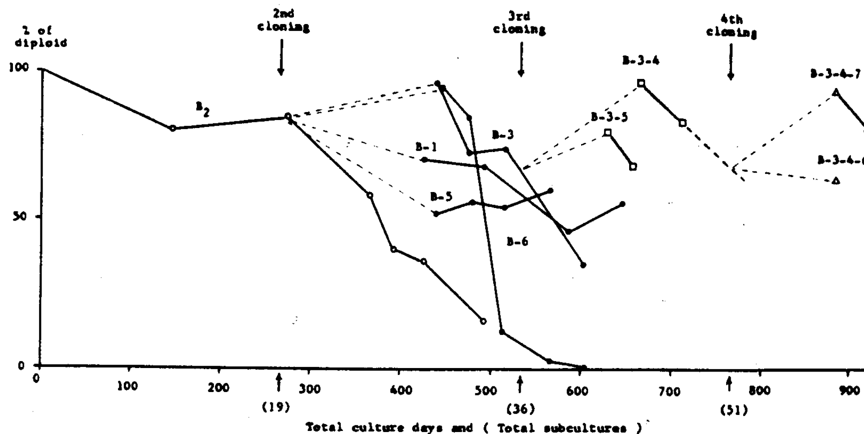
diploid karyotype. In RLN-J line, the cells of J-5-2-1 and J-5-2-5 line have 82% diploid at day 914 *in zitro*, and 90% diploid at day 920 *in vitro*, respectively. J-5-2-7 line cells have 41 chromosomes containing one large size metacentric marker chromosome as a stem cell.

After cloning, the obtained lines varied in the degree, from the lines with high rate of diploid (B-3-4-7, J-5-2-1) to those with no diploid (B-3-5-8, J-5-2-7). Even after cloning, the normal diploid cells tended to decrease invariably in number with time, while the cells with abnormal karyotype increased, and eventually the latter overgrew the former in every diploid cell lines such as in J-5-2 and J-5-2-3 (Table 2). Such a karyological instability was commonly observed in both cell lines not only after the first cloning, but also after the fourth cloning as in Figs. 1 and 2. But so long as the colonial clone method is repeated, these cell lines were able to maintain



broken lines : first chromosome analysis after cloning
solid line with open circles : first clone line
solid line with filled circles : second clone line
solid line with squares : third clone line
solid line with triangles : fourth clone line

Fig. 1 Culture history of rin-B line

This line was derived from a colonial clone of primary culture and thereafter cloned at day 260 (19th subculture), day 520 (36th subculture) and day 760 (51st subculture). By colonial method, this line maintained normal diploid karyotype in more than 80% of cells for over 900 days *in vitro*.

normal diploid karyotype in more than 80% of cells for over 30 months *in vitro* such as in B-3-4-7, J-5-2, J-5-2-1 and J-5-2-5.

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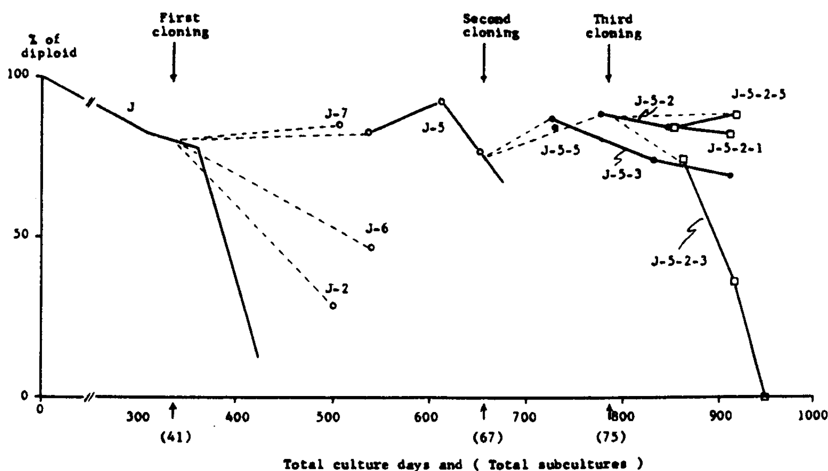


broken lines : first chromosome analysis after cloning
solid line with open circles : first clone line
solid line with filled circles : second clone line
solid line with squares : third clone line

Fig. 2. Culture history of rin-J line
This cell line, being different from RLN-B line, has been subcultured in mass-culture from primary culture before the first cloning at day 330, and thereafter cloned at day 660 (67th subculture) and day 780 (75th subculture). Three sublines of this clone line maintained more than 80% diploid cells for over 900 days *in vitro*.

