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Kyoichiro Nishina*

*Okayama University,

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KEYWORDS: Alpha-fetoprotein producing, ascites hepatoma AH70B

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ALPHA-FETOPROTEIN PRODUCING CLONES DERIVED FROM ASCITES HEPATOMA AH70B IN CULTURE

Kyoichiro NISHINA

Division of Pathology, Cancer Institute, Okayama University Medical School, Okayama, Japan (Director: Prof. J. Sato)

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Abstract: The establishment of permanent cell line that can produce an α-fetoprotein has made tissue culture a powerful tool for the study of α-fetoprotein. For this reason, the hepatoma cells of rat ascites hepatoma AH70B were cultured *in vitro* and some biological characters of the isolated six clones examined. The cultured cells were morphologically epithelial and the mode of chromosome number in hypotetraploid range, and possessed tumorigenicity. The cells secreted α-fetoprotein at the high level and a few components of serum proteins in the culture medium for more than one year. α-Fetoprotein was also detected in cytoplasm by fluorescent antibody technique. The examined character was little different among the six colonial clones. From the present cloning procedure, it was suggested that the cultured cells derived from a single cell were secreting α-fetoprotein and several components of serum proteins together.

It was demonstrated in 1963 by ABELEV *et al.*, that transplantable mouse hepatoma synthesized α-fetoprotein (AFP), which was not synthesized by the liver cells of normal adult mice (1). From this report, many discussions have been carried on concerning a possible correlation between hepatoma and AFP (2, 3, 4, 5). Systems synthesizing AFP *in vitro* will be of a great value for experimental studies of the biological and biochemical characters of AFP. Hepatoma cells have been shown to retain the ability to synthesize AFP in tissue culture (1, 6, 7, 8, 9, 10, 11); but it is not certain that AFP-production has been maintained for a long-term in culture. More information on the AFP synthesis site in cancer of the liver has been obtained by immunofluorescence (9, 12, 13, 14). On the other hand, all hepatomas have not synthesized AFP (2, 3, 5, 15, 16).

The author attempted to culture rat ascites hepatoma AH70B synthesizing AFP at the high level, for the purpose of elucidating the biological and biochemical characters of AFP.

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MATERIALS AND METHODS

Origin of the hepatoma: The tumor cells used to establish a cell line were the rat ascites hepatoma AH70B (17). A tumor-bearing host animal was kindly supplied by Dr. Hiroshi SATOH (Sasaki Inst.). This hepatoma is transplantable in rats of Donryu strain in our laboratory.

Culture method: Ascites fluid was collected under sterile conditions from the rat on the 13th day after inoculation of the tumor cells. The cells were sedimented by centrifugation for five minutes at 1,000 rpm. The pellet was washed in the medium three times. The washed cells were pipetted after treatment with 0.2% trypsin (Difco) solution in Mg^{++}-free and Ca^{++}-free phosphate-buffered saline (PBS) for 10 minutes at 37°C to obtain a single cell suspension. The isolated cells were again suspended in the culture medium to obtain a concentration of 10^4 cells per ml, and three ml of cell suspension were inoculated into a Petri-dish, then incubated in CO_2 incubator. Synthetic culture medium, Eagle's MEM (Chiba Pref. Serum Inst.), was supplemented with 10% heat-inactivated bovine serum (BS) and 10% fetal calf serum (FCS, BDH Chemicals) in primary and cloning or with 20% heat-inactivated BS in the usual subculture. Forty-eight hours after the primary culture, fresh medium introduced into the dishes, and the medium was renewed in a similar manner two to three times every week. Cells were subcultured after incubation with 0.2% trypsin solusion in PBS for five to seven minutes at 37°C. The cell suspension was diluted 1 : 3 to 1 : 5 and the cells were plated individual new petri-dishes or test tubes.

Cloning procedure: Clonal isolation was performed by trypsinized filter paper according to PUCK *et al.* (18).

Measurements of growth rate and function: The simplified replicate tissue culture method described by KATSUTA *et al.* was employed for the estimation of cell proliferation (19). The population doubling time was calculated from the growth curve at logarithmic phase. The morphology of cultured cells was observed by an inverse phase contrast microscope or staining of Giemsa solusion. PAS staining was performed according to Lillie's method (20).

Detection of rat serum protein in culture medium: The cells were inoculated in TD_{40} bottles. When the cells were preconfluent, the culture medium was discarded and the serum-free culture medium used. After 48 hours, the culture medium was harvested and centrifuged. The supernatant was condensed by ultrafiltration in collodium bags (Sartorius membrane filter). The condensed culture medium was tested by double diffusion or immunoelectrophoresis.

