THE IN VITRO INDUCTION OF AN INCREASE IN CELL MULTIPLICATION AND CELLULAR LIFE SPAN BY THE WATER-SOLUBLE CARCINOGEN DIMETHYLNITROSAMINE

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It has been shown in previous studies on chemical carcinogenesis in tissue culture[1, 2] that carcinogenic hydrocarbons can directly induce *in vitro* the transformation of normal hamster embryo cells into tumor cells. In contrast to normal cells which have a limited life span in culture,[2, 3] cells transformed by these chemical carcinogens show an increase in cellular life span and can be continuously cultured.[2] Transformed cells with similar properties have also been obtained after treatment of normal mouse prostate organ cultures with carcinogenic hydrocarbons,[4] and after *in vitro* treatment of normal hamster embryo cells with 4-nitroquinoline N-oxide and its derivatives.[5]

The present experiments with normal hamster embryo cells and carcinogenic nitroso compounds were undertaken (1) to extend the study of chemical carcinogenesis in tissue culture to water-soluble carcinogens, (2) to find a water-soluble carcinogen that can induce an increase in cell multiplication after treatment of normal cells, and (3) to determine whether treatment of normal cells with such a carcinogen can induce an increase in cellular life span. The results in the present communication are primarily concerned with dimethylnitrosamine,[6] a carcinogen that is 100 per cent soluble in water.[6, 7]

Materials and Methods.—Cell cultures and cell cloning: Minced whole embryos from golden hamsters were used as a source of normal cells. Mass cultures of primaries were seeded in Eagle's medium with a fourfold concentration of amino acids and vitamins (EM) with 10 per cent nonfetal calf serum. Mass cultures of secondary and subsequent cultures were seeded in EM with 10 per cent fetal calf serum. Cells were cloned at 10^3 cells per plate on X-irradiated (4000 r) rat embryo feeder cells in EM with 10 per cent fetal calf serum, and the plates were fixed in methanol and stained with May-Grünwald-Giemsa at nine days after seeding.[8]

Treatment with dimethylnitrosamine (DMNA) and subsequent subcultures: DMNA was kindly supplied by Dr. P. N. Magee and Dr. R. Preussmann, and a third sample was obtained from Eastman Kodak Co. Similar results were obtained with the samples obtained from all three sources. The DMNA was dissolved in EM without serum, warmed to 37°C. The carcinogen solutions were made and added to the cultures in a room with blacked-out windows which allowed the entrance of only a small amount of diffuse light necessary for visibility. Primary cultures were treated in closed beakers, and secondary cultures either in Petri dishes (Falcon Co.) one day after they had been plated for cloning, or in flasks on a Gyrotory shaker. The beakers used in these experiments were 125-ml conical beakers (Corning Glass Works, catalogue no. 1080), whose tops had been made circular so that they could be readily closed with stoppers.

In experiments on the treatment of cells with carcinogen in closed beakers, the DMNA was added to two-day-old primary cultures whose medium had been changed to EM with 10 per cent fetal calf serum. When cells were treated on a shaker, they were washed with phosphate-buffered-saline, and then incubated on the shaker for two hours in EM without serum at a cell density of 10×10^5 cells per ml. The cells were incubated on the shaker in 250-ml Erlenmeyer flasks in a 37°C room in the dark. After this treatment the cells were washed with EM, resuspended in new EM with 10 per cent fetal calf serum, and seeded in closed beakers at $20–25 \times 10^5$ cells per beaker. The shaker used was a model S3-S Gyrotory shaker (New Brunswick Scientific Co.) with the speed adjusted to 100 oscillations per minute.

Subcultures of the cells after carcinogen treatment in beakers were always made in 50-mm Petri dishes. Cells were subcultured by seeding 5×10^5 cells per plate usually at 4–6-day intervals, and if the growth rate decreased so that there were less than 5×10^5 cells per plate, they were subcultured at lower cell densities. The end of the life span was taken as the time when the number of cells had decreased to about 10^4 cells per plate, and the cells in these cultures appeared to be all giant cells. Counts of the total number of cells were made on two beakers or two plates per point, and the counts given are the averages of the two samples.

Treatment with N-nitrosomethylurea: This carcinogen[7] is about 1 per cent (w/v) soluble in water, and the sample used was kindly supplied by Dr. R. Preussmann. The material was dissolved and added to primary cultures in closed beakers or to secondary culture cells in Petri dishes in the same way as DMNA.

