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A SHORT-TERM, SIMPLE METHOD FOR DETECTION OF N-NITROSOCOMPOUNDS PRODUCED FROM SODIUM NITRITE AND MORPHOLINE IN STOMACH

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SUMMARY: Syrian hamsters in the 11th or 12th day of pregnancy were given sodium nitrite and morpholine simultaneously by stomach tube. The embryonic cells were cultured for 72 h in normal MEM medium plus 10% fetal calf serum and then transferred into medium containing 8-azaguanine. After cultivation in the selection medium, number of 8 azaguanine-resistant colonies was scored. As the results, this oral concurrent transplacental application of sodium nitrite and morpholine can cause 8 azaguanine-resistant mutants on the cultured embryonic cells from mothers that received these chemicals. Nitrosomorpholine was only detected in stomach of animals treated with sodium nitrite and morpholine.

INTRODUCTION

One of the most important problems in cancer research is to be able to rapidly detect causative agents of cancer. Indeed thousands of chemical compounds in our environment are known to cause malignant neoplasma and somatic mutation in experimental animals and probably in human beings. For detection of these substances, many testing systems have been demonstrated using a range of organisms ranging from bacteria to experimental animals (1-6). It seems necessary that more rapid, simple and sensitive methods be developed for screening the hazardous chemicals, es-

Abbreviations: $NaNO_2$, Sodium nitrite; Mo, morpholine; 8AG, 8-azaguanine; N-Mo, N-nitrosomorpholine.

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pecially carcinogenic substances being produced from two or more non-carcinogenic or non-mutagenic chemicals in vivo. On the other hand, abundant evidence has been accumulated on the in vivo formation of carcinogenic N-nitrosocompounds by concurrent administration of nitrite and some appropriate amines (7-10). And some dialkaline nitrosamines have also been reported to produce tumors transplacentally (11, 12). But to detect these biologically produced carcinogenic products, only chemical carcinogenesis using rats, hamsters, and mice was reported (7-9, 13, 14). These facts led us to search for a way to establish simple, sensitive and rapid testing systems for these products.

MATERIALS AND METHODS

Details of experimental procedures have been described in a previous paper (15). Syrian golden hamsters which have been carefully bred in a clean barrier system room in our laboratory to avoid contaminations of microorganisms, in the 11th or 12th day of pregnancy were given 500 mg/kg morpholine (Mo), 500 mg/kg sodium nitrite ($NaNO_2$), 100 and 200 mg/kg N-nitrosomorpholine (N-Mo), and each 500 mg/kg Mo and $NaNO_2$ simultaneously with a stomach tube. Single application of 500 mg/kg $NaNO_2$ was over LD_{50}, and on the other hand, concurrent application of each