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In vitro Malignant Transformation by Methylcholanthrene of the Progeny of Single Cells Derived from C3H Mouse Prostate*

Sukdeb Mondal† and Charles Heidelberger‡

MCARDLE LABORATORY FOR CANCER RESEARCH, UNIVERSITY OF WISCONSIN, MADISON

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Abstract. Cell lines derived from C3H mouse ventral prostate grow to a monolayer, do not pile up, and do not give tumors on inoculation into isologous mice. When treated with carcinogenic hydrocarbons, these cells produce piled-up colonies that give rise to fibrosarcomas in mice; these colonies are termed transformed. Individual single cells were treated with 0.5 per cent dimethyl sulfoxide (DMSO), and 2 per cent of them gave rise to clones containing transformed colonies. Under optimal conditions, treatment of individual single cells with 3-methylcholanthrene (MCA) led to the formation of transformed colonies in 100 per cent of the clones. From recloning experiments, it appears probable that all the progeny of the MCA-treated cells were potentially transformed, although the time required for the transformation to be scored was variable. It is concluded that in this system the carcinogen does not select for pre-existing malignant cells.

Introduction. The mechanisms of chemical carcinogenesis are at present unknown. One cellular mechanism that has been proposed, particularly by Prehn,[1] is that the carcinogen somehow selects for pre-existing malignant cells. This possibility is examined in the present study.

In this laboratory we have long been interested in the mechanisms whereby polycylic aromatic hydrocarbons initiate the process of carcinogenesis.[2, 3] Toward this end we have developed a quantitative system for hydrocarbon carcinogenesis *in vitro*.[4–7] Aneuploid lines of cells derived from C3H mouse ventral prostate can be cultured indefinitely, grow to a monolayer without piling up in the dishes, and do not give rise to tumors on subcutaneous inoculation of 10^6 to 10^7 cells into irradiated C3H mice. The incidence of spontaneous transformation in these cells is low, and occurs only after very prolonged cultivation.[5] When such cells are treated for one day in culture with methylcholanthrene, they pile up after reaching a monolayer and give rise to progressively growing, transplantable, and metastasizing fibrosarcomas on subcutaneous injection of 10^3 cells into nonirradiated C3H mice.[6] When the control cells are plated sparsely and treated with carcinogen piled-up colonies (each one of which is capable of producing tumors in mice[7]) can easily be scored. The number of these colonies is proportional to the carcinogenic activity of the hydrocarbon and is unrelated to

the toxicity the compounds exert.[7] Other investigators working with cells from sources different form ours have also successfully achieved systems for obtaining chemical carcinogenesis *in vitro*.[8-10]

We shall herewith define the word "transformation" to denote the phenotypic state in which cells form piled-up colonies that give rise to fibrosarcomas on inoculation into isologous mice.

Materials and Methods. All cultures were done in Eagle's BME medium plus 10% fetal calf serum (Gibco, Grand Island, N.Y.) and antibiotics at 37°C in humidified incubators in an atmosphere of air and 5% CO_2. Methylcholanthrene solutions were made up as previously described,[6] and after the period of hydrocarbon treatment the medium was removed and the cells were washed twice with medium before the culture was continued. When the cells were treated for one day with 0.5% DMSO or various concentrations of MCA, the cells were washed as above, fresh medium (without carcinogen or solvent) was added, and subsequently the medium was changed twice weekly. When the treatment was for 6 days, fresh carcinogen-containing medium was added at 3 days, and at 6 days the cells were washed, noncarcinogen-containing medium was added, and subsequently the medium was changed twice weekly. The DMSO-containing media were handled similarly. Ring-isolation of colonies was done by the method of Puck *et al.*[11]

Isolation of single cells: This was done by modification of methods previously described by Schenk and Moskowitz[12] and Freeman *et al.*[13] Four hundred cells from the 25th subculture of a line of fibroblasts derived from adult C3H mouse prostate[5] were plated in 35-mm Falcon plastic culture dishes containing 20–25 small pieces of coverslips about 1 mm^2 in area. After incubation for 16 hr, the dishes were examined carefully with an inverted microscope (Zeiss), and the fragments of coverslips with only one cell attached were located and marked on the underside of the dish with a wax pencil. Such pieces of coverslip were then removed from the dishes with a pair of fine curved forceps, washed gently in BME medium to remove any cells that might have adhered to the sides of the coverslip fragment, and put individually into new culture dishes of the same size containing 0.8 ml of medium to which was added 0.5% dimethyl sulfoxide (DMSO) or 3-methylcholanthrene (MCA) in 0.5% DMSO. The dishes were once again carefully examined microscopically to make sure that each coverslip fragment still had only one cell attached. The dishes were then transferred to the incubator and examined daily; the medium was changed twice weekly. Great care was taken to avoid contamination or transfer of cells from one dish to another; individual pipets were used for each dish. In about 10 days, when cells originating from the single cell covered the piece of coverslip, the medium was removed, the coverslip fragment was transferred to another dish, one drop of 0.05% trypsin was placed on it, and after 10 min, 4 ml of medium was added. This was pipetted gently 4 times, and 2 ml of the cell suspension was then plated in a new dish bearing the same label. Culturing was continued in the same dish until eventually it was fixed and stained, as described previously.[5-7]

