Carcinoma after Malignant Conversion *in Vitro* of Epithelial-like Cells from Rat Liver following Exposure to Chemical Carcinogens

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**SUMMARY**

Epithelial-like cells from rat liver were exposed in cell culture to aflatoxin B<sub>1</sub>, dimethylnitrosamine, N-nitrosomethylurea, N-hydroxy-N-2-fluorenylacetamide, or 7,12-dimethylbenz(a)anthracene. Microscopic observation revealed several morphological changes in almost all of the treated sublines but revealed no uniform, characteristic alterations common to all of the cultures. The injection of 5 to 20 × 10<sup>6</sup> treated cells into newborn or X-irradiated syngeneic rats yielded tumors, usually after latent periods of 2 to 8 months or an average of 7.8 months. The tumors displayed epithelial aspects and were diagnosed as carcinomas.

**INTRODUCTION**

A cell culture system capable of malignant conversion by a variety of chemical carcinogens would be of considerable use in the testing of carcinogens and in the study of the mechanism of their action. The presently available systems have generally displayed susceptibility to limited classes of carcinogens, mainly polycyclic hydrocarbons (2, 4, 12, 14). One reason for this may be the absence in these cultures of the enzymes required for the metabolism of many carcinogens to their active derivatives. The liver has the broadest capabilities of this kind and, therefore, cultured liver cells would be potentially the most likely to possess wide responsiveness. We previously reported a method for the reproducible initiation of epithelial-like cell cultures from rat liver (23). This communication describes both the malignant conversion of these cultures after exposure to a variety of classes of chemical carcinogens and the development of carcinomas from treated cells transplanted into syngeneic hosts (24).

**MATERIALS AND METHODS**

TRL<sub>2</sub> and TRL<sub>6</sub>, which were used in these studies, are among the lines previously reported, and culture conditions were as described (23). The sources of the carcinogens were as follows: AFB<sub>1</sub>, Dr. G. N. Wogan, Massachusetts Institute of Technology, Cambridge, Mass.; DMN and DMBA, Eastman Organic Chemicals Rochester, N. Y.; NMU, synthesized by Dr. E. K. Weisburger; N-OHFAA, from Dr. E. K. Weisburger and obtained through the courtesy of Dr. H. B. Wood, Jr., National Cancer Institute. The water-soluble DMN was pipetted directly into the culture medium, while the solid carcinogens were dissolved in dimethylformamide:dimethyl sulfoxide (1:1) at concentrations yielding a final concentration of less than 0.002% vehicle in the medium. As a control for the presence of solvent, a subline of TRL<sub>2</sub> was continuously exposed to 0.0025% vehicle. The objective of the treatment was to induce malignant conversion, and accordingly maximum exposure to the carcinogens was made. Thus, after preliminary toxicity studies, the highest dose not resulting in overt cell death or decreased plating efficiency was administered continuously. In a few cases, however, when cultures were growing poorly for unknown reasons, control medium was substituted for a period of days during the course of exposure. NMU, which hydrolyzes spontaneously, was given as a single exposure over a 24-hr period. In all cases of continuing treatment, the carcinogen was mixed with the medium prior to each medium change, usually twice per week. The details of the exposure regimens are listed in Table 1.

Tumorigenicity was determined by periodic s.c. or i.p. injections of a counted number of cells into syngeneic rats. In initial testing, the carcinogen was always omitted from the medium for several days before its injection. The cells were dislodged by mechanical scraping from the culture flasks after brief incubation with a 0.25% trypsin solution and, after sedimentation by low-speed centrifugation, they were resuspended in 0.1 to 0.4 ml of phosphate-buffered saline solution. At the beginning of the study, newborn Fischer rats were used as recipients, but later weanling animals pretreated with 350 to 400 R whole-body irradiation were used, so that a larger injection volume containing a greater number of cells could be accommodated. At first, rats given injections were killed as negative 6 months after injection, but later recipients were (and continue to be) observed for their lifetimes.

The abbreviations used are: TRL, line of Ten-day-old Rat Liver cells; AFB<sub>1</sub>, aflatoxin B<sub>1</sub>; DMN, dimethylnitrosamine (or N-nitrosodimethylamine); DMBA, 7,12-dimethylbenz(a)anthracene; NMU, nitrosomethylurea; N-OHFAA, N-hydroxy-N-2-fluorenylacetamide (or N-2-fluorenylacetohydroxamic acid, or N-hydroxy-2-acetylamino-fluorene).
Once tumorigenicity was detected in animals, the carcinogen treatment of the cells in vitro was discontinued, and the cells were repeatedly retested after numerous passages and growth in control medium for several months.

Control lines TRL2 or TRL6 and the solvent-treated TRL2 subline were periodically injected in the same manner as were experimental sublines. These served as controls for a number of experimental, treated sublines and thus were injected simultaneously with one or another of the experimental sublines.