Antiserum against rat serum and α-fetoprotein: Ammonium-sulphate-precipitated crude rat AFP from tumor-bearing rat serum was used as the antigen. A mixture of one ml antigen and one ml of Freund s complete adjuvant (Difco) was injected subcutaneously two times at intervals of seven days. After one month, two-ml antigen was injected intraperitoneally. The rabbits were bled at seventh day after last injection. The sensitized rabbit serum was absorbed at the same volume as adult rat serum. The properties of antigen (AFP) and

antibody were examined according to immunoelectrophoresis. Antiserum against adult rat serum was obtained by a similar immunization procedure.

Fluorescent antibody technique: The labelling of the antiserum with fluorescein isothiocyanate (FITC, Sigma) was performed according to KAWAMURA (21). The final molecular ratio of FITC to protein was about 1:1.9. The cultured cells on the cover-slip were fixed with 1% acetic acid alcohol solusion or acetone for 15 minutes in cold, and stained for 60 minutes in a moist chamber (21). The specimen was observed by a Nikon fluorescent-microscope, and a UV-filter was employed for observation and photomicrography.

Tumorigenicity of cultured cells: Cultured cells after trypsinization were suspended in PBS to obtain a concentration of 10^5 cells per ml. One ml of cell suspension per rat was intraperitoneally backtransplanted into adult male Donryu rats.

Chromosome preparation: Chromosome preparation of cultured cells was performed by the air-drying method, according to MOORHEAD *et al.* (22).

RESULTS

Primary culture and cloning: After the primary inoculation of ascetic cells, colonial proliferation was obtained (Photo 1). During this period, multilayered or aggregate growth of epithelial cells, and monolayered or scattered growth of fibroblastic cells were observed (Photo 2). The former cells were quickly shed from the glass and remained alive in the liquid phase. Epithelial cells had prominent nuclei, with one, two, and occasionally more, nucleoli, and the relatively scant cytoplasm contained numerous granules.

Three weeks later, 15 isolated colonies which originated from a single cell were picked up by trypsinized filter papers. Ten clones proliferated (8 epithelial, two fibroblastic). Colonial isolations were consecutively done two more times in the same manner. Fibroblastic cells ceased proliferation after the second cloning. Finally six epithelial clones were obtained. These clones were designated AH70Btc clone 10-1, 10-4, 10-5, 10-6, 15-1, and 15-2 respectively. These clones were little different morphologically (Photos 3, 4, Table 1). The growth rate of clone 10-6 was not so high until after three passages, but, after three passages the cells proliferated steadily. Other clones also possessed the same growth character and growth rate (Table 1).

PAS positive granules were observed in the cytoplasm of a few cells, but they were incompletely prevented after the digestion with diastase.

α-Fetoprotein production of cultured cells: AFP secretion of cultured cells into the culture medium was examined by double diffusion against anti-rat-AFP serum. In the epithelial clones and parent culture (AH70Btc), AFP was

