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INDUCTION OF DIFFERENTIATION IN CULTURED MOUSE NEUROBLASTOMA CELLS BY N-METHYL-N'-NITRO-N-NITROSOGUANIDINE*

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SUMMARY

N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), a potent mutagen and carcinogen, induces differentiation uniquely in N-18 mouse neuroblastoma cells, when compared with that of dibutyryl cyclic adenosine monophosphate. After treatment with 10 μM MNNG for only 2 h, RNA and protein synthesis are stimulated together with neurite formation, while DNA synthesis and growth of the cells are inhibited indefinitely. Induction of neurite formation by MNNG is irreversible, being inhibited by actinomycin D or cycloheximide but recovering after withdrawal of these inhibitors.

INTRODUCTION

Cultured C-1300 mouse neuroblastoma cells can be differentiated and show morphologically, enzymatically and physiologically similar phenotypes to mature neurones in the presence of various agents such as dibutyryl cyclic adenosine monophosphate (B_2cAMP) (1-3), bromodeoxyuridine (4), cytosine arabinoside (5), haemin (6) or serum-deprived medium (7,8). However, this differentiation can be reversed by feeding the cells with drug-free or serum-supplemented medium.

Oral administration of MNNG, a potent mutagen and characteristic carcinogen, produces stomach cancer, and also is seen to induce intestinal metaplasia in the stomach mucosa of experimental animals (9,10). In addition, in our preliminary experiment, certain enzyme activities in cultured cells derived from human oat cell carcinoma (11) increased after treatment with MNNG.

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We wish to report here that MNNG induced "irreversible" differentiation of N-18 cells (12), one of the clonal lines of C-1300 mouse neuroblastoma cells.

MATERIALS AND METHODS

Cell culture and drug treatment; N-18 cells were obtained from Dr. T. Amano (Mitsubishi-Kasei Institute of Life Science, Tokyo) and grown in Falcon plastic flasks (25 cm^2) containing 5 ml of Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum (Flow), penicillin (100 U/ml) and streptomycin (100 µg/ml) in a humidified atmosphere of 10% CO_2-90% air at 37°. Confluent cells were trypsinized and $1X10^5$ harvested cells were placed in a flask. After 24 h cultivation, the cells were treated with 10 µM MNNG (Aldrich Chemical Co.) in 0.05% ethyl alcohol-medium for 2 h and then the medium was replaced with fresh normal-medium. In another flask, the cells were grown for 3 days in the presence of 1 mM B_2cAMP (Sigma) and then the medium was replaced with fresh normal-medium. As control, cells were treated with 0.05% ethyl alcohol-medium for 2 h. In all cultures, the medium was changed every day according to the protocol indicated above.

Morphological differentiation; More than 500 cells were determined microscopically by the length of their neurites and the percentage of the cells bearing neurites (longer than 40 nm) (13) was expressed as a function of cultivation time.

Growth curve; The monolayered cells were trypsinized and the cell number was counted using a hemocytometer, at each time indicated in Fig. 2, with each point representing an average of the two samples.

Incorporation of labeled precursors into cells; Every 4 h during cultivation, control or MNNG treated cells were incubated with 2 µCi/ml of [6-^{3}H]thymidine (23 Ci/mmol) for 2 h or with both 2 µCi/ml of [5-^{3}H]uridine (26 Ci/mmol) and 0.2 µCi/ml of L-[U-^{14}C]leucine (356 mCi/mmol) for 1 h. After incubation, the cells in flasks were washed twice with phosphate-buffered saline, harvested, and then the radioactivity in acid-insoluble material per cell was determined as described previously (14).

Autoradiography; Culture conditions were the same as described above except that Lab-Tek culture chambers (#4804) were used. Thirty-seven h after initiation of the treatment with MNNG or B_2cAMP, 2 µCi of [6-^{3}H]thymidine (23 Ci/mmol) or 2 µCi of [5-^{3}H]uridine (29 Ci/mmol) was added to each chamber and incubated for 2 h or 1 h, respectively. Then, the cells in the chamber were washed with phosphate-buffered saline 3 times and were fixed in 3% glutal aldehyde. Autoradiography was performed by using Sakura autoradiographic emulsion (Konishiroku Photo Ind. Co. Ltd., Japan) with exposure for 5 days and by staining with hematoxylin and eosine.