Results.—Induction of an increase in cell multiplication: In the first experiments to test the effect of treatment with dimethylnitrosamine (DMNA), different concentrations of the carcinogen were added to cells in Petri dishes one day after they were plated for cloning. The results (Table 1) indicate that although a 1 per cent concentration of DMNA showed a cytotoxic effect, there appeared to be no cytotoxicity at concentrations up to 0.1 per cent. An examination of the colonies produced in these DMNA-treated cultures showed no colonies with a piled-up random pattern of cell growth.

Since treatment of cells in Petri dishes may not have provided the right con-

TABLE 1. *Cloning efficiency of cells treated with DMNA.*

	Medium Change 2 Hr after Addition of DMNA		No Medium Change after Addition of DMNA	
Conc. of DMNA (%)	Total no. colonies counted	Cloning efficiency (%)	Total no. colonies counted	Cloning efficiency (%)
Control	454	5.8	470	5.9
1	0	0	0	0
0.1	513	6.4	532	6.7
0.01	418	6.9	471	5.9
0.001	189	6.3	448	5.6

Secondary culture cells were treated with DMNA in Petri dishes 1 day after they were plated for cloning. When the medium was changed after 2 hr, the cells were washed once with EM before the addition of new medium. Colonies were stained at 9 days after plating.

in the total number of cells, compared to the controls. The use of different seeding levels indicated that treatment of two-day-old primary cultures containing 6–8 $\times$ 10^5 cells per beaker usually gave the clearest indication of an increase in cell number. The data obtained in an experiment with different seeding levels are shown in Table 2. Results obtained with the treatment of ditions for DMNA to induce hereditary changes *in vitro*, in the next series of experiments cells were treated with the carcinogen in closed beakers. The cloning of cells that had been cultured with DMNA for four days in closed beakers also showed no colonies with a piled-up random pattern of cell growth. However, it was found in the experiments with closed beakers that not only was there no decrease in total cell number at concentrations up to 0.1 per cent, but that DMNA incubated with mass cultures for four or five days can induce an increase cells with DMNA on a Gyrotory shaker have indicated that an increase in cell number similar to that found in cells treated without shaking was obtained after treatment on the shaker for two hours.

In addition to these experiments with DMNA, two-day-old primary cultures were treated in closed beakers with N-nitrosomethylurea. An increase in cell number, compared to the controls, was also found with this carcinogen at four days after treatment with 1–10 μg/ml. No colonies with a piled-up random pattern of cell growth were observed after cloning of these cells treated in closed beakers with 1–10 μg/ml, or after treatment of secondary culture cells in Petri dishes with 1–100 μg/ml of N-nitrosomethylurea.

In order to test whether the increase in cell multiplication induced by DMNA could be maintained in subsequent subcultures, cells incubated with DMNA for four days in closed beakers were passaged in Petri dishes. The results indicate that the increase in cell multiplication found after treatment was maintained in subsequent subcultures (Fig. 1). In the experiment given in Figure 1, the increase in cell multiplication was maintained in all the passages shown. In other experiments in which the increase in cell multiplication was maintained on subculture, an occasional passage gave a similar cell number as the control, whereas subsequent passages then again showed an increase compared to the control cultures.

Induction of an increase in cellular life span: The continued passaging of cultures with an increase in cell multiplication after treatment with DMNA has indicated that treatment with this carcinogen also induced an increase

TABLE 2. *Number of cells in mass cultures treated with DMNA.*

No. of cells at 0 time ($\times$ 10^5)	No. of Cells at 5 Days after the Addition of DMNA ($\times$ 10^5)		
		Conc. of DMNA (%)	
	Control	0.1	0.01
4	4	3	4
5	9	14	15
7	24	36	40
18	47	47	46

Two-day-old primary cultures were treated with DMNA in closed beakers. No. of cells at 0 time = no. of cells per beaker at the time DMNA was added. In order to obtain this number of cells at 2 days after seeding, the primary cultures were seeded at 10, 15, 20, and 40 $\times$ 10^5 cells per beaker respectively.