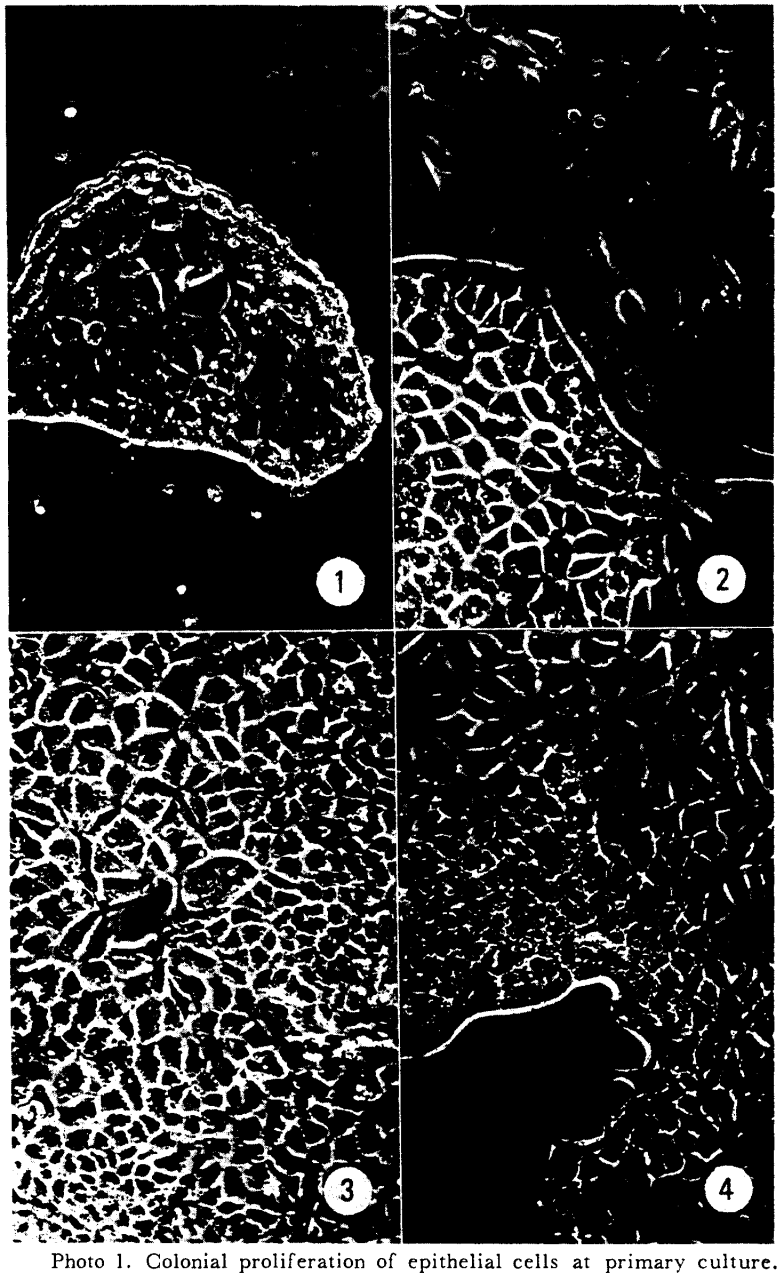

Photo 1. Colonial proliferation of epithelial cells at primary culture. Phase contrast micrograph. ×100

Photo 2. Epithelial and fibroblastic cells at primary culture. Phase contrast micrograph. ×100

Photo 3. Clone 10-5 cells. Phase contrast micrograph. ×100

Photo 4. Single cloned cells. Phase contrast micrograph. ×100

TABLE 1 THE COMPARISON OF SIX CLONES

clones	morphology	doubling time[a]	tumorigenicity[b]	PAS staining[c]	serum protein secretion
10-1	epithelial	40.8 hr	+	+	+
10-4	epithelial	37.4 hr	+	±	+
10-5	epithelial	36.0 hr	+	+	+
10-6	epithelial	39.6 hr	+	+	+
15-1	epithelial	42.0 hr	+	±	+
15-2	epithelial	38.2 hr	+	±	+

a : calculated from growth curve. hr ; hours. b : details are shown in Table 3. c : + ; PAS positive granules were seen in more than 5% of the cells. ± ; PAS positive granules were seen in less than 5% of the cells.

detected in the culture medium at 2- to 4-fold concentrations. But in fibroblastic clones which ceased proliferation after the second cloning, AFP was not detected at 10- to 20-fold concentrations. Follow-up study is needed to determine whether or not AFP is maintained at the secretion level in long-term cultivation. It seems that the amount of AFP secretion was not changed for about 500 days, as the AFP secretion was checked at the time of the serial subculture (Fig. 1). The precipitation line of culture medium with the anti-

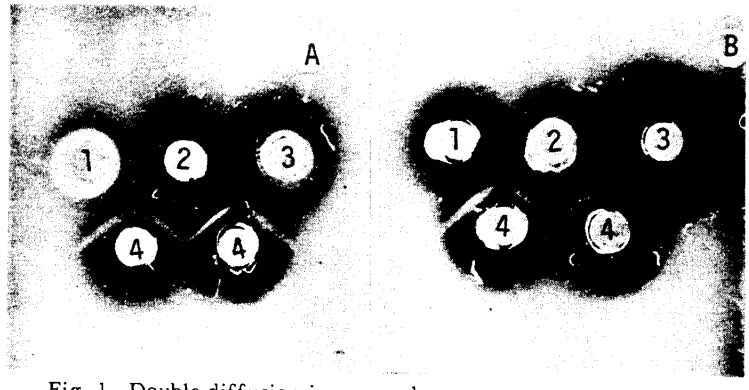

Fig. 1. Double diffusion in agar gel.
A : The comparison of α-fetoprotein in culture medium, fetal serum and hepatoma serum. 1. rat embryo serum. 2. culture medium of clone 10-6. 3. The serum of rat ascites hepatoma AH7OB. 4. anti-rat-AFP serum.
B : The AFP section at different culture days. 1. 150 days. 2. 300 days. 3. 500 days. 4. anti-rat-AFP serum.

rat-AFP serum, fused in fetal and tumor-bearing rat sera (Fig. 1). Fresh growth medium containing FCS and BS gave no precipitation line with the same antiserum.

