Acta Medica Okayama

Volume 28, *Issue* 2 1974 *Article* 4

APRIL 1974

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Abstract

Cultured rat liver cells which were cloned from a single cell were transformed into malignant cells by a chemical carcinogen, 4-dimethylaminoazobenzene (DAB). The DAB-transformed cells produced tumors when back-transplanted into new-born rats but the carcinogen-untreated control cells did not. Characteristics of the transformed liver cells were compared to those of DAB-untreated control cells in regard to the morphology, the consumption of DAB from the culture medium by the cells, the incorporation of 3H.DAB into the cells, and the aggregate.forming ability of the cells in rotation culture. The results showed that no significant parameter of malig. nant transformation in culture was detectable except the tumorigenicity of the transformed cells upon the inoculation into animals.

*PMID: 4277389 [PubMed - indexed for MEDLINE]

Acta Med. Okayama **28**, 99—109 (1974

CARCINOGENESIS IN TISSUE CULTURE 22. MALIGNANT TRANSFORMATION OF CLONED RAT LIVER CELLS TREATED IN CULTURE WITH 4-DIMETHYLAMINOAZOBENZENE AND PROPERTIES OF THE TRANSFORMED CELLS

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Received for publication, November 8, 1973

Abstract: Cultured rat liver cells which were cloned from a single cell were transformed into malignant cells by a chemical carcinogen, 4-dimethylaminoazobenzene (DAB). The DAB-transformed cells produced tumors when back-transplanted into new-born rats but the carcinogen-untreated control cells did not. Characteristics of the transformed liver cells were compared to those of DAB-untreated control cells in regard to the morphology, the consumption of DAB from the culture medium by the cells, the incorporation of ^{3}H-DAB into the cells, and the aggregate-forming ability of the cells in rotation culture. The results showed that no significant parameter of malignant transformation in culture was detectable except the tumorigenicity of the transformed cells upon the inoculation into animals.

Either solid hepatoma or ascites hepatoma was produced by 4-dimethylaminoazobenzene (DAB) in rats (1, 2, 3). MILLER recently reviewed the biochemical mechanisms of the action of DAB on liver cells (4). There arise many difficulties in the studies of the mechanism concerned with chemical carcinogenesis *in vivo*; namely, 1) the selection of cells by treatment with drugs, 2) the monocellular mechanism of DAB-carinogenesis, and 3) the immune response of animals. For these reasons, the experiments regarding malignant transformation have been carried out by tissue cultures at the cellular level (5-15). In most of these studies were used fibroblast-like cells from mice and hamsters.

KATSUTA and TAKAOKA treated liver cells with DAB in culture and found that the DAB-treatment during the first four days after explant induced a proliferation of resting rat liver cells (16). SATO confirmed a similar "proliferation-inducing activity" of 3'-methyl-DAB on rat liver cells in culture (17). He also stated that the liver cells transformed in culture by 3'-methyl-DAB produced tumors in the rats on back-transplantation of the cells (18, 19).

However, the control cells culture for a long period of time spontaneously transform themselves into the malignant form and form tumors when back-transplanted into new-born rats (20). They suggested that the dye accelerated a malignant transformation of cultured liver cells, but in these experiments they have employed mass cultured liver cells. It is, however, more useful to clarify the mechanisms of carcinogenesis in tissue cultured cells originating from a single cell clone rather than from the cell in mass culture.

This report describes malignant transformation of cloned rat liver cells by DAB and some characteristics of the transformed cells. To determine DAB target sites in cells, we investigated also whether or not some specifically active sites of DAB remained in the transformed cells following the initial exposure of cells to DAB.

MATERIALS AND METHODS

Cells and tissue culture: A rat liver cell line, PC-2, which was cloned from a single cell of a rat liver cell line (RLN-E7) at 543rd day, was used for the present study. Characteristics of PC-2 cells and cloning methods were reported elsewhere (21). The parent line, RLN-E7, was established from livers of 5-day-old male Donryu rats as previously reported (14). The cells were grown in monolayers in Eagle's Minimal Essential Medium (MEM) supplemented with 20% bovine serum. This medium was used as the control medium. Subculture of cells was performed by treating the cells with 0.2% trypsin (Difco) solution in phosphate buffered saline.

Treatment of cells with 4-dimethylaminoazobenzene (DAB): DAB (Merck) was dissolved in ethyl alcohol at a concentration of 5 mg/ml and stored until use. The DAB solution was diluted with bovine serum and was added to Eagle's MEM at a final concentration of 5 or 20 μg/ml. The cells were treated continuously with DAB by replacing the DAB-containing culture medium twice a week.