Another cell line was also used in some of these experiments; it was obtained from C3H mouse ventral prostates in the same way as described before[5] except that fetal calf serum was used instead of horse serum in the initial organ culture phase.

Test for malignant transformation: Cells were tested for malignant transformation by examination of the piled-up colonies on the inverted microscope and in fixed and stained dishes, as described previously.[7] In some experiments, each clone of cells was injected subcutaneously into isologous C3H mice (A. R. Schmidt Co., Madison, Wis.). All mice were observed for at least four months before being scored as negative for tumors. Most tumors appeared in the mice within 3 weeks.

Results. Cloning efficiencies of the single cells: Single cells were isolated by inspection of bits of coverslips and were then cultured in individual dishes as described in **Materials and Methods.** The cloning efficiency is hereby defined

as the percentage successful growth of clones of cells compared with the number of single cells plated in individual dishes. In the experiment shown in Table 1, 18 clones were successfully grown from 25 individual single cells treated for six days with 0.5 per cent DMSO as the solvent controls. This corresponds to a cloning efficiency of 72 per cent. Table 1 also gives the results of the cloning efficiencies obtained when 172 individual single cells were treated with various concentrations of MCA. There was little effect of the carcinogen on the cloning efficiency of these individual single cells at levels of 0.25 to 2.5 μg/ml for six days. However, slight toxicity was observed at 5.0 and 10.0 μg/ml for six days and at 10 μg/ml for one day.

TABLE 1. *Cloning efficiency of single cells treated with 3-methylcholanthrene.**

Conc. MCA (μg/ml)	Duration of treatment (days)	No. of cells treated	No. of clones obtained	Cloning efficiency (%)
0.0	6	25	18	72
0.25	6	10	6	60
0.50	6	14	9	64
1.0	6	36	26	72
1.0	1	21	13	62
2.5	6	16	11	68
5.0	6	22	12	55
10.0	6	28	12	43
10.0	1	25	11	44

* During the treatment period all cultures contained 0.5% DMSO.

TABLE 2. *Transformation frequency of clones obtained from single cells treated with 3-methylcholanthrene.**

Conc. MCA (μg/ml)	Duration of treatment (days)	No. of clones studied	No. of clones with transformed colonies	Percentage clones with transformed colonies	Avg. no. of days for transformation
0	6	18	1†	6	75
0.25	6	6	2	33	64
0.50	6	9	8	88	81
1.0	6	26	26	100	52
1.0	1	13	13	100	55
2.5	6	11	11	100	56
5.0	6	12	8	67	72
10.0	6	12	8	67	62
10.0	1	11	8	72	55

* These are the same cells as those described in Table 1. During the treatment period all cultures contained 0.5% DMSO.

† These cultures were observed for 120 days and then discarded.

Transformation frequencies: The successful clones derived from the individual single cells described in Table 1 were watched daily for the appearance of piled-up colonies. The results are shown in Table 2. Although the clones derived from the individual single cells treated with DMSO were kept more than 100 days, only one of these showed piled-up colonies. Thus, in this experiment (using the older cells[5]) 6 per cent of the clones derived from individual single cells (1 clone) underwent spontaneous transformation. On the other hand, under

optimal conditions of MCA treatment (1.0–2.5 μg/ml), 100 per cent of the clones derived from individual single cells gave rise to piled-up transformed colonies. At concentrations of MCA lower and higher than the optimal, the percentage of clones exhibiting transformation ranged from 33 to 88 (Table 2).

In order to verify that the piled-up transformed colonies were composed of malignant cells, the clones picked from control and treated dishes were injected into groups of three adult C3H male mice, with the results shown in Table 3.