Effects of inhibitors; All the experimental conditions including MNNG-treatment were the same as described above except that the treatment with either 0.05 µg/ml actinomycin D (Act.D) (Sigma) for 3 h or 5 µg/ml cycloheximide (Sigma) for 24 h was added as indicated in Fig. 4. The number of cells bearing neurites longer than 40 nm among the 500 cells was counted 2, 3, and 5 days after initiation of the experiment.

RESULTS AND DISCUSSION

When N-18 cells were grown in the medium containing 1 mM B_2cAMP, neurite-like processes were extended promptly. After 10 h cultivation, the number of

cells bearing neurites longer than 40 nm increased up to the maximal level of 36% of the total cells. Extension of neurites occurred only in the presence of B_2cAMP and was readily reversed to the point of complete retraction by withdrawing the drug from the medium (Fig. 1) as reported before (1). In contrast, with the treatment by 10 μM MNNG for only 2 h, some of the cells increased their own cellular and nuclear size, became flat, and finally, after about a 24 h period, started to extend processes similar to, and even greater in length, those induced by B_2cAMP or X-ray irradiation (15). Although the frequency of the induction of neurites by MNNG (22%) was a little lower than that by B_2cAMP, it was characteristic that the neurites once induced by MNNG could not be retracted even with the imposed absence of MNNG. Ten μM of concentration and 2 h of treatment with MNNG were enough to induce the maximum frequency. Under these conditions, according to trypan blue exclusion assay, MNNG did not affect cell viability during the experiment. Higher concentration and prolonged treatment with MNNG had no effect on the frequency of the induction of neurites, but rather an adverse effect on the cells as judged by the appearance of cell debris and release of the cells from the surface of the flask.

The multiplication of cells was inhibited immediately after the addition of MNNG and thereafter indefinitely, even in the absence of MNNG (Fig. 2). On the other hand, B_2cAMP did not inhibit cell growth as much as MNNG and after the withdrawal of the drug the cells recovered completely and proceeded to continue to grow much in the manner of the control cells (Fig. 2).

Acetylcholine esterase activity, which ordinarily increases gradually to the maximum in the stationary phase during the growth of N-18 cells (16), increased rapidly after treatment with MNNG concomitant with the neurite formation and after 3 days showed a 10-fold increase, reaching the same level as in the stationary phase of control cells (data not shown). Whether the increase of acetylcholine esterase activity is related to differentiation (17) or growth inhibition (16) is not clear as yet in regard to the treatment with MNNG.

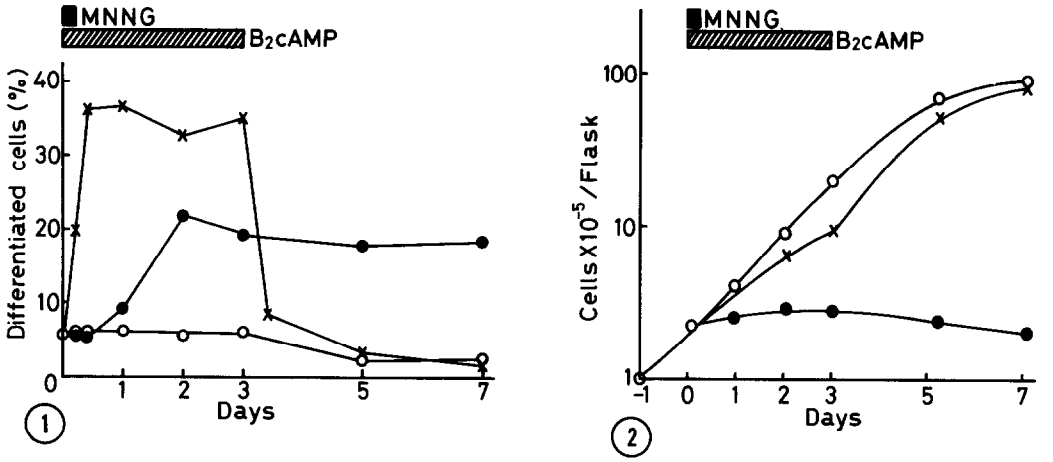

Fig. 1 Induction of morphological differentiation of N-18 cells by MNNG or B_2cAMP. The cells were treated with 10 μM MNNG for 2 h (■) or 1 mM B_2cAMP for 3 days (▨). The percentage of cells bearing neurites longer than 40 nm was expressed as a function of time. o—o, control; •—•, MNNG; x—x, B_2cAMP.

