Jpn. J. Cancer Res. (Gann), 79, 734–741 ; June, 1988

Resistance to Serum-induced Terminal Differentiation in Normal Human Tracheobronchial Epithelial Cells after *in vivo* Exposure to 7,12-Dimethylbenz[*a*]anthracene

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Normal human tracheobronchial epithelial cells (NHTBECs) from nine donors were used to repopulate de-epithelialized rat tracheas. After transplantation into nude mice and treatment with 7,12-dimethylbenz[*a*]anthracene (DMBA), the transplants were removed at 3, 4, 5 and 6 months. Epithelial cells from DMBA-treated tracheas were subculturable. Epithelial cells from most untreated tracheas were not subculturable. After treatment with 0.5, 1, 2, 4, 6 and 8% serum, cells exhibited increased subculturability after *in vivo* treatment with DMBA, did not terminally differentiate and were still proliferating even in medium containing 8% serum. Karyotypes from these cells showed considerable aneuploidy. Although these cells did not survive for more than 10 subcultures (42 weeks), this was considerably longer than the survival of control cells. Because of their longer survival, resistance to serum-induced terminal differentiation and chromosome alterations, they were considered to be phenotypically altered or partially transformed cells produced by *in vivo* treatment of human cells with a chemical carcinogen.

Key words: Human lung — Bronchi — Carcinogenesis — 7,12-Dimethylbenz[*a*]anthracene — *In vivo* exposure

Numerous studies have unambiguously linked cigarette smoke with lung cancer, one of the most frequent causes of cancer death in the world (reviewed in ref. 1). Several polycyclic aromatic hydrocarbons (PAH), major components of cigarette smoke, have been shown to induce lung tumors in several experimental animal models (reviewed in refs. 2–4). In addition, several investigators succeeded in producing preneoplastic and probably neoplastic lesions in xenotransplanted human bronchi treated with PAHs.[5–8] Since the donor tissues used in those experiments originated from lung cancer patients, we proposed an experimental model utilizing human infant normal tracheobronchial epithelium. In previous reports[9, 10] we described the procedure for epithelial cell amplification, based on the techniques described by Lecher *et al.*,[11, 12] as well as the xenotransplantation of these cells in devitalized rat tracheas, subsequent treatment with carcinogen and production of metaplastic dysplastic lesion. In order to demonstrate the carcinogenic effect of PAHs on the human tracheobronchial epithelium, an investigation of the *in vivo* induced altrerations of *in vitro* growth behavior was conducted. Alteration of *in vitro* behavior, especially resistance to terminal differentiation in the presence of an increasing serum concentration, is a hallmark of several lung carcinoma cell lines as well as of *in vitro* transformation of human bronchial epithelial cells.[13–15] Similarly, rat tracheal epithelial cells treated *in vivo* with PAHs have been shown to change in their nutritional requirements once placed *in vitro* and are able to generate variants that eventually become immortal and tumorigenic.[16, 17] It is within this context that we have attempted to produce a transformed cell line of human origin using *in vivo* PAH-treated cells, in order to demonstrate by a

Abbreviations used are: DMBA, 7,12-dimethylbenz[*a*]anthracene; NHTBEC, normal human tracheobronchial epithelial cell.

combination of *in vivo* and *in vitro* techniques that PAHs are able to exert their carcinogenic properties on NHTBECs.