RESULTS

At the beginning of the studies, untreated control lines TRL2 (Figs. 1 and 2) and TRL6 exhibited the typical aspect of the morphology of cultures initiated by the method previously reported (23). The cells had a regular polygonal shape and grew closely adherent in mosaic, pavement-like sheets. In subconfluent cultures, the cells grew in islands with smooth peripheral contours (Fig. 2). The vehicle-treated subline appeared no different from the parent line. Subcultivation was performed every 10 to 14 days.

The treated cultures displayed no immediate alterations and, in some cases, there was little change even after protracted exposure. However, eventually, in all but the DMN-treated subline, definite morphological changes arose before tumorigenicity was detected. The most prominent alteration was the development of markedly irregular cell outlines (Fig. 3). In such cultures, some cells also had enlarged nuclei and more prominent and numerous nucleoli. In subconfluent cultures, the irregular cell shapes, as well as a less cohesive growth pattern, resulted in an irregular peripheral contour of growing islands (Fig. 3) which contrasted sharply with the smooth outline of islands in the control cultures. In confluent cultures, pleomorphism was still apparent (Figs. 4 and 5). When cultures became crowded, there was some extent of cell overlapping (Fig. 5), but there was none of the piled-up, criss-crossed aspect associated with the transformation of fibroblast-like lines (15). The appearance of sublines treated with different carcinogens varied from one to another (Figs. 4 and 5). The morphology of each treated subline was fairly constant and permanent, even after carcinogen treatment was discontinued. However, there seemed to be no specific feature other than pleomorphism that was common to all sublines, even after the acquisition of tumorigenicity. Usually, the carcinogens initially produced a slight retardation in growth, which prolonged the interval between subcultivations by several days. Later, the growth rates exceeded those of the control cultures, and subcultivation had to be performed every 7 to 10 days.

TRL2 has now been in culture 30 months. The cells are smaller and more compact than they appeared to be at the beginning of the study. However, there are no nuclear changes and no deviations in growth pattern such as developed in the treated sublines. The solvent-exposed subline of TRL2 is similar. TRL6, which has been in culture 22 months, appears unchanged.

The conditions of treatment which were followed by the acquisition of tumorigenicity are presented in Table 1. In some cases (AFB1, DMN, and NMU), exposure had been discontinued before tumorigenicity was demonstrated while, in others (N-OHFAA and DMBA), conversion occurred while carcinogen was present. The durations of treatments preceding the development of tumorigenicity were quite varied. Numerous negative injections into rats preceded injections that first yielded tumors. For the various sublines derived from TRL2, the total time from initiation of treatment until detection of tumorigenicity varied considerably in these experiments. A small difference in age would not be significant, since it might reflect only the period between injections but, in the intervals between the ages at conversion of 27 weeks (N-OHFAA), 46 weeks (NMU), and 61 weeks (DMBA), several injections were made of the sublines that gave evidence of tumorigenicity only later. Except in the case of N-OHFAA, tumorigenicity was demonstrated after removal of the carcinogen and growth for over 1 month in control medium involving at least 3 subcultivations and 8 changes of medium.

Consistent with the absence of morphological change in the parent lines and vehicle-treated subline was the fact that their injection into rats never produced tumors, although tests continued to be made at times well after the treated sublines had become tumorigenic. The total number of injections of the control lines were as follows: TRL2, 4; vehicle-exposed

<table>
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<tr>
<th>Carcinogen</th>
<th>Concentration of carcinogen (µg/ml)</th>
<th>Age of cultures at beginning of treatment (wk)</th>
<th>Duration of treatment (wk)</th>
<th>Interval after cessation of treatment before tumorigenic (wk)</th>
<th>Total time from initiation of treatment until tumorigenic (wk)</th>
<th>Total time in culture before tumorigenic (wk)</th>
</tr>
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<tr>
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<td>4</td>
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<td>37</td>
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a All cultures involved TRL2, except series treated with DMN which involved TRL6. Age given is from time of primary explant.
b Maintained in culture on control medium without carcinogen for time shown, when injection of carcinogen into syngeneic rats led to tumors.
c Dose increased after 34 weeks.
TRL2, 9; and TRL6, 5. No tumor was seen in any of the injection sites, and thus the data are presented in combined form, parallel to that used for treated cell lines (Table 2).