Back-transplantation of cultured cells into rats: Before back-transplantation the cells were cultured in the control medium. The cells were transplanted intraperitoneally or intracerebrally into new-born male or female rats which were less than 48-hour-old. The inocula ranged from 10^6 to 10^7 cells per animal. The rats were observed for 6 months.

Assay of consumption of DAB from culture medium: On the second day of the culture, the control medium was replaced by medium containing DAB at a final concentration of 5 to 20 μg/ml, and the cultures were maintained further for 3 days. After 3 days, 1 ml of the culture medium containing DAB was mixed with 3 ml of toluene to extract the DAB remaining in the medium. The amount of DAB was measured spectrophotometrically at 410 mμ.

Incorporation of ^{3}H-DAB from culture medium: The ^{3}H-DAB was obtained from Sinloihi Co., Japan (specific activity: 10.6 mCi/mg) and was dissolved in the same way as DAB. Monolayered cells on cover-slips were cultured in

the presence of 50 to 100 μCi/ml of ^{3}H-DAB (10 to 20 μg/ml of DAB) for 3 days. After 3 days the cover-slips were washed 3 times with phosphate-bufferd saline solution, then treated with 5% trichloroacetic acid at 4°C for one hr, and were washed 3 times with acetone, and dried. The incorporation of the ^{3}H-DAB into cells was determined radioautographically by a conventional method using Sakura nuclear emulsion NR-M2. The exposure time was 18 days.

Rotation culture: According to the procedure reported by MOSCONA (22), 3×10^6 cells in 3 ml cultre medium were incubated in a 25 ml Erlenmeyer flask rotating on a gyratory shaker at 70 rpm and 37°C for 24 hr. The aggregation of cells was recorded photomicrographically and the aggregation size was determined by measuring the diameter of 100 aggregates.

RESULTS

I. *Malignant transformation of cultured rat liver cells with DAB.*

Malignant transformation of rat liver cells which were cloned from a single cell line was obtained by the treatment of cells in culture with DAB. As shown in Fig. 1, the cells treated with 5 μg/ml of DAB produced tumors

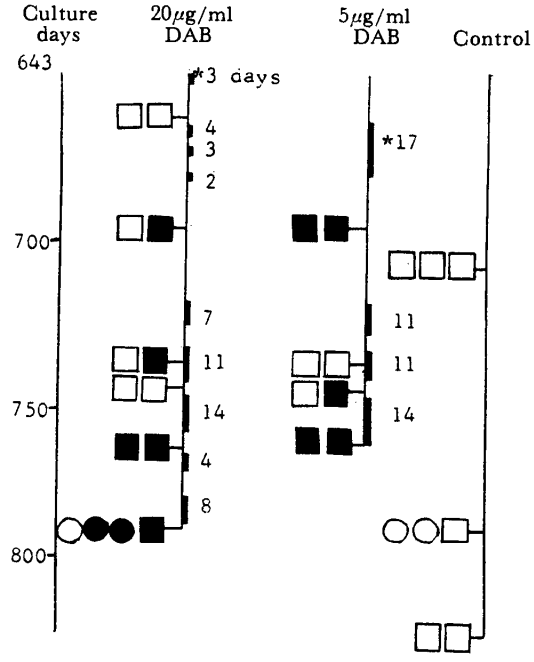

Fig. 1. Intraperitoneal or intracerebral backtransplantation of the cloned rat liver cells (PC-2) treated with DAB in culture into newborn rats
* The numerals in the figure indicate the culture days with DAB-containing medium
—▬— Treatment with DAB, □ Intraperitoneal inoculation, ○ Intracerebral inoculation Black squares and circles show tumor 'take'

when back-transplanted into new-born rats. Similar results were obtained with cells treated with 20 μg/ml of DAB. There was no significant difference in carcinogenesis between the cells treated with 5 μg/ml of DAB and those with 20 μg/ml of DAB. On the other hand, the cells cultured in the control medium did not produce any tumors when back-transplanted. The tumorigenicity in terms of back-transplantability rate and survival days of tumor-bearing animals increased in proportion to the length of time with DAB-treatment. Histologically, tumors were diagnosed as an undifferentiated carcinoma as shown in Photo 3.