MATERIALS AND METHODS

Normal Newborn Bronchial Tissue and Culture Procedures Normal human newborn bronchial tissue was obtained from intermediate (24–48 hr after death) autopsies of infants 1–12 months of age. Specimens were submerged in L-15 medium containing penicillin (50 μg/ml), streptomycin (100 μg/ml), amphotericin B (0.5 μg/ml), and gentamycin (50 μg/ml) immediately after removal, and transported at 4° to our laboratory. The surrounding connective tissue was removed as thoroughly as possible and the bronchial tissue was cut into 5×10 mm explants. To eliminate mucus and promote reversal of ischemic damage these pieces were incubated at 37° in ACB-1 medium[10] in a gassed (50% O_2, 5% CO_2, and 45% N_2) chamber (Bellco Glass, Inc., Vineland, NJ) and agitated 6–8 times/minute on a rocker platform.[11] ACB-1 medium was a mixture of one part of F-12 medium and one part of MCDB 151 medium supplemented with 1.25% fetal bovine serum, epidermal growth factor (10 ng/ml), insulin (2.6 μg/ml), transferrin (5 μg/ml), hydrocortisone ($5\times10^{-7}M$), phosphoethanolamine and ethanolamine ($1.5\times10^{-7}M$), and trace elements.[12]

After 3–5 days in culture the tissue fragments were cut into smaller (2×2 mm) pieces. Six to seven pieces were put epithelial side up on a coated 60 mm tissue culture dish. The dishes were coated with a mixture of human fibronectin (10 μg/ml); collagen (30 μg/ml); and crystallized bovine serum albumin (10 μg/ml), dissolved in MCDB 151 medium. The dishes containing these explant cultures were incubated in growth medium ACB-1 at 36.5° in humidified, gassed (95% air + 5% CO_2) incubators. The medium was replaced twice weekly. After 6–8 days of incubation, when epithelial outgrowth from the pieces had reached more than 1 cm in diameter, the explants were transferred to new culture dishes and the growth medium ACB-1 was replaced with a modified serum-free medium LHC-9.[11] The monolayer cells were incubated in LHC-9 at 36.5° in an atmosphere of 5% CO_2. After 5–7 days in culture, up to 5×10^5 cells could be enzymatically harvested from each 60 mm culture dish.

In this fashion, we processed tracheobronchial tissues from 9 different donors. All donors had died from sudden infant death syndrome, and no other specific disease or genetic abnormality was found in these individuals.

Xenotransplantation Once the monolayer cultures reached 80% confluency, the cells were harvested by incubation in Hepes-buffered saline containing 0.25% trypsin (1:250, Gibco) and 0.02% EDTA, washed and resuspended in ACB-1. The cells ($3–4\times10^5$ cells in 0.1 ml of medium) were seeded into the lumina of de-epithelialized Fischer 344 rat tracheas (Charles River Breeding Lab, Inc., Wilmington, MA). De-epithelialization was achieved by repetitive freezing and thawing of the tracheas[10, 18]; before de-epithelialization the tracheas were mounted on polyethylene tubing to maintain their original shape and length. After cell inoculation, the tracheas were sealed and transplanted into the dorsal subcutaneous tissues of specific pathogen-free 8-week-old female BALB/c nude mice (Life Science, St. Petersburg, FL) (2 tracheas per nude mouse).

Evaluation of Cell Repopulation in the De-epithelialized Rat Tracheas and Exposure to DMBA One, 2, 3, 4 weeks after xenotransplantation of the normal human tracheobronchial cells into nude mice using de-epithelialized rat tracheas, two to four xenotransplanted rat tracheas were examined histologically.

Four weeks after xenotransplantation, tracheal transplants were exposed to beeswax pellets containing 100 μg of DMBA or beeswax blank pellets without DMBA. After insertion of the pellets, the effects of the carcinogen and beeswax blank pellets were determined at 3, 4, 5 and 6 months. Two to four tracheal transplants were used in most cases per time point.

***In vitro* Procedures for the Study of Phenotypically Altered Cells after *in vivo* Exposure to DMBA** Three, four, five and six months after inserting the pellets, tracheal transplants were removed, and explant cultures were established as follows.

The removed tracheas were cut into 1×1 mm pieces and put epithelial side up in 60 mm coated tissue culture dishes (6–7 pieces per dish). These pieces were incubated in ACB-1 medium. Once outgrowths from the pieces reached 1 cm diameter, the growing pieces were transferred into a new coated culture dish (one piece per dish). This procedure was repeated until the pieces stopped producing outgrowth of epithelial cells. The epithelial cells remaining in the dishes were subcultured, if and when the cultures reached 80% confluency.