Fig. 2 Growth curve of N-18 cells after initiation of differentiation by MNNG or B_2cAMP. The number of cells per flask was expressed as a function of time. o—o, control; •—•, MNNG-treated for 2 h (■); x—x, B_2cAMP-treated for 3 days (▨).

Incorporation of [^{3}H]thymidine into the cells was inhibited immediately after the addition of MNNG and this inhibition continued even after withdrawing MNNG. Thus, 24 h after 2 h of treatment with MNNG, the incorporation corresponded to only 29% of the control. On the other hand, incorporation of [^{3}H]uridine or L-[^{14}C]leucine was stimulated by MNNG-treatment and after 28 h or 32 h showed a maximum of 198% or 228% of the control, respectively. In autoradiography, control round cells (Fig. 3a) and the large differentiated cells with neurites induced by B_2cAMP (Fig. 3b) incorporated [^{3}H]thymidine into their nuclei so thoroughly that individual grains could no longer be distinguished. However, after the treatment with MNNG, grains were very few in the differentiated cells although the same amount of radioactivity as in the case of control and B_2cAMP was used (Fig. 3c). In a further experiment, cytosine arabinoside (0.3 μg/ml) did not induce neurite extension in N-18 cells in spite of inhibition of DNA synthesis and cell growth to an extent

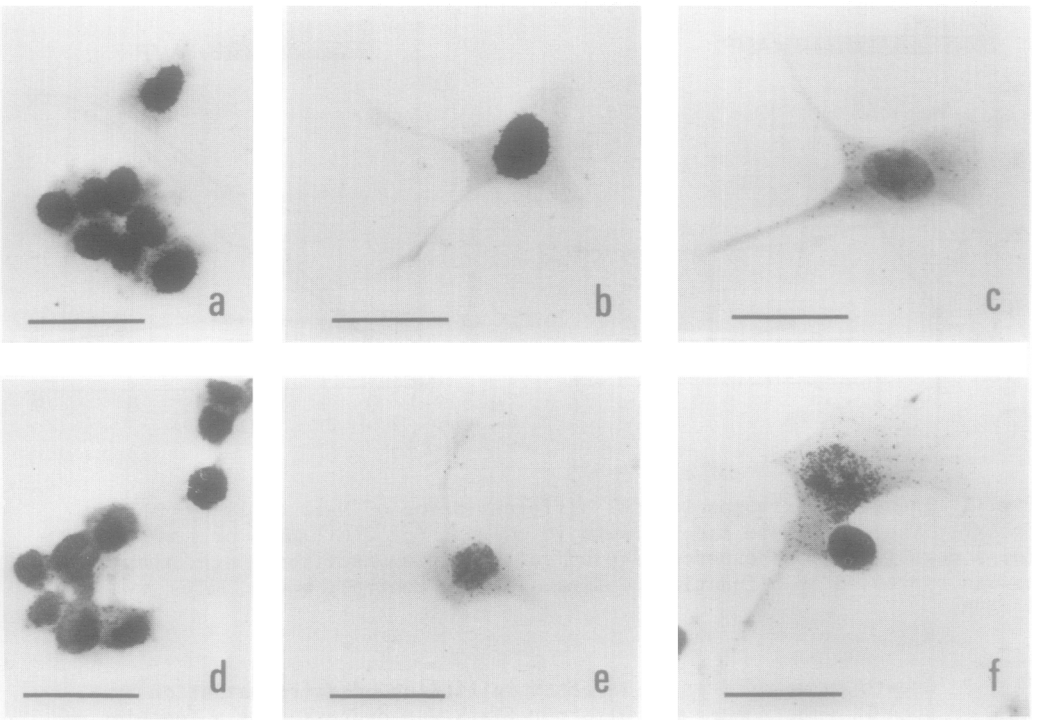

Fig. 3 Autoradiographs of N-18 cells differentiated by either MNNG or B_2cAMP. a) control, b) B_2cAMP-treated and c) MNNG-treated cells incorporating [^{3}H]-thymidine. d) control, e) B_2cAMP-treated and f) MNNG-treated cells incorporating [^{3}H]uridine. The lines in figures represent 40 nm.