TABLE 3. *Induction of fibrosarcomas in C3H mice by subcutaneous inoculation of prostate cells treated with 0.5% DMSO or 1 μg/ml of MCA for 6 days.**

No. of clones injected	Treatment	No. cells injected	No. tumors/ no. mice	Tumors (%)
18	DMSO	$1.2–1.5 \times 10^6$	3†/54	5
26	MCA	$1.0–1.5 \times 10^6$	73‡/76	96

* These mice were injected with cells from each clone. These are the same clones described in Tables 1 and 2.

† These tumors were all derived from cells of the clone that had a piled-up colony (Table 2).

‡ Cells from all clones gave rise to tumors.

In the mice injected with cells from clones derived from the individual single cells treated with DMSO, three fibrosarcomas were found. These were all produced from the only DMSO-treated clone that contained transformed colonies. No other tumors were found in mice inoculated with cells from DMSO-treated clones. By contrast, 73 of the 76 mice injected with cells from the clones derived from MCA treated individual single cells developed fibrosarcomas, and all the MCA-treated clones gave rise to tumors. These tumors could not have arisen from the carry-over of carcinogen in the cells because of the length of time (55–81 days) that the cells were in culture after washing out the minute amount of MCA that was added on the first day of culture. This experiment confirms the previous ones[5–7] that the cells derived from piled-up colonies are malignant.

A comparison of the cloning efficiencies (Table 1) and the transformation frequencies (Table 2) at different concentrations of MCA reinforces the conclusion drawn from our earlier work[7] and that of Huberman and Sachs[14] that the toxicity produced by carcinogenic hydrocarbons is not directly related to their ability to produce transformations. This stems from the fact that the transformation frequencies were less than 100 per cent at concentrations of MCA both lower and higher than optimal, whereas minor toxicity was only observed at levels higher than those producing optimal transformation frequencies. This experiment demonstrates conclusively that malignant transformation in our system does not involve the selection of pre-existing malignant cells.

At the optimal concentration of MCA for the transformation of individual single cells (1.0–2.5 μg/ml), 100 per cent of the clones developed piled-up transformed colonies (Tables 2 and 3). Does this mean that only a few of the progeny were transformed, or were all progeny cells potentially transformed?

Since in our experiments transformed clones piled up on a monolayer, we wished to know whether there were really two populations of cells in the dishes, or whether the monolayer areas contained potentially transformed cells that would eventually form piled-up colonies. In order to approach this question

we wished to find out whether cloned long-term cultures of transformed cells would give rise to such typical piled-up colonies or whether they would grow in multilayered array. Accordingly, a single piled-up colony was isolated from a dish of prostate cells that had been treated with MCA at the 26th subculture, and was subsequently kept in continuous culture for 260 days. At this time the cells were placed at 400/dish in 20 dishes (35 mm). After eight days, 30 clones were ring-isolated from these 20 dishes and cultured individually in 30 dishes. After another eight days, 35 colonies were ring isolated and re-cloned in separate dishes. In contrast to the usual appearance of the piled-up colonies on a background of strictly monolayered cells these dishes filled with multilayers of cells arranged randomly and not in distinct colonies. Within 28 days, all the dishes had the same appearance. Thus, cultures of cells cloned and re-cloned from a population that had been grown for a long time after transformation had a different appearance than newly transformed cultures. Hence, it can be concluded that *all* the cells in long-term cultures of MCA-treated cells are transformed.

We then asked whether all the progeny of single cells that were treated with MCA and re-cloned before transformation was evident would give rise to transformed colonies. Thus, an individual single cell in a dish was treated with 1 μg/ml of MCA for one day, and the cells were grown for 36 days, at which time there were no piled-up colonies. The cells were then plated sparsely (400/dish) in 10 dishes (35 mm). Eight days later, 36 clones were isolated by the ring-isolation technique[11] and cultured separately in 36 dishes. Of these, 33 clones were successfully grown. At 14 days, nine of the dishes had piled-up colonies, and within the next 30 days all the remaining 24 dishes showed similar changes.

The conclusion drawn from this experiment is that all the progeny of the cell that had been treated with MCA had the potential of transformation, but became scoreable as piled-up transformed colonies at different times. However, the formal argument is not excluded (but appears to us very unlikely) that the original clones consisted of a mixture of transformed and nontransformed cells, and that the latter did not grow at all on re-cloning. The improbability of this argument is underscored by an experiment in which a number of ring-isolations were carried out on monolayer areas of treated dishes that contained piled-up colonies. Those clones when re-plated in other dishes all eventually gave rise to piled-up colonies.