Histology and Determination of the Human Nature of Tissues and Cells Two tracheal rings separated by 5–6 mm were sampled from each transplant, fixed in neutral buffered formalin, embedded in paraffin and stained with hematoxylin-eosin for histological examination. To determine the human derivation of the repopulated epithelia, sections from each case were processed for *in situ* hybridization with ^{35}S-labeled sonicated human DNA probes and ^{3}H-labeled *Alu* probes.[19] Likewise *in situ* hybridization and chromosomal analysis were

also done to determine the human derivation of cells in culture.

Results

***In vivo* Exposure to Carcinogen** Xenotransplanted NHTBECs from six donors were exposed to 100 μg of DMBA for 3 to 6 months. At these time points the tracheal transplants containing the carcinogen-treated NHTBECs as well as another group of transplants exposed to control pellets without carcinogen were removed from the animals and placed in explant culture. A histological examination of three tracheal rings per tracheal transplant showed that the tracheal transplants exposed to control pellets without carcinogen contained normal mucociliary epithelium (Fig. 1 A). The carcinogen-treated tracheal transplants contained predominantly a hyperplastic mucociliary epithelium (Fig. 1B) with some areas in which the epithelium was devoid of ciliated cells and showed a tendency to stratify (2–3 layers of columnar or cuboidal cells) (Fig. 1C). Some of these stratified epithelial patches gradually changed into incipient squamous metaplasias or into hyperplastic or