2. *Some properties of normal diploid clone lines*

a) Analysis of chromosomes in these diploid cells by G-banding pattern

Up to date, normal diploid cells were identified on the basis of morphology of chromosomes such as shape and size. By this method, however, it was impossible to identify all of individual chromosomes of a rat (18). Recently, a number of methods have been developed to differentiate mammalian, particularly human, chromosomes longitudinally into regions or bands by use of various staining procedures. Especially, Giemsa-banding procedure, the simplest and most economical way of staining, has made the identification of each normal chromosome of rats possible (19, 21–24). G-banding pattern of these repeated-colonial clone line cells was also examined. The banding techniques also permitted each chromosome of Donryu rat to be identified individually (Figs. 3 and 4) and G-banding pattern of these diploid cells were in a close agreement with normal pattern from bone marrow cells of normal rats (Figs. 5 and 6).

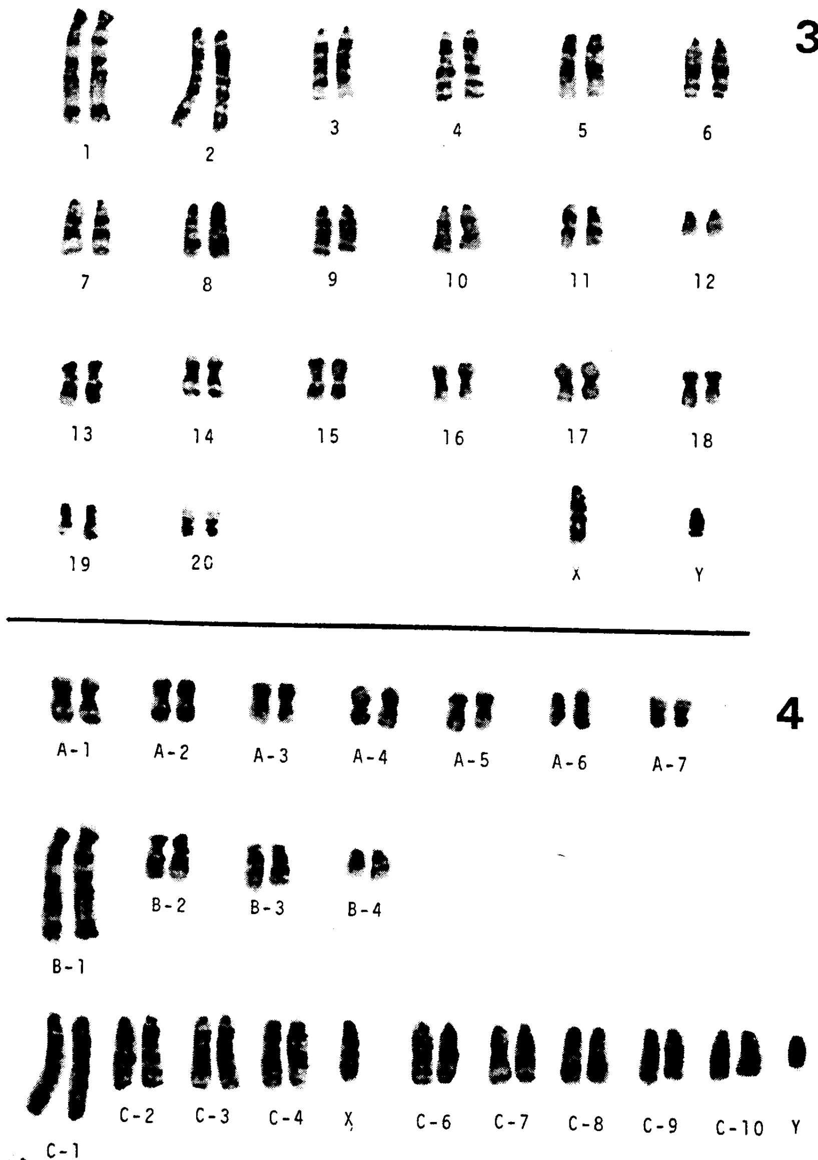


Fig. 3. Trypsin-Giemsa-banded, normal diploid karyotype of a cell from bone marrow cells of a normal male Donryu rat. The karyotype was arranged according to the standard numbering system (24). Each chromosome was identified individually with Trypsin-Giemsa technique. Note that chromosome 3 in Donryu strain are a telocentric type differing from other strains such as Norway rats.

Fig. 4. The same karyotype as in Fig. 3 was rearranged by our numbering system (18).