similar to the addition of 10 μM of MNNG (unpublished). Thus, it is conceivable that inhibition of DNA synthesis is not necessarily related to neurite formation. After MNNG-treatment, incorporation of [^{3}H]uridine into the nuclei of the differentiated cells (Fig. 3f) was much greater than that either in the control cells (Fig. 3d) or in the differentiated cells induced by B_2cAMP (Fig. 3e). Incorporation of L-[^{14}C]leucine into the differentiated cells induced by MNNG was also much greater (data not shown). Therefore we studied the effect of inhibition of RNA and protein synthesis on the neurite formation induced by MNNG.

When the cells were incubated with 0.05 μg/ml Act. D or 5 μg/ml cycloheximide at the same time as the MNNG-treatment (at 0 h), at the initiation of neurite formation (after 24 h) or after neurite formation (after 48 h) as

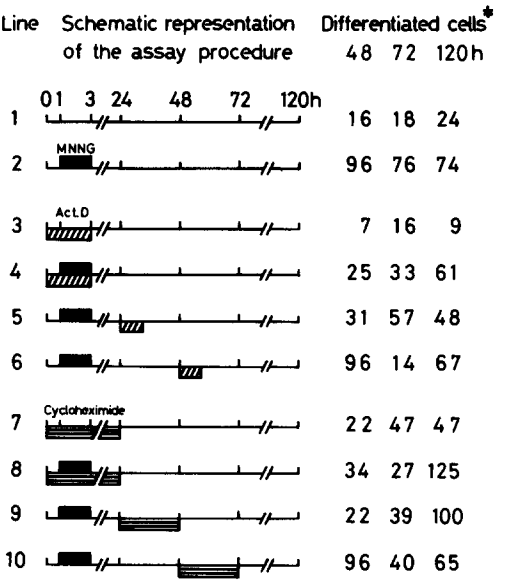

Fig. 4 Effect of actinomycin D and cycloheximide on neurite formation induced by MNNG in N-18 cells. The cells were treated with 10 μM MNNG for 2 h (■), 0.05 μg/ml Act. D for 3 h (▨) and/or 5 μg/ml cycloheximide for 24 h (▤) at various times as indicated. *, the number of cells bearing neurites longer than 40 nm among 500 cells counted at each time indicated.

shown in Fig. 4, extension of neurites was inhibited independently of the stage of drug-addition. However, the cells again began to form neurites after withdrawal of the inhibitors (Fig. 4). At this concentration of Act. D or cycloheximide, RNA or protein synthesis in N-18 cells was inhibited at 54% or 93% of the control, respectively. However, any cytotoxic effect was not observed for at least 3 days after each treatment. Hence, it is suggested that neurite formation induced by MNNG required RNA and protein synthesis. Meanwhile, it has been observed that the neurite extension induced by B_2cAMP is inhibited by cycloheximide (15) but is not inhibited by Act. D (our unpublished data).

The differentiation induced by MNNG is unique and irreversibly fixed, with the neurites never undergoing retraction even with the cessation of MNNG administration. RNA and protein synthesis were stimulated concomitantly with neurite formation which was temporarily inhibited by Act. D or cycloheximide but recovered after withdrawal of the inhibitors. Furthermore, when the monolayered cells bearing neurites induced by MNNG were trypsinized, replated and

cultivated, the cells re-extended neurites, as was reported before (18). Thus, in spite of the limited response of the cells to MNNG in induction of differentiation, neurite formation could not be merely an expression of non-specific cytotoxity.

A similar irreversible differentiation was also seen after treatment with alkyl derivatives of MNNG, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea or hydroxyurea (unpublished).

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