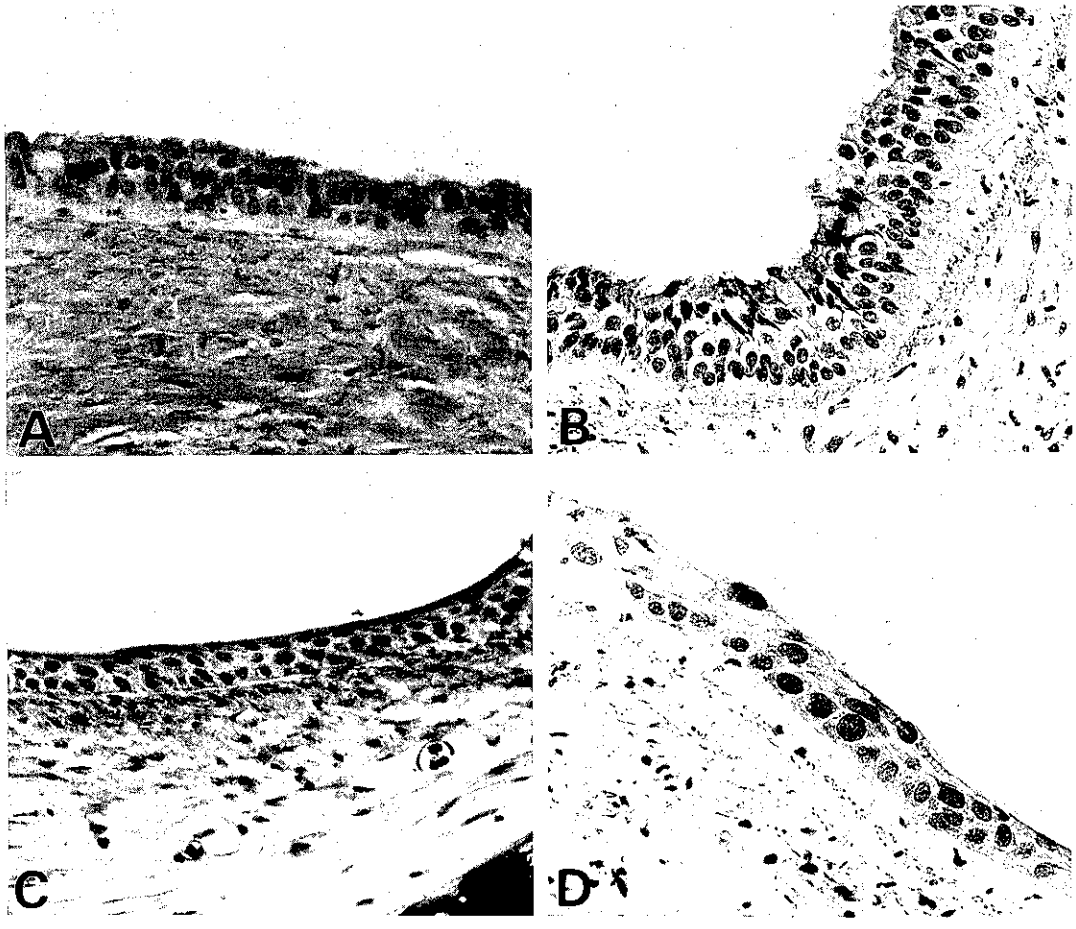

Fig. 1. Epithelia found in tracheal transplants containing human xenotransplanted epithelium. A) Normal mucociliary epithelium after 12 weeks of treatment with beeswax pellet without carcinogen. B) Hyperplastic mucociliary epithelium found 12 weeks after DMBA treatment. C) Stratified epithelium composed of 2–3 layers of deciliated poorly differentiated cells. From the same donor, 4 months after DMBA treatment. D) Stratified epithelium with moderate atypia in another ring of the same transplant. All repopulated epithelia originated from donor 5. Hematoxylin-eosin, ×190.

normal mucociliary epithelium. A few lesions of dysplastic nature, classified as squamous or stratified metaplasias with moderate atypia (Fig. 1D), could be seen in all carcinogen-exposed tracheal transplants. In contrast, the latter lesions were never seen in the control tracheal transplants. Normal or hypoplastic mucociliary epithelium was the predominant feature of the NTHE treated with beeswax control pellets. *In situ* hybridization with human specific DNA probes demonstrated the human origin of all repopulated epithelia.

***In vitro* Growth of NHTBECs Exposed *in vivo* to DMBA** Explanted fragments from tracheal transplants treated with control pellets had a very short *in vitro* survival. Although most tissue pieces exhibited epithelial outgrowths in ACB-1 medium, the cells showed involutional features shortly after the first week in culture. Except for two cases (donors 4 and 9), the epithelial cells could not be subcultured as a single cell suspension, nor could the fragments produce epithelial outgrowths when replaced on new dishes (Table I). Explants from carcinogen-treated tracheal transplants showed more variation in *in vitro* growth behavior (Table II). Tissues from three donors could not be successfully replated or subcultured and had an *in vitro* survival time of 2–4 weeks. Tissues from another three donors could be subcultured 2–3 times and had a longer *in vitro* survival. Only tissues from one donor (donor 5) showed a remarkable increase in *in vitro* survival (42 weeks), and the epithelial cells were subculturable 10 times.

Characteristics of D5 Cells Explants from donor 5 produced epithelial outgrowths for 4

Table I. Tracheal Transplants Repopulated with Normal Human Tracheobronchial Epithelium (Beeswax Controls)

Donor	Duration of *in vivo* experiment (months)	Number of transplants	*In vitro* survival (weeks)	Number of subcultures
1	4	4	2	0
2	4	2	4	0
3	5	4	3	0
4	6	4	8	3
8	4	2	4	0
9	4	1	6	2

Table II. Tracheal Transplants Repopulated with Normal Human Tracheobronchial Epithelium Exposed *in vivo* to 100 μg of DMBA

Donor	Duration of *in vivo* experiment (months)	Number of transplants	*In vitro* survival (weeks)	Number of subcultures
2	3	9	2	0
3	5	1	2	0
4	6	4	4	1
5	4	4	42	10
6	4	2	8	3
7	4	1	6	2
8	4	1	6	3