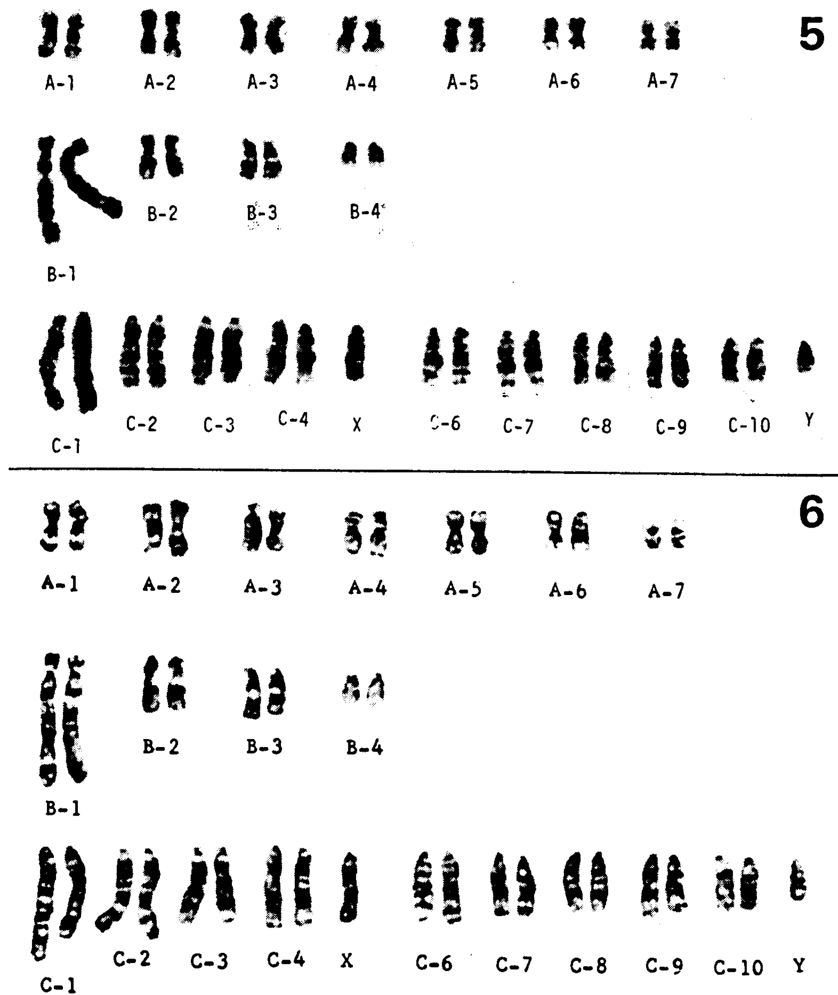
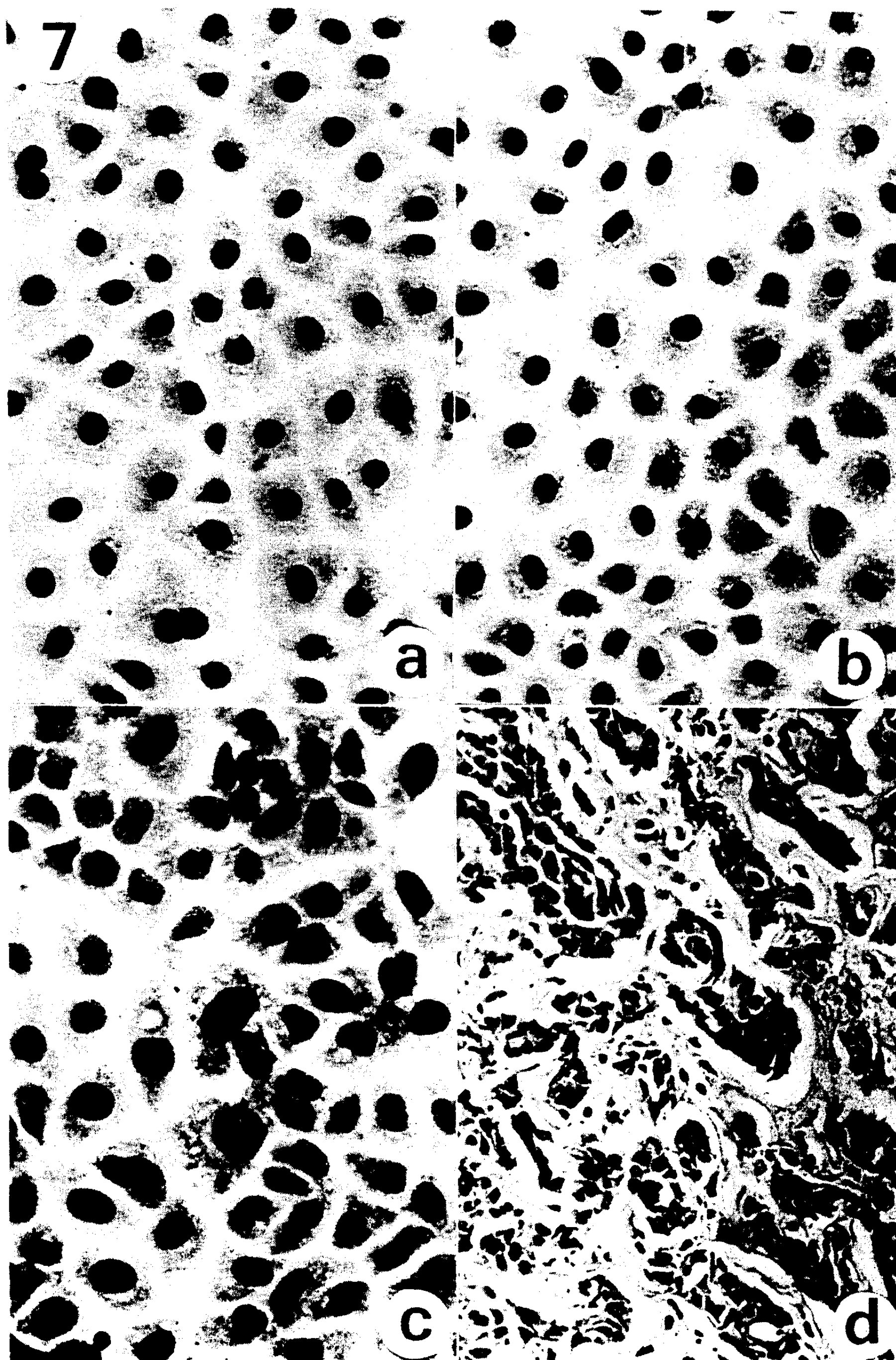


Fig. 5. Karyotype of a cell from a repeatedly cloned diploid line, J-5-2-5 line, at day 920 *in vitro*. The banding patterns are similar to those seen in Figs. 3 and 4.