II. *Characteristics of transformed cells.*

Inoculation into a new-born rat of PC-2 cells, which were treated with 5 μg/ml of DAB for 53 days in culture, gave rise to an ascites hepatoma by 117th day. The ascites hepatoma cells were again submitted to tissue culture and were named as a line DT-2.

a) *Morphology*: The control culture of PC-2 cells showd a sheet of epithelial cells. Individual cells spread widely over a glass surface and had a large cytoplasm and oval nucleus with several nucleoli (Photo 1). The transformed cells showed pleomorphism of the cytoplasm and nucleus and the number of polynucleated giant cells increased. Areas consisting of piling-up cells were very infrequent (Photo 2).

b) *The effect of DAB on cell proliferation*: The effect of DAB on the proliferation of PC-2 and DT-2 cells was investigated. On the second day of the culture in the control medium, the culture medium was replaced by DAB-containing medium and the cells were treated with DAB for succeding 3 days. As shown in Fig. 2, the presence of 5 to 20 μg/ml of DAB did not show any cytotoxic effect. As illustrated in Fig. 3, various concentrations of cells of both lines were treated with DAB, but cytotoxic effects did not differ much in either line with concentrations less than 18.2 μg/ml of DAB. The effect of DAB on the plating efficiency was compared between the two cell lines, *i.e.* PC-2 and DT-2. As shown in Table 1, the plating efficiency of both lines was reduced by about 30% when they were cultured in the DAB-containing medium. All these results suggest that there is no difference between the control and the transformed cells in the sensitivity of cells to the toxic action of DAB and no resistance to the cytotoxic effect of DAB in theDAB-transformed cells.

c) *DAB-consumption from culture medium and incorporation of ^{3}H-DAB into cells*: DAB consumption measured from the medium in which cells are growing exponentially is represented by "cell life unit". This unit is the amount of DAB-consumption from the medium per cell during one cell cycle. The consumed amount of DAB per cell life unit was 52×10^{-6} μg by the