Typical patterns of the resulting tumors are illustrated in Figs. 6 through 11. Some tumors consisted mainly of disorganized fields of irregular polygonal cells with sparse cytoplasm and indistinct cell membranes (Fig. 6). Nuclei were large and irregularly oval or round (never elongated) and generally had a pale nucleoplasm with distinct nucleoli. Such tumors were diagnosed as undifferentiated malignant tumors. Most tumors displayed epithelial cells with larger amounts of denser cytoplasm and distinct cell membranes (Fig. 7). These cells were arranged in nests or cords. Some tumors displayed irregular spaces lined by the malignant epithelioid cells (Fig. 8) or areas of papillary growth (Fig. 9). Tumors with these characteristics were diagnosed as carcinomas. In approximately 15% of the tumors, areas of malignant gland formation were present (Fig. 10), permitting their classification as adenocarcinomas. The tumors often contained some minor component of nonneoplastic connective tissue (Figs. 6, 7, and 10); this was most conspicuous in tumors resulting from NMU-treated cells (Fig. 6) and is considered to be a desmoplastic reaction. One tumor displayed a malignant epithelial component accompanied by a matrix of undifferentiated malignant cells (Fig. 11) reminiscent of a malignant teratoma or hepatoblastoma.

Several of the tumors that developed from either s.c. or i.p. implantation were accompanied by histologically identical metastases in lungs and lymph nodes. All of the tumors grew progressively, debilitated, and would have killed the hosts, if permitted.

Tumors arising from the injection of differently treated sublines were excised and restored to cell culture in those cases in which the morphology of the predominating cell type was identical to that of the parent subline, which had been maintained in culture. However, extensive piling up of the cells was now clearly evident (Fig. 12). In the initial plating, a few colonies of fibroblast-like cells were present, probably derived from the reactive connective tissue present in some tumors. These cells were rapidly overgrown by the malignant, epithelial-like cells, and they were not preserved in subsequent subcultures. Upon reinsertion of these cultures into syngeneic rats, tumors uniformly resulted, usually with a shorter latent period (as short as 14 days). These growths exhibited the same microscopic aspect as did the original lesion. Also, portions of the original tumor directly transplanted into newborn rats rapidly developed into new tumors.

**DISCUSSION**

Cultures of epithelial-like cells from rat liver were treated with a variety of chemical carcinogens, including several procarcinogens. The treated cultures (but not the controls) became tumorigenic. The malignant character of the tumors formed was manifested by their morphology, progressive and invasive growth, issuance of metastases, and ability to kill the hosts. The tumorigenicity of the treated lines was always confirmed after several passages and at least a month of growth in the absence of carcinogen. In addition to the fact that 1 dose of the carcinogen (even in the amount used for exposure) would not cause local tumors, this precaution almost assures that the tumors arose from injected cells. Also, DMN would never induce local tumors, and a single dose of N-OHFAA would do so infrequently.

Regarding the results, the major uncertainty is whether tumorigenicity was induced or spontaneous. This is an important consideration, since spontaneous conversion has been observed in other cultured cells derived from rat liver (16, 18), as well as in several of the lines cultured by the present method (Williams, Elliott, and Weisburger). The ideal method for demonstrating induction is by treatment of a single cell, as was done by Mondal and Heidelberger (13). In this experiment, the evidence for induced conversion is primarily the absence of tumorigenicity in the control parent lines. Also supporting this conclusion is the finding that, in different sublines derived from a single parent line, conversion occurred at quite varied ages in culture. This suggests that different treatments influenced the time at which cancer developed.

The most significant result of this study was the finding that, regardless of the cause of the malignant conversion, the tumors that resulted from injection of treated cells were of an epithelial character and were diagnosed as carcinomas. This contrasts with most of the preceding cell culture studies in which carcinogen-treated cells gave rise to mesenchymal-type, spindle-cell sarcomas. There are several previous reports of “carcinomas” arising from spontaneously converted (6, 16) or treated (9, 14) liver cells, but none of these illustrate the presence of malignant glandular tissue (Fig. 10) diagnosable as adenocarcinoma. Namba et al. (14) and Koshiba et al. (10)
obtained carcinomas after they treated rat liver cells with 4-nitroquinoline-N-oxide, but there are no previous reports of such results for different classes of carcinogens, including those that require metabolism for activation. In the study by Namba et al. (14) and in this study, a variety of histological tumor types resulted from treatment with a single agent. This could be due either to heterogeneity of the cultures or the presence of multipotential cells. The presence of malignant glandular tissue in about 15% of the tumors raises the possibility that cells of bile duct origin were present or were even predominant in the cultures. It does not, however, necessarily preclude the possibility that cells derive from hepatocytes, since hepatocellular carcinomas can develop areas of malignant gland formation (15).