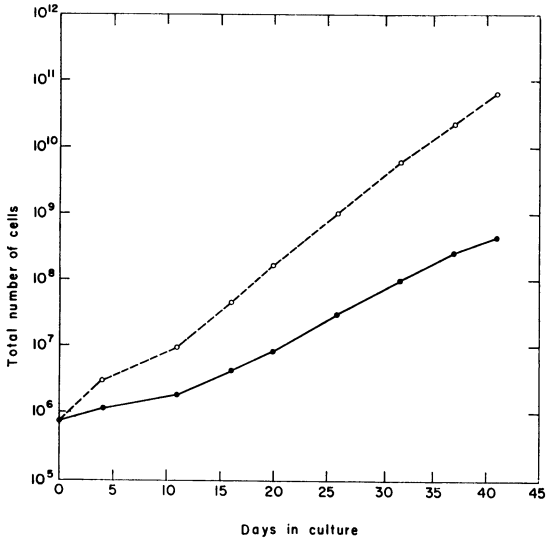

FIG. 1.—Increase in total number of cells in cultures treated with DMNA. Two-day-old primary cultures containing 7×10^5 cells were treated with 0.1% DMNA in closed beakers, and 4 days later the cells were subcultured in Petri dishes by seeding 5×10^5 cells per plate. Further passages at the same seeding level were then made at 4–7-day intervals. Cell counts were made at the time the passages were subcultured. The total number of cells was calculated from the over-all increase in cell number. ●—● = Control cultures; O—O = DMNA-treated cultures.

in cellular life span. Towards the end of the second month *in vitro* the control cultures started to decrease in cell number, and the cells terminated their life span at about 80 days. But in the DMNA-treated cultures with an increase in cell multiplication there was, towards the end of the second month, only a decrease in growth rate for about three to four weeks followed by a rapid increase in growth rate. These cultures produced cell lines that have now been growing continuously in culture for 11 months.

When cultures which developed into cell lines were cloned at about two weeks after the addition of DMNA, the colonies did not consist of cells with a piled-up random pattern of growth. However, when cloned at about five months after the addition of DMNA, the colonies of cells from these lines showed a piled-up pattern of growth. At this time, the cells of these lines did not form colonies in soft agar[9, 10] even when seeded at 1×10^5 cells per plate, and did not form progressively growing tumors when four-week-old hamsters (9 animals) were inoculated subcutaneously with 5×10^6 cells per animal. However, about three months later, cells of these lines gave about 5 per cent colonies in soft agar and formed progressively growing fibrosarcomas in 17/17 animals after subcutaneous inoculation of 5×10^6 cells per animal into four-week-old hamsters. The continued growth of the DMNA-treated cultures after the end of the life span of the controls was observed before the appearance of cells with a piled-up random pattern of growth.

Discussion.—The present results have indicated that treatment of normal hamster cells with the water-soluble carcinogen DMNA can induce an increase in cell multiplication, and that this increase can be maintained in subsequent cell passages. The continued increase in subsequent subcultures indicates that treatment with DMNA had induced in some cells in the population a hereditary ability for an increased rate of cell multiplication under the conditions tested. It is of interest that an increase in cell multiplication was found both with

DMNA that is 100 per cent water-soluble and assumed to be enzymatically decomposed to form an active carcinogenic intermediate, and with the less stable N-nitrosomethylurea, that is about 1 per cent (w/v) water-soluble and for which it is assumed that the intermediate can be formed without enzyme action.[6, 7]

The results with DMNA have further indicated that this carcinogen can induce both an increase in cell multiplication and an increase in cellular life span. The increased capacity for cell multiplication may also be the cause of the increase in life span, and it can thus be suggested that both increases may be due to the same change in the cellular control mechanism.

In contrast to the finding of a considerable frequency of colonies with a piled-up random pattern of cell growth a short time after treatment of normal hamster embryo cells with carcinogenic hydrocarbons[1, 2] and X-irradiation,[11] or infection with polyoma virus,[8] no such colonies were observed a short time after treatment with DMNA or N-nitrosomethylurea. Colonies with a piled-up random pattern of growth were only found in the later passages of the DMNA-treated cultures, after the increase in cellular life span had been observed. This was then followed at a still later time in culture by an ability to produce colonies in soft agar, and to form progressively growing tumors after subcutaneous inoculation into adult animals. The late appearance of these cells in the DMNA-treated cultures indicates that they may have arisen by a secondary change in cells with an increased life span.

Summary.—It has been shown that treatment of normal hamster cell cultures with the completely water-soluble carcinogen dimethylnitrosamine (DMNA) can result in an increase in cell multiplication, that this increase can be maintained in subsequent cell passages, and that lines of continuously growing cells can be obtained from these DMNA-treated cultures. Colonies with a piled-up random pattern of cell growth were only found in later passages after the increase in life span had been observed. This was then followed by an ability to produce colonies in soft agar, and to form progressively growing tumors after subcutaneous inoculation into adult animals. The late appearance of these cells in the DMNA-treated cultures indicates that they have arisen by a secondary change in cells with an increased life span. The observation that DMNA can induce *in vitro* both an increase in cell multiplication and an increase in cellular life span suggests that both increases may be due to the same change in the cellular control mechanism.

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