Immunofluorescent antibody technique was performed for observing

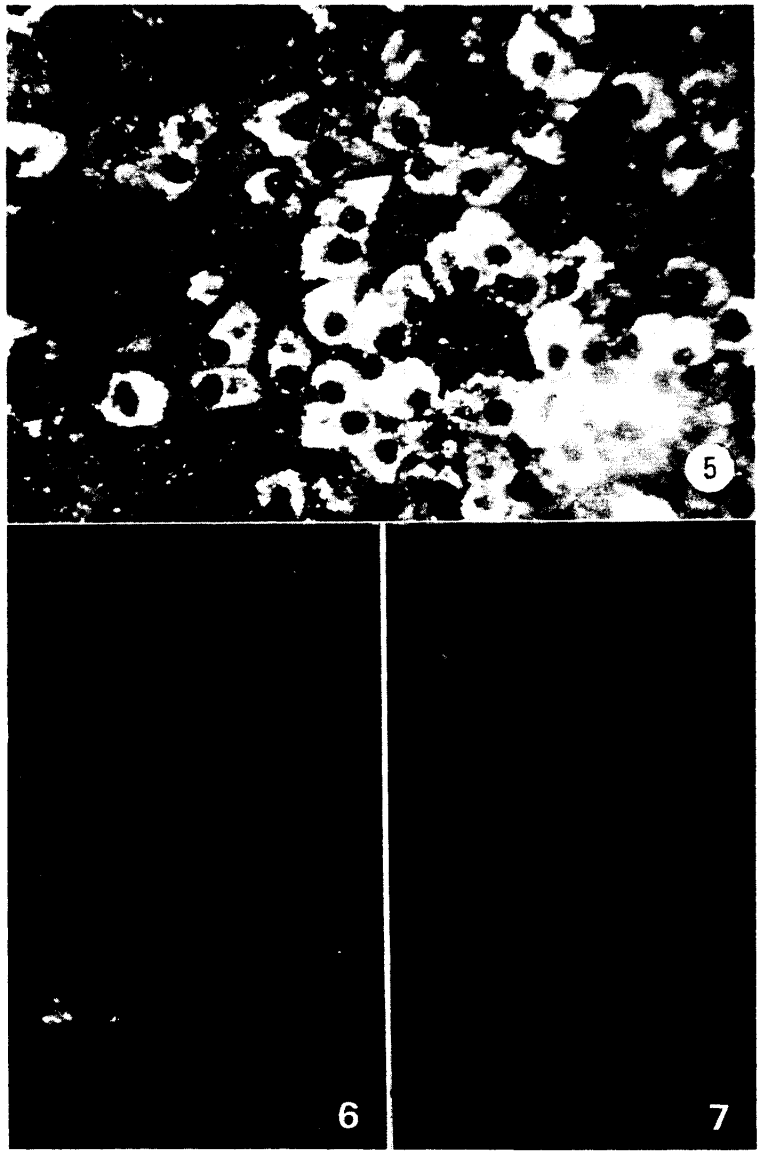

Photo 5. The cells of clone 10-6; diffuse fluorescence of cytoplasm ×400

Photo 6. The cells of clone 10-6; fluorescence is markedly reduced after prior exposure of non-labeled anti-rat-AFP serum. ×400

Photo 7. Adult rat liver cells in culture; no fluorescence is seen ×400 (This cell line was kindly supplied by Dr. Tsutamune in our laboratory)

intracellular localization of AFP in clone 10-6 cells. Cytoplasmic fluorescence was seen in about 50% of the cells in the same specimen. Yellow-green fluorescence was observed homogeneously in the cytoplasm of almost all positive cells, or fine granular fluorescence was observed in the cytoplasm of a few cells (Photo 5). The fluorescence was markedly reduced after prior exposure to non-labeled anti-rat-AFP serum and no fluorescence was seen in cultured fibroblastic cells or in adult liver cells (Photos 6, 7). No morphological difference was seen between AFP-positive and -negative cells in the same specimen.