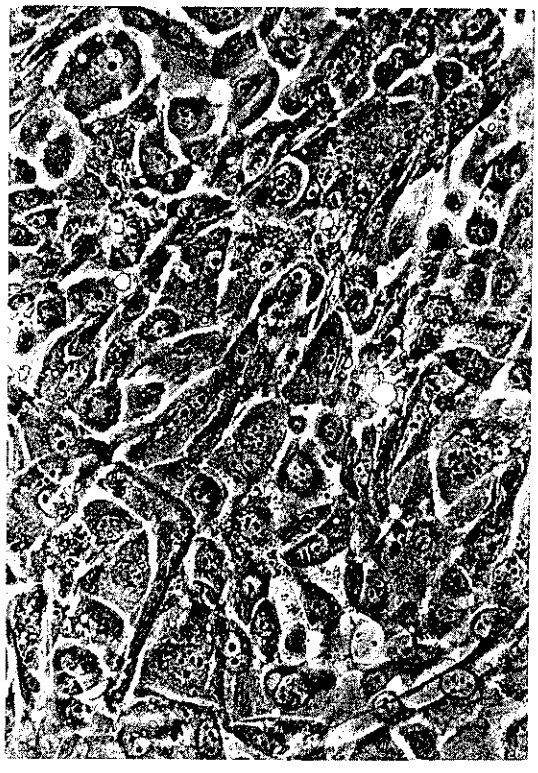

Fig. 2. D5 cells grown in ACB-1 medium exhibited a marked variation in size, shape and nuclear density, that contrasted markedly with the uniform cellular pattern of normal untreated human tracheobronchial epithelial cultures. Phase contrast, ×230.

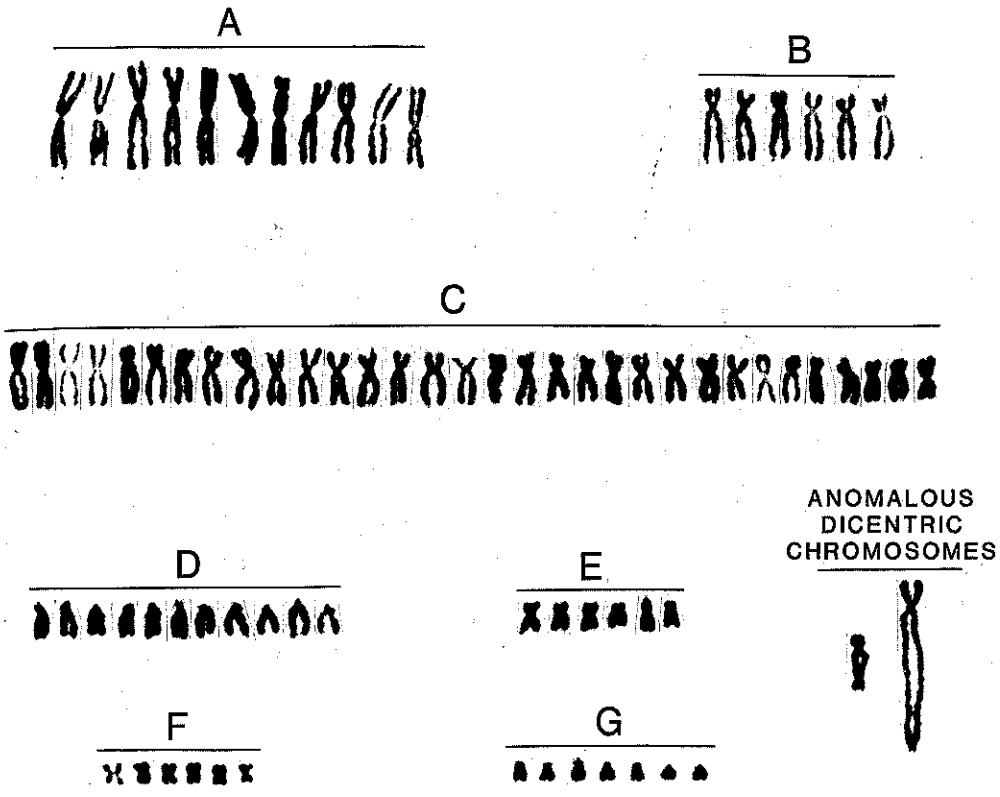

Fig. 3. Chromosome composition of D5 cells at passage six showing in this case 79 chromosomes with 2 anomalous dicentrics.