Fig. 6. Karyotype of a cell from a repeatedly cloned diploid line, B-3-4-7 line, at day 922 *in vitro*. The banding patterns are similar to those seen in Figs. 3 and 4.

Fig. 7. a, Confluent-culture of RLN-B line at day 349, subculture 26. Giemsa, ×400. The cells are uniform in size, and the growth pattern is pavement-like. Binucleated cells are not abundant. b, Confluent-culture of repeatedly cloned diploid line, J-5-2-1 line, at day 908 *in vitro*. subculture 82, Giemsa, ×400. c, Confluent-culture of RLN-B_2 line, a highly heteroploid and malignant transformed line, at day 899 *in vitro*, subculture 143. Giemsa, ×400. Note that appearance differs from diploid line. The cells have assumed a random orientation, pleomorphism and lost contact inhibition. The cellular cytoplasm is more basophilic. d, A section of a tumor produced by intraperitoneal injection of the highly heteroploid cells of RLN-B_2. The malignant cells have abundant cytoplasma and distinct cell borders and are arranged in nests. The tumor was diagnosed as poorly differentiated hepatocarcinoma. H. and E. ×250


7
a
b
c
d

b) Morphology and growth pattern

For approximately 900 days *in vitro* the diploid cells had retained epithelial features in morphology and had an oval or round nucleus with one or more nucleoli of various sizes; in the average of 2.4 nucleoli per cell and having 2 nucleoli in about a half of the cells. Binucleated cells were not abundant and cellular cytoplasm of the cells appeared less granular (Figs. 7a and 7b). The cells grew in islands, eventually coalesced and were subcultured by dividing cells equally in each 2 bottles every 7-10 days. Plating efficiency of these cell line was about 0.5 to 1.0%. The characters mentioned above in the diploid cells which have been maintained for over 900 days *in vitro* are equivalent to those of the diploid cells in early passage. But when cells with abnormal karyotype increased in cell population, the cell morphology became heterogeneous, and plating efficiency and growth rate increased rapidly.

DISCUSSION

Two rat liver epithelial cell lines derived from a 7-day-old male rat are described. They have maintained normal diploid karyotype in more than 80% of cells for over 30 months *in vitro* by the repeated-colonial cloning based on the morphological characteristics. Their normal diploid karyotypes were identified with conventional Giemsa and also G-banding techniques. They also showed little difference in cell morphology and growth rate between early and late passages. These facts suggested that they would continue to proliferate and maintain normal karyotype as long as the cloning is repeated, and seem to grow indefinitely without " senescence " contrary to what HAYFLICK had mentioned (25, 26). The question that these differences depend on either species or cell types requires further studies.

Karyological instability was observed in these colonial clone lines as well as in mass-cultured cell lines obtained by roller tube method (15). The question whether such phenomenon resulted from spontaneous mutagenicity of cells or environmental difference of *in vitro* and *in vivo* remains unanswered. A detailed report of chromosomal alteration related to an establishment of these diploid clone line lines will be described in other reports.

Up to now, there have been two rat diploid fibroblastoid cell strains in the literatures which seem persistent and in which cells are predominantly euploid *i. e.* PETURSSON's (27) and KROOTH's (28) cases. The former is a diploid rat strain, derived from rat embryos, and has remained diploid for 40 transfers or approximately 18 months *in vitro*. The latter was derived from rat striated muscle tissue, serially propagated for over 15 months, subcultured 88 times, and was over 85% of euploid. But both cell lines were fibro-

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blastoid in morphology, and further the duration of diploid cells maintained *in vitro* is shorter than ours. With regard to diploid epithelial cell lines derived from normal rat liver cells, there have been only two cell lines up to now. COON (1, 2) reported on a rat liver epithelial clone line derived from 2-week to 12-month-old rats, and the cell lines were diploid for 70 cell generations and have some differentiated properties of liver specificity. BOREK (8, 22) reported on a differentiated epithelial cell line which was derived from normal liver of a 3-month-old Buffalo rat and was isolated by three consecutive clones. The cell line expressed some liver specific properties and had remained 50% diploid at the 38th month *in vitro*, but the cell line has not yet been tested for producing carcinoma by inoculating cells cultured *in vitro*.