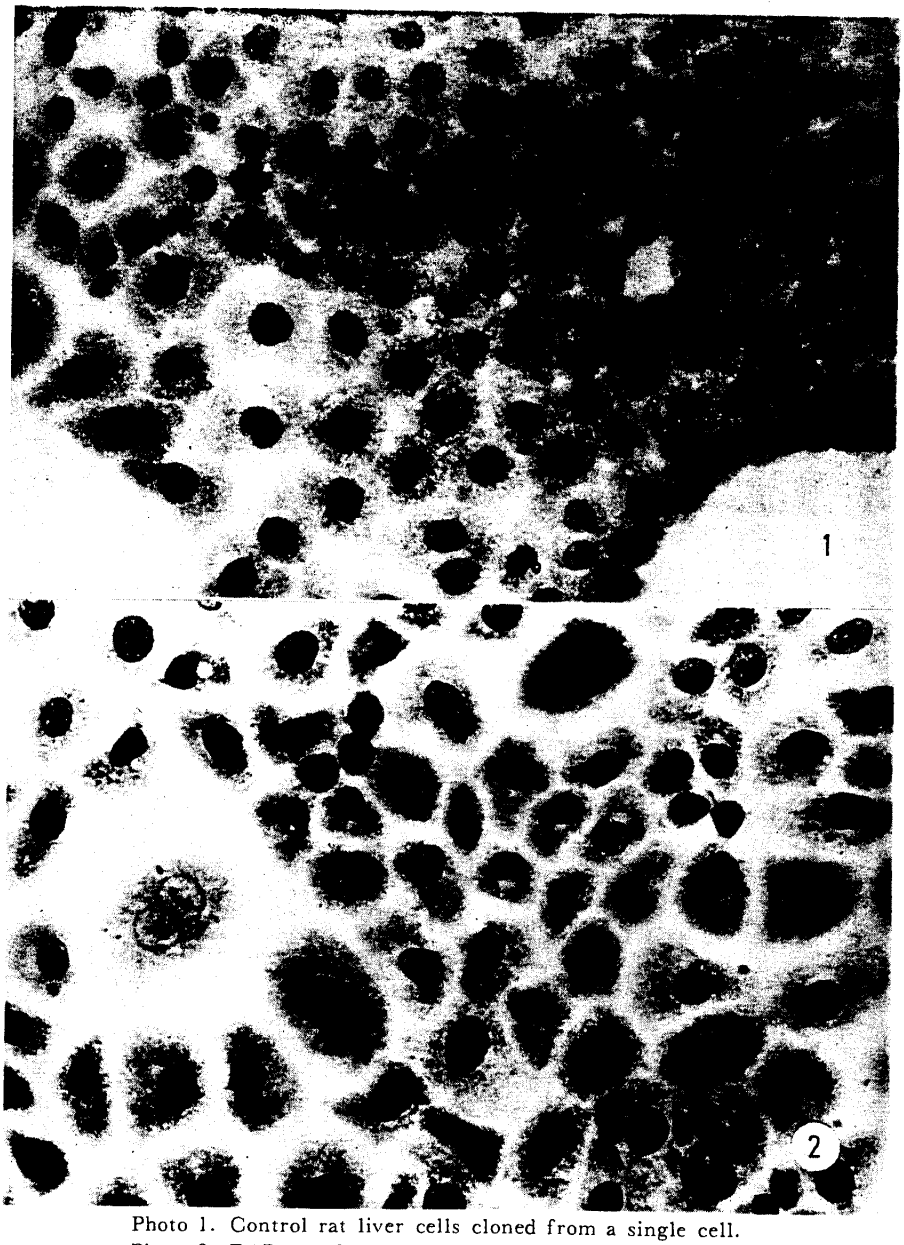

Photo 1. Control rat liver cells cloned from a single cell.
Photo 2. DAB-transformed cells.

control cells and 44×10^{-6} μg by the transformed cells when the cells were cultured in the medium containing 10-20 μg/ml of DAB. When the cells became confluent, *i. e.* in culture for 48 hr, 99% and 100% of DAB were lost

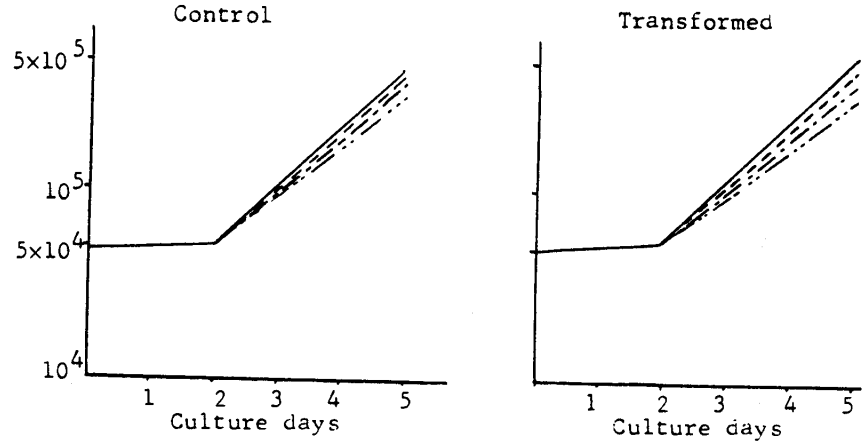

Fig. 2. The effect of DAB at various concentrations on the proliferation of the control and the transformed liver cells
——— Control medium, - - - - - 5μg/ml DAB medium, —·—· 10μg/ml DAB medium, —··—·· 20μg/ml DAB medium

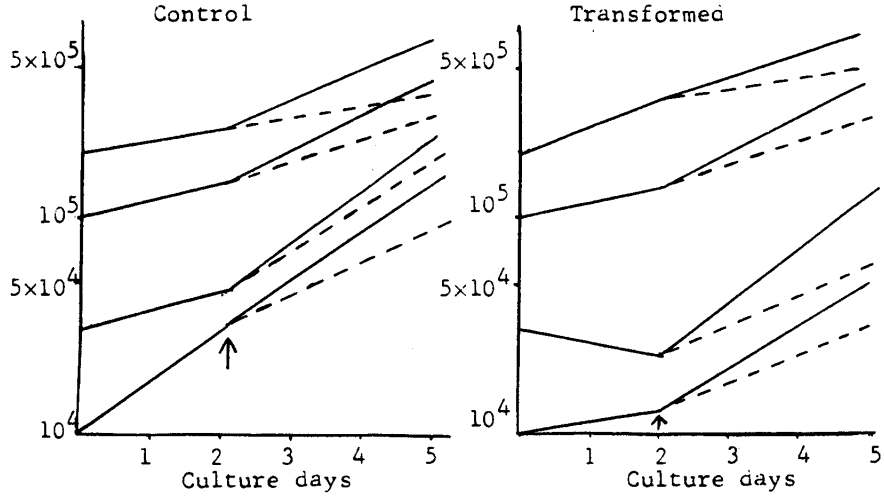

Fig. 3. The effect of DAB on the proliferation of the control and the transformed liver cells
↑ DAB treatment performed for 3 days, —— Control medium, - - - - - - DAB medium (18.2μg/ml)

from the medium by control and transformed cells, respectively; this is not so significant.