months and could be replated five times. Epithelial cells from donor 5 (D5) cells were maintained for 10 months in ACB medium containing either 1.25 or 2% serum. They proliferated actively, forming confluent cell sheets composed of heterogeneous large, medium and small cuboidal or polyhedral cells with large nuclei and prominent nucleoli (Fig. 2). Mitotic figures were frequent. Chromosome analysis of D5 cells (first done on the sixth passage) showed predominantly hyperdiploid and hypertetraploid cells (mean chromosome number $\pm$SD$=107\pm46$) with anomalous dicentric chromosomes (Fig. 3) as well as occasional chromosome fragments and double minutes.

Chromosomal preparations from normal as well as nontreated xenotransplanted NTHE kept in culture for 2 to 6 months showed predominantly diploid cells.

Resistance to Serum-induced Terminal Differentiation In order to determine if D5 cells had acquired new characteristics compatible with partially transformed or phenotypically altered cells we examined their *in vitro* survival and terminal differentiation after exposing the cells to ACB medium containing various amounts of serum. The D5 cells at passage 5 grew very slowly in ACB medium without serum, but showed increased growth in 0.5% serum as well as in 1, 2, 4, 6 and even 8% serum-containing medium (Fig. 4). Cells from donor 4 at passage 2 exposed *in vivo* to beeswax pellets without carcinogen were similarly tested for *in vitro* survival. These cells, as well as tracheobronchial epithelial cells from 3 normal donors that were not xenografted and were never exposed to either carcinogen or blank pellets, grew well in mediun with 0.5–1% serum, but failed to