In the present study, a colony in which the cells were small, polygonal and uniform in morphology, was isolated as a normal diploid colony according to our experiences (11-17) of cultivation of rat liver cells. Our cloned epithelial cells seem to have been originated from hepatocytes as WILLIAMS (5, 9) and MONTESANO (10) and CASANELLO (4) reported. It is because B and J clone lines have a morphological similarity to the cell lines mentioned above, and further the cultured cells derived from the same pool of B and J cells respectively and deviated highly from normal karyotype, produced undifferentiated carcinoma in syngeneic newborn rats by inoculating 1×10^7 cells intraperitoneally after several months (Fig. 7d). These diploid cells, especially B line, in an early stage of cultivation contained some activity of tyrosine transaminase and the liver type of aldolase and glucokinase (29).

In vivo, the cells exist in constructing various tissues with cell masses and are under the control of various hormones. When these cell forming tissues are dissociated into a single cell and transferred into *in vitro* condition, the cultured cells lose the differentiated function and structure and revert to an immature or fetal state, this process being reversible by some unknown factors (29). We have been making efforts to maintain the diploid rat liver epithelial cell *in vitro* based on chromosome and cell morphology. In future we plan to maintain other normal hepatocytes with differentiated properties at least in immature or fetal type in culture, and then revert them to mature or differentiated type by altering culture conditions, especially by improving culture medium.

REFERENCES

1. COON, H. G.: Clonal culture of differentiated rat liver cell. *J. Cell. Biol.* **39**, 29A, 1968
2. COON, H. G.: Clonal culture of differentiated cells from mammals: Rat liver cell culture. *Carnegie Institution of Washington Year Book* **67**, 419-421, 1969
3. TOYOSHIMA, K., HIASA, Y., ITO, N. and TSUBURA, Y.: *In vitro* malignant transformation of cells derived from rat liver by means of Aflatoxin B_1. *Gann* **61**, 557-561, 1970