The cells incorporated ^{3}H-DAB homogeneously into cytoplasm and nucleus. Grain numbers were 1 to 93 in the control cells and 1 to more than

300 in the transformed cells. The rate of incorporation per cell varied with cell size. If the incorporated ^{3}H-DAB is bound to cell proteins, the grain numbers per area of cell represent the concentration of dye-bound proteins in the cells. Table 2 shows that the number of grain per μm^2 were greater in

TABLE 1 COMPARISON OF THE SENSITIVITY OF CELLS TO DAB BETWEEN THE DAB-UNTREATED AND THE DAB-TREATED TRANSFORMED CELLS

Cell line	No. of cells seeded per plt.	No. of colonies Medium without DAB (mean no.)	No. of colonies Medium with DAB (mean no.)	No. of colonies in medium with DAB / No. of colonies in medium without DAB %
Control cells PC 2	200	100 83 67 (83)	61 55 62 (59)	71
Tumor cells DT-2	800	198 193 184 (192)	150 160 (155)	70

* DAB was contained in the medium at a final concentration of 18.4μg/ml

TABLE 2 THE INCORPORATION OF ^{3}H-DAB IN THE CONTROL AND THE TUMOR CELLS

	No. of grains per cell	Cell area (μm^2)	No. of grains per area μm^2
Control cells PC-2	27.7	896	3.09
Tumor cells DT-2	54.0	1140	4.72

the transformed cells than in the control cells.

d) *Rotation culture*: The aggregate-forming ability of the two cell lines in rotation culture was examined. As illustrated in Photos 4 and 5, the mean value of diameter of the aggregates was 0.041 nm with the control and 0.047 nm with the transformed cells, respectively, indicating that the transformed cells formed slightly larger aggregates than the control cells (Photos 4 and 5).