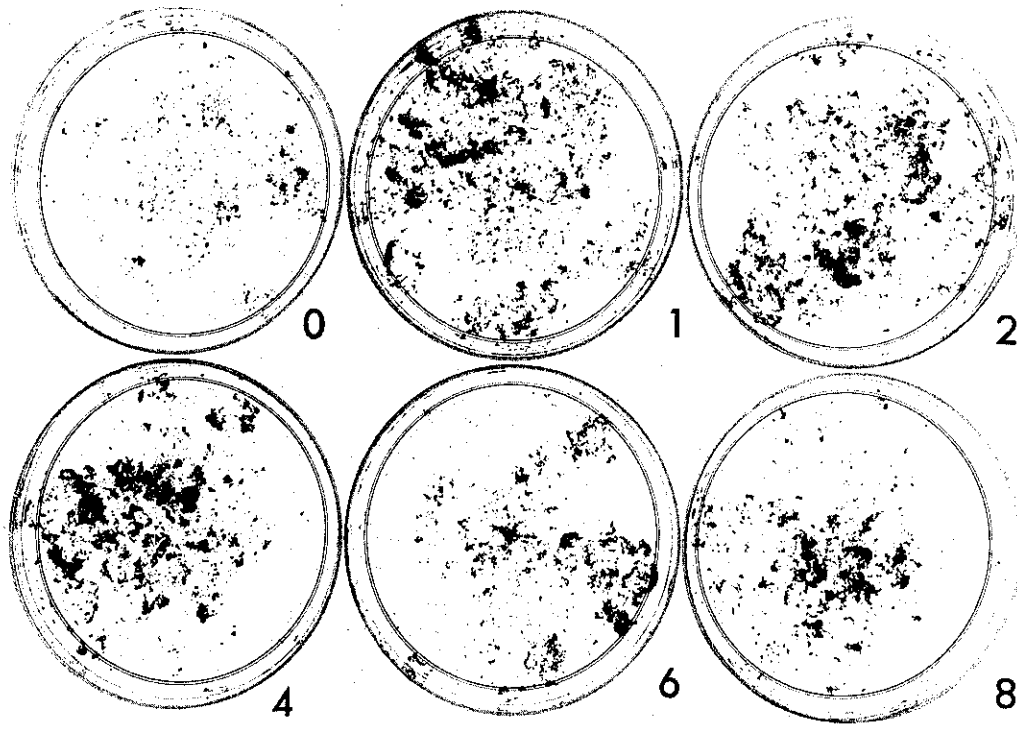

Fig. 4. Plates containing D5 cells 2 weeks after seeding 5×10^4 cells per plate containing ACB-1 medium with various concentrations of fetal bovine serum (as indicated in the right corner under each plate). Minimal cell proliferation could be seen in medium devoid of serum (0) whereas a maximum number of cells was seen with 4%. All other concentrations tested allowed for cell proliferation and survival for a two-week period. Normal cells did not survive for more than a few days. (Formalin fixation and crystal violet stain.)

proliferate and terminally differentiated in medium containing higher concentrations of serum.

Discussion

Exposure of xenotransplanted NHTBECs to the carcinogen DMBA has produced consistent hyperplastic and metaplastic-dysplastic lesions in all tracheal transplants. This includes 23 transplants reported herein as well as several dozen transplants previously treated with the same dose of carcinogen.[9, 10] In contrast to this rather homogeneous response only the tracheas from one donor (out of seven) exhibited a significantly longer *in vitro* survival (42 weeks) than the cells from four donors treated *in vivo* with beeswax control pellets (2–8 weeks). In addition these D5 cells could be subcultured up to 10 times. This contrasted with the cells from all other donors which were either not subculturable or could only be subcultured 1–3 times. Although originally we prepared beeswax blank and beeswax carcinogen-exposed tracheal transplants with all donor cells, unfortunately in some cases *in vivo* repopulation was not achieved. Despite the fact that this has resulted in incompletely paired data, the difference between the DMBA-exposed and the control groups seems to be relevant. Not only the longer survival and subculturability of carcinogen-treated cells but also their morphological and chromosomal abnormalities made D5 cells very different from all the other cells seen in this study. Finally, the capacity of D5 cells to survive and proliferate in tissue culture medium with a high concentration of serum sets them clearly apart from normal cells that

differentiate and senesce under similar conditions. Since only lung tumor cell lines,[13] oncogene-transfected[14] and some virus-infected tracheobronchial epithelial cells (Harris and Reddel, personal communication) exhibit resistance to serum-induced terminal differentiation, it is logical to consider D5 cells as at least partially transformed or phenotypically altered cells. These cells are probably equivalent to some of the rodent tracheal epithelial carcinogen-altered cells described as enhanced growth variants or non-subculturable epithelial cells forming foci[17, 20] that have been considered as early stages in the process of carcinogenesis.[21] These cells could, under appropriate conditions or additional stimuli, progress to more advanced stages malignancy. At this stage of our investigations it is impossible to determine with certainty if the changes detected in D5 cells correspond to actual stages of effective human carcinogenesis or if they correspond to an abortive pathway of limited cell divisions that have many common features with successful malignant transformation. Since the effectiveness in detecting these changes is apparently low, we are now testing other *in vitro* procedures for selection of *in vivo* carcinogen-altered cells. In addition, in order to decrease the probable fluctuations in the effect of carcinogens due to the individual variability in their metabolism, we are screening direct-acting carcinogens for ability to induce lesions in xenotransplants. It is hoped that with these technical innovations a larger variety of phenotypically altered cells in different stages of tumor development will be found.

Acknowledgments

The authors especially thank D. Trono and B. Tallardy for their excellent technical assistance and M. Yasuda for preparing the manuscript. This work was supported by PHS grants CA-35562 and CA-44981 awarded by the National Cancer Institute, USA, and by Grants-in-Aid for Scientific Research 62570630 (M.B.), 62440053 (Y.Y.) from the Japanese Ministry of Education, Science and Culture.

(Received March 3, 1988/Accepted April 27, 1988)

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