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4. CASANELLO, D. E., and GERSCHENSON, L. E.: Some morphological and biochemical characteristics of isolated rat liver cells dissociated with sodium tetraphenylboron and cultured in suspension. *Exptl. Cell Res.* **59**, 283-290, 1970
5. WILLIAMS, G. M., WEISBURGEE, E. K. and WEISBURGEF, J. H.: Isolation and long-term cell culture of epithelial-like cells from rat liver. *Exptl. Cell Res.* **69**, 106-112, 1971
6. IYPE, P. T.: Cultures from adult rat liver cells I. Establishment of monolayer cell-cultures from normal liver. J. *Cell. Physiol.* **78**, 281-288, 1971
7. KATSUTA, H. and TAKAOKA, T.: Carcinogenesis in tissue culture XIV. Malignant transformation of rat liver parenchymal cells treated with 4-nitroquinoline-1-oxide in tissue culture. *J. Natl. Cancer Inst.* **49**, 1563-1576, 1972
8. BOREK, C.: Neoplastic transformation *in vitro* of a clone of adult liver epithelial cells into differentiated hepatoma-like cells under conditions of nutritional stress. *Proc. Nat. Acad. Sci.* U. S. A. **69**, 956-959, 1972
9. WILLIAMS, G. M., ELLIOTT, J. M. and WEISBURGER, J. H.: Carcinoma after malignant conversion *in vitro* of epithelial-like cells from rat liver following exposure to chemical carcinogdns. *Cancer Res.* **33**, 606-612, 1973
10. MONTESANO, R., SAINT VINCENT, L. and TOMATIS, L.: Malignant transformation *in vitro* of rat liver cells by dimethylnitrosamine and n-metyl-n'-nitro-n-nitrosoguanidine. *Br.* J. *Cancer* **28**, 215-220, 1973
11. KATSUTA, H. and TAKAOKA, T.: Carcinogenesis in tissue culture. I. Cultivation of normal rat liver cells. *Japan J. Exp. Med.* **33**, 265-275, 1963
12. KATSUTA, H. and TAKAOKA, T.: Carcinogenesis in tissue culture. II. Proliferation-inducing effect of 4-dimethylaminoazobenzene on normal rat liver cells in culture. *Japan J. Exp. Med.* **35**, 209-230, 1965
13. SATO, J. and YABE, T.: Carcinogenesis in tissue culture. V. Effects of long-term addition of 4-dimethylaminoazobenzene and 3'-methyl-4-dimethylaminoazobenzene on liver cells in culture *Japan J. Exp. Med.* **35**, 445-462, 1965
14. NAMBA, M., MASUJI, H. and SATO, J.: Carcinogenesis in tissue culture. IX. Malignant transformation of cultured rat cells treated with 4-nitroquinoline-1-oxide. *Jap. J. Exp. Med.* **39**. 253-265, 1969
15. MASUJI, H.: Somatic chromosomal changes of rat liver cells during *in vitro* cultivation. *Acta Med. Okayama* **25**, 87-100, 1971
16. SATO, J., NAMBA, M., USUI, K. and NAGANO, D.: Carcinogenesis in tissue culture. VIII. Spontaneous malignant transformation of rat liver cells in long-term culture. *Japan J. Eip. Med.* **38**, 105-118, 1968
17. MIYAHARA, Y., NAMBA, M., and SATO. J.: Malignant transformation of cloned rat liver cells treated in culture with 4-dimethyl-aminoazobenzene and properties of the transformed cells. *Acta Med. Okayama* **28**, 99-109, 1974
18. MASUJI, H.: Autosomal polymorphism in Donryu strain rats. *Acta Med. Okayama* **24**, 81-91, 1970
19. UNAKUL, W. and HSU, T. C.: The C- and G-banding Pattern of Rattus norvegicus chromosomes. *J. Natl. Cancer Inst.* **49**, 1425-1431, 1972
20. SEABRIGHT, M.: A rapid banding technique for human chromosomes. *Lancet* **ii**, 971, 1971
21. SCHNEDL, W., and SCHNEDL, M: Banding patterns in rat chromosomes (Rattus norvegicus). *Cytogenetics* **11**, 188-196, 1972
22. MILLER, D. A., DEV, V. G., BOREK, C. and MILLER, O. J.: The quinacrine fluorescent and Giemsa banding karyotype of the rat, Rattus norvegicus, and banded chromosome analysis of transformed and malignant rat liver cell lines. *Cancer Res.* **32**, 2375-2382, 1972
23. YOSIDA, T. H. and SAGAI, T.: Similarity of Giemsa banding patterns of chromosomes in several species of the genus Rattus. *Chromosoma* (Berl.) **41**, 93-101, 1973

24. COMMITTEE FOR A STANDARDIZED KARYOTYPE OF RATTUS NORVEGICUS: Standard karyotype of the Norway rat, Rattus norvegicus. *Cytogenet. Cell Genet* **12**, 199-205, 1973
25. HAYFLICK, L. and MOORHEAD, P. S.: The serial cultivation of human diploid cell strains. *Exp. Cell Res.* **25**, 585-621, 1961
26. HAYFLICK, L.: The limited *in vitro* lifetime of human diploid cell straims. *Exp. Cell Res.* **37**, 614-636, 1965
27. PETURSSON, G., COUGHLIN, J. I. and MEYLAN, C.: Long-term cultivation of diploid rat cells. *Exp. Cell Res.* **33**, 60-67, 1964
28. KROOTH, R. S., SHAW, M. W. and CAMPBELL, B. K.: A persistent strain of diploid fibroblasts. *J. Natl. Cancer Inst.* **32**, 1031-1044, 1964
29. OGAWA, K., ICHIHARA, A., MASUJI, H. and SATO, J.: Isozyme patterns of branched-chain amino acid transaminase in cultured rat hepatocytes. *Cancer Res.* **33**, 449-453, 1973