Serum protein secretion of cultured cells: The macromolecules in the serum-free culture medium were concentrated about 100-fold. The concentrated culture medium gave two to three precipitation lines against anti-rat serum by immunoelectrophoresis. The immunoelectrophoretic pattern of the protein components in the culture medium of clone 10-6 corresponded largely to the pattern of the serum protein. However, only a tentative identification was possible. In the albumin region, one line was seen (Fig. 2), but it is uncertain whether this line corresponded to albumin or not. Other precipitation lines were seen in α- and β-globulin regions (Fig. 2). These precipitation lines were also seen in the other five clones (Table 2). Fresh growth medium containing FCS and BS did not show any precipitation line.

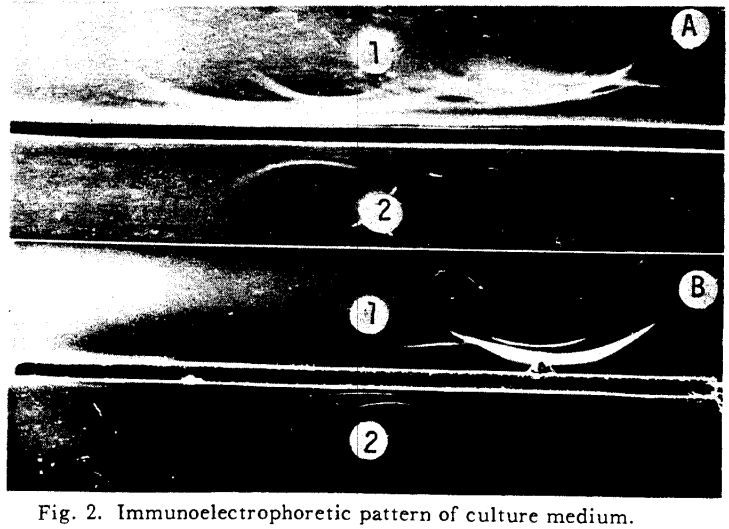

Fig. 2. Immunoelectrophoretic pattern of culture medium.
A; well 1. rat serum. 2. condensed culture medium of clone 10-1. trough. anti-rat serum
B; well 1. rat albumin Fr. 5 (Miles). well 2. condensed culture medium of clone 10-6. trough. anti-rat serum. anode is right.

TABLE 2 THE SERUM PROTEIN SECRETION OF SIX CLONES

clones	alpha-fetoprotein		other proteins[c]
	medium[a]	cellular site[b]	
10-1	+	cytoplasm	2
10-4	+	cytoplasm	2
10-5	+	cytoplasm	3
10-6	+	cytoplasm	3
15-1	+	cytoplasm	2
15-2	+	cytoplasm	2

a : by double diffusion using 2-4 times concentrated culture medium
b : by immunofluorescent antibody techinique
c : a numeral is number of precipitation lines of immunoelectrophoresis against anti-rat serum

TABLE 3 TUMORIGENICITY OF SIX CLONES

clones	culture days	inoculum size	transplantability ♯	survival days
10-1	109	1×10^6	5/5	32.2
10-4	105	1×10^6	5/5	34.6
10-5	105	1×10^6	5/5	30.6
10-6	105	1×10^6	5/5	31.8
15-1	109	1×10^6	5/5	27.6
15-2	109	1×10^6	5/5	30.8

The cultured cells were intraperitoneally inoculated into adult male donryu rat.
♯ ; numerator : number of rats inoculated denominator : number of rats died of tumor

Tumorigenicity of cultured cells : The rats into which cultured cells were intraperitoneally inoculated, died of tumors. Table 3 shows the survival days of the rats. The rats which died of tumors had bloody ascites and a similar metastatic condition of the original AH70B rat. In the serum and ascites of rats died of tumors, AFP was detected.

The chromosome numbers : The chromosome number of cultured cells exhibited the mode in hypotetraploid range (Fig. 3). The chromosome number of the AH70B cells *in vivo* also exhibited the mode in the same range.