Functioning parenchymal liver cells would be expected to be responsive to a wide variety of carcinogens in culture, since liver possesses the enzyme systems required for activation of the majority of chemical carcinogens. Possibly because of a deficiency of these activation reactions, most other types of cell cultures have responded with malignant conversion to mainly polycyclic hydrocarbons (2, 4, 12, 13). Recently, AF1 (5, 22) and N-OHFAA (5) have also been found to be active in cell culture. A proliferative response in rat liver cultures has been observed after exposure to DMN (21), NMU (7), and azo dyes (8, 19). In our studies, conversion was obtained with NMU, which does not require host participation for activation, N-OHFAA, which may need at least 1 further step of biochemical conversion, and with DMBA, AF1, and DMN, each of which requires a different specific activation reaction known to occur in liver. The response obtained with all of these agents suggests that the exposed lines possessed several of the various enzymatic systems of liver for securing active intermediates. The response to DMBA was not unexpected, since other studies have revealed that the lines possess the constitutive and inducible aryl hydrocarbon hydroxylase which metabolizes polycyclic hydrocarbon carcinogens (G. M. Williams, J. B. Idoine, and J. H. Weisburger, unpublished observations).

The changes in morphology observed after carcinogen treatment seemed rather nonspecific, and there was no uniformity of appearance between variously treated sublines. It is difficult to determine whether these changes resulted from toxicity, but their persistence long after carcinogen removal suggests that they are hereditary. Even if they are considered toxic, they are still indicative of a response to the carcinogens. Sato et al. (18, 19) and Namba et al. (14) also observed mainly pleomorphism and cytological atypia accompanying malignant conversion in rat liver cells. The most prominent alteration in the treated cultures in this study was the markedly irregular outline of islands in subconfluent cultures, but the disorganized criss-crossed growth pattern associated with transformation in fibroblast-type cultures, which apparently sometime is adopted by epithelial-like cells (4, 17), was not observed in this study, nor was it reported in studies of other transformed liver cell cultures (3, 9, 14, 16, 18). The “piling up” observed by Borek (3) following nutritional stress was not prominent in the treated sublines but was very marked after the reculture of tumors arising from injected cells; this could result if the treated sublines were mixtures of normal and neoplastic cells in which the normal cells affected the growth of the malignant cells (1, 2, 11, 20). The recultured tumors might then be a population of entirely malignant cells.

These initial findings indicate that it may be possible to use rat liver cultures to investigate metabolism and response to carcinogens. Studies of this type with human material would be of great importance especially in elucidating the handling of procarcinogens by humans.

ACKNOWLEDGMENTS

We thank Dr. Robert Kroes, Rijks Instituut voor de Volksgezondheid, Bilthoven, The Netherlands, for consulting with us on the diagnosis of the tumors.

REFERENCES

Williams, Elliott, and Weisburger


Fig. 1. Confluent culture of control line TRL2. Cells are uniform in size, and the growth pattern is in a regular, pavement-like sheet. Phase contrast, × 400.

Fig. 2. Subconfluent culture of TRL2. Islands of cells have smooth peripheral contours. Phase contrast, × 400.

Fig. 3. Subconfluent culture of DMBA-treated line. Growing islands have irregular peripheral contours due to pleomorphic cells. Phase contrast, × 400.

Fig. 4. Confluent culture of DMBA-treated line. Pleomorphism is apparent, and there is considerable variation in nuclear size and shape; growth pattern is irregular. Phase contrast, × 400.

Fig. 5. Confluent culture of N-OHFAA-treated subline. Cells are smaller and more pleomorphic than control line (Fig. 1), but appearance differs from that of DMBA-treated subline (Fig. 4). A small degree of cell overlapping is present in the center of the field. Phase contrast, × 400.

Fig. 6. Section of a tumor arising from the s.c. injection of NMU-treated cells. The malignant cells have irregular, round, or oval nuclei varying considerably in size. The cytoplasm is indistinct. The cells lack any particular growth pattern. There are a few nonmalignant cells with elongated nuclei typical of fibroblastic cells. H & E, × 250.

Fig. 7. Section of a tumor arising from the s.c. injection of DMN-treated cells. The malignant cells have abundant cytoplasm and distinct cell borders and are arranged in nests. Spindle-shaped cells lie between the nests. H & E, × 100.

Fig. 8. Section of a tumor arising from the s.c. injection of AFB₁-treated cells. Numerous irregular spaces are lined by low cuboidal or plump, malignant, epithelial-like cells. H & E, × 100.

Fig. 9. Section of a tumor arising from the s.c. injection of AFB₁-treated cells. Extensive papillary configurations covered by the malignant cells are present. H & E, × 63.

Fig. 10. Same tumor as Fig. 9. Malignant gland formation permitting diagnosis of adenocarcinoma. H & E, × 100.

Fig. 11. Section of a tumor arising from the s.c. injection of DMBA-treated cells. Matrix of undifferentiated malignant cells interspersed with numerous clefts lined by malignant epithelioid cells. H & E, × 40.

Fig. 12. Culture of tumor which developed from injection of AFB₁-treated cells. Note piled-up cells in right center. Phase contrast, × 400.
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