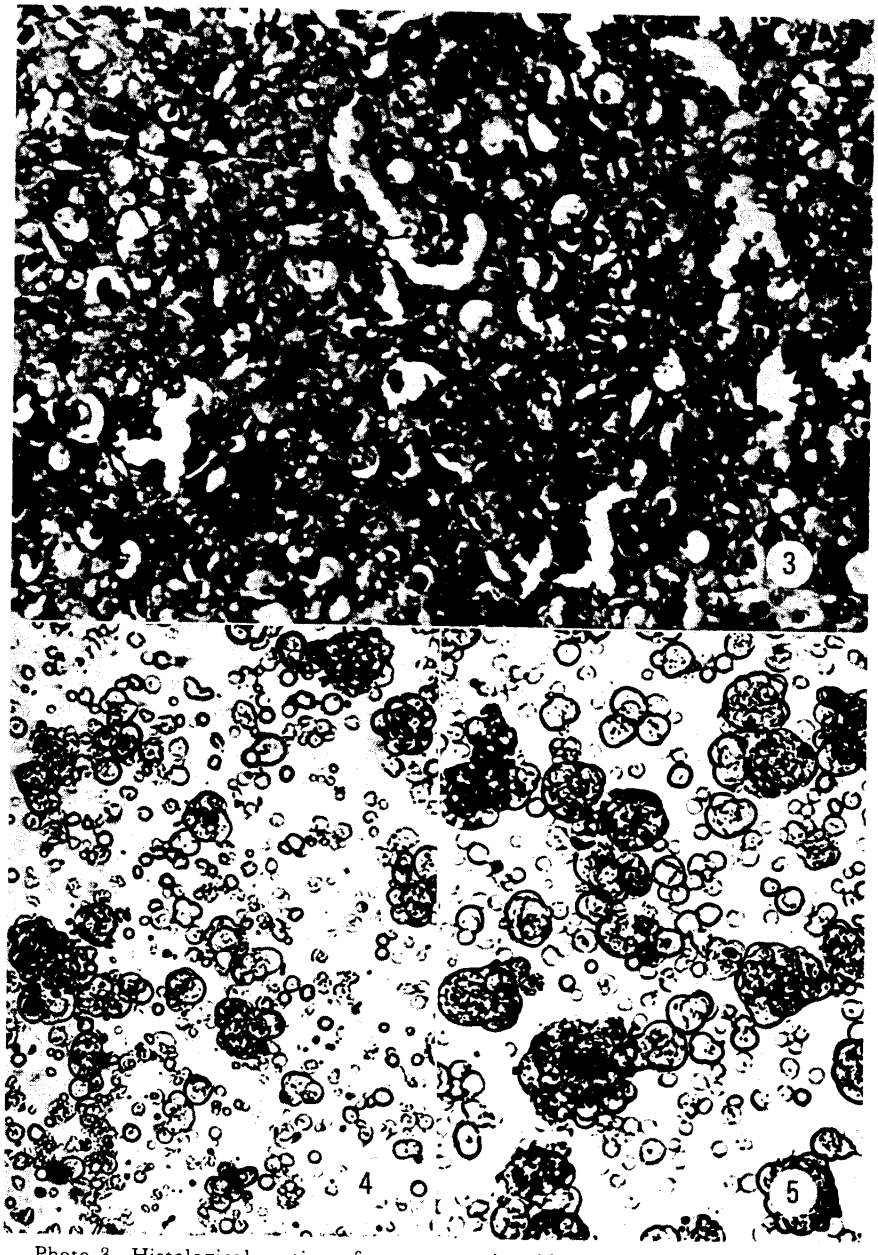

Photo 3. Histological section of a tumor produced by the intraperitoneal transplantation of rat liver cells treated with DAB.

Photo 4. Aggregates of the cloned rat liver cells.

Photo 5. Aggregates of the DAB transformed cells.

DISCUSSION

As well known, 4-dimethylaminoazobenzene is a potent carcinogenic agent for the rat liver when the agent is administered *in vivo*. It is suggested that the rat liver can specifically metabolize DAB to proximate carcinogens.

KATSUTA and TAKAOKA (16) and SATO (17) reported that the azo dyes had an inducing activity of the proliferation of rat liver cells in culture. It was also reported that the cells treated with the agent altered their biochemical properties and morphological characteristics, but the cells did not from tumors when they were backtransplanted in rats (23, 24). SATO reported that a treatment of rat liver strains with 3'-methyl-DAB caused an acceleration of malignancy of the cells in terms of the backtransplantability rate of cells and a shortening of the survival days of tumor-bearing rats (18). At the same time, spontaneous transformation of nontreated control liver cells occurred in the experiments. He confirmed that a long-term culture from several rats' liver caused a spontaneous malignant transformation (20). The present paper demonstrated that the DAB-treatment of cloned rat liver cells caused malignant transformation and the nontreated control cells did not form tumors during the period of the experiment (6 months). But single-cell cloned cell line showed spontaneous transformation in long period of time after the completion of our experiments. Thus a question is raised as to whether or not DAB will cause spontaneous transformation of the cultured liver cells.

As cited in the results, the control cells consumed DAB in a high rate at the concentration of 10-20 μg/ml of DAB. This was also observed by the transformed cells and the rate of DAB consumption by the transformed cell did not show any appreciable difference as compared with the control cells. These suggest that this single-cloned cell line has a character of hepatocyte (25) and the effect of DAB on the consumption is not detectable in the transformed cells.

The autoradiographic observation of ^{3}H-DAB incorporation revealed no appreciable difference between the transformed and the control cells. This suggests that the malignant transformation by DAB does not reflect an alteration of the amount of DAB-binding protein in the transformed cell. Similar result was obtained by the cultured cells from rats fed DAB for various days (57, 191 and 312 days) (26). These results are not consistent with the results reported by MILLER *et al.* (27, 28), in that the content of cellular proteins which bind to DAB is lower in DAB-transformed hepatomas than that in the normal liver. Recently, SUGIMOTO and TERAYAMA reported that the nature of dye-binding protein isolated from rat's livers changed irreversibly during continuous feeding of the dye (29). This suggests that the cellular protein which

binds DAB might differ in its nature between the control and the transformed cells used in our experiment.

This paper shows that the single-cloned liver cells treated with DAB form hepatoma when the cells are inoculated into new-born rats, suggesting that DAB does not select any specific cell line having strong tumorigenicity. It is to be not excluded that DAB might select the cells undergoing spontaneous malignant mutation. But it remains to be solved whether or not the cells used as a control in the present investigation are completely normal, because it is difficult at present time to prepare a single clone from cultured rat liver cells. It is often observed that the established cells have some polymorphism in chromosome numbers and karyotypes (in press). A further study is required to devise a culture method appropriate for maintaining the cells completely normal.

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