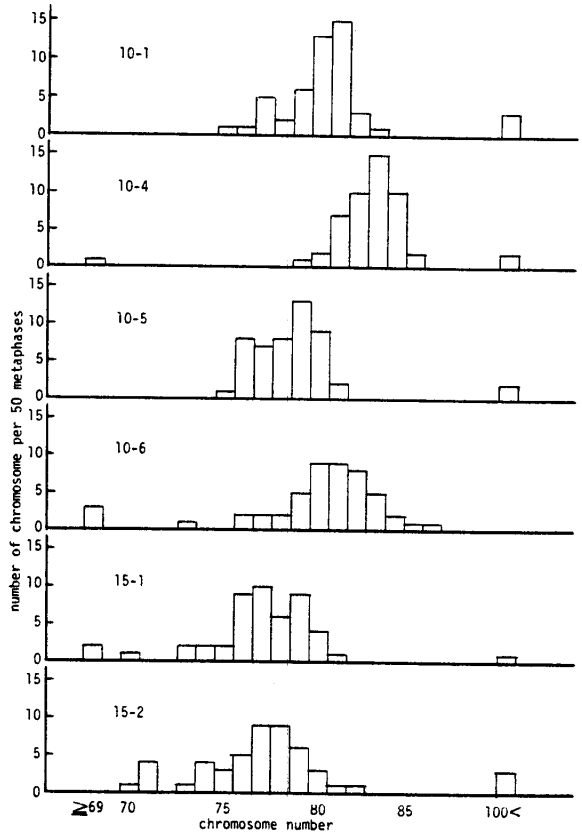

Fig. 3. Distribution of chromosome number of six clones

DISCUSSION

The AFP-producing cell line was established from rat ascites hepatoma AH70B. The AFP secreted from the cultured cells was thought to be the identical AFP from embryo and hepatoma sera by immunological experiments.

IRLIN *et al.* demonstrated that *in vitro* AFP-production ceased or decreased during long-term cultivation using mouse hepatoma cells (6). Otherwise, in experiments of HULL *et al.* using monkey hepatoma cells, AFP was produced continuously in culture (7). The AH70Btc cells, including clonal lines, produced AFP for over one year. The maintenance of AFP-production in culture may be due to the differences in species or strains of host animal, or to the differences in culture conditions.

In the experiment of fluorescent antibody technique using clone 10-6 cells, cytoplasmic fluorescence of AFP was seen in about 50% of the cells in

the same specimen. NISHIOKA *et al.* demonstrated that AFP in hepatoma tissue was detected in 20 percent by immunofluorescent antibody technique (14). This finding may mainly be related to the cell cycle, though at what stage AFP is synthesized and released in the cell cycle, must be determined by using synchronous culture.

Serum protein secretion in cultured liver cells was demonstrated by several authors (6, 23, 24, 25, 26, 27, 28), but it is very difficult for cultured cells to maintain differential functions. AH70Btc cells also secreted serum proteins. However, it was considerably less than the AFP secretion, so it is not clear what kind of serum protein this is. Further studies on this problem are necessary.

GAUDERNACK *et al.* (29) assumed that individual hepatocytes produced most of the different serum proteins, by the experiments of clonal hepatoma cell line established by RICHARDSON *et al.* (25). TSUKADA *et al.* obtained the similar result using a colonial cloned cell line from AH66 in tissue culture (10). However, since the colonial cloned cells do not always engender from a single cell, a single cell cloning was performed from clone 10-6 cells by the capillary method. The cells engendered from a single cell also secreted AFP and a few kinds of serum proteins together. This result strongly supports the above mentioned Gaudenack's hypothesis.

ISAKA *et al.* demonstrated AFP-producing and non-producing clones from Yoshida sarcoma cells (30). And TSUKADA *et al.* showed low-producing clones of AFP by the experiment of placing AH66 in tissue culture (10). IRLIN *et al.* demonstrated that AFP-synthesis ceased in a long-term cultivation of mouse hepatoma cells (6). In our experiment, low-producing clones were not isolated from AH70Btc cells in the case of six colonial clones and other single cell clones. If more trials of clonal isolation or change of culture condition are performed, non- or low-producing clones may be obtained. Otherwise, the examined characters of six clones in our experiment differ little. From this fact, it seems that individual hepatoma cells possess a similar biosynthetic potential.

Recently AFP was detected in regenerating liver cells of patients with liver disease and in pregnancy by radioimmunoassay (3, 31, 32). However, the serum level of AFP in the majority of hepatomas is considerably higher than that observed in non-hepatoma states. It is also considered that all cultured cells derived from hepatocytes produced AFP, as is said, the cultured cells come to indicate an embryonal pattern. Some of the many cell lines derived from rat liver in our laboratory are producing AFP at low concentration by radioimmunoassay (33). AH7OBtc cells secreted about 100 times more than the other AFP-producing lines in our laboratory.

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