We then examined the behavior of cells that were cloned before, and re-cloned after treatment, in order to determine whether under these conditions, selection could be involved in the malignant process.

Cells from a different line of mouse prostate cells (where fetal calf serum was used for all phases of its growth) were taken from a mass culture at its 12th passage. These cells were plated sparsely (400/dish) in 20 dishes (35 mm). Eight days later, 60 clones were picked by the ring-isolation method and plated in individual dishes. One day later, 30 of the clones were treated for 24 hours with 1 μg/ml of MCA, and the other 30 were treated for 24 hours with 0.5 per cent DMSO. After an additional eight days, 35 colonies were isolated from the 30 MCA-treated dishes, and 34 clones were similarly isolated from the DMSO-treated clones. Thus, the cells were cloned before treatment and re-cloned after

treatment. In the dishes with the re-cloned MCA-treated cells, the first piled-up colony became evident on the 21st day after the second cloning, and piled-up colonies were observed with an approximately linear accumulation with time until all 35 clones had given piled-up colonies by the 77th day. The dishes were fixed and stained and re-examined for confirmation of the piled-up colonies several days after they were first detected. On the other hand, no piled-up colonies were seen in any of the 34 clones derived from the DMSO-treated cells. On the 77th day these dishes were also fixed and stained, and careful examination failed to reveal any piled-up colonies. Therefore, there was no spontaneous transformation. This experiment reinforces the conclusion drawn from the experiments with individual single cells that the malignant change produced in our system does not involve the selection of pre-existing malignant cells. It also demonstrates that all 35 of the treated clones have transformed progeny.

Discussion. In the present work we have demonstrated that with lines of cells derived from mouse prostate, single cells can be isolated by a modified coverslip technique and grown in individual dishes into clones with a high efficiency (72%). When these individual cells were treated for one day with 1.0 μg/ml of methylcholanthrene or for six days with 1.0 or 2.5 μg/ml of MCA, 100 per cent of the clones that grew from these cells contained malignant transformed cells as determined by the presence of piled-up colonies in the dish and their ability to produce tumors in isologous mice after subcutaneous inoculation. One out of 18 single cells treated with the solvent gave rise to a transformed clone, but in a subsequent experiment in which the cells were never in contact with horse serum (which has been shown by Parshad and Sanford[15] to increase spontaneous malignant transformation in mouse embryonic fibroblasts) *no* spontaneous transformation was observed in 34 clones treated with DMSO. In this experiment, as well, 100 per cent of the MCA-treated clones contained transformed cells.

Our results indicate that there is no direct relationship between the toxicity of the carcinogenic hydrocarbon and its ability to produce transformation. This is in agreement with the findings of Huberman and Sachs in their *in vitro* carcinogenesis system using embryonic hamster fibroblasts.[14] However, we are in disagreement with those other workers who have inferred a direct relationship between toxicity and transformation, which has been used as one of the main arguments in the clonal selection theory of carcinogenesis.[1, 10, 16–18]

We believe that our experiments rule out the possibility that the hydrocarbon is producing the malignant change by means of a clonal selection of a pre-existing population of malignant cells. The only selection that could have occurred in our individual single cell experiments involves the use of cells that attach to the coverslip. However, the vast majority of cells in our line attach very readily. Since the cloning efficiencies were 62–72 per cent in the MCA-treated cells, if selection were the explanation for the malignant change, it would have to mean that 62–72 per cent of the cells in the population were malignant, and we know that this is not so for many reasons, including the very low incidence of spontaneous transformation in our solvent-treated cells.

It appears likely from the re-cloning experiments that all the progeny of the

treated single cells are potentially transformed. However, the reason for the variation in time required for the expression of the transformed state remains obscure. This matter is under investigation.

Since the selection of pre-existing malignant cells has been ruled out as an explanation for *in vitro* carcinogenesis in our system, it appears that the hydrocarbon acts to convert nonmalignant control cells into malignant cells. Before the molecular mechanism of this apparently direct transformation can be elucidated it will be necessary to determine whether the chemical carcinogen transforms the cells by itself, or whether there is the intervention of an oncogenic virus. This question is under intensive study in this laboratory.

Our *in vitro* system is being used to study a number of fundamental biological problems of chemical carcinogenesis, some of which have been described here. The question of whether this system is really a valid model of *in vivo* chemical carcinogenesis must be kept under continual scrutiny.

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† Holder of National Institutes of Health International Postdoctoral Research Fellowship FO5 TW 1341.

‡ American Cancer Society Professor of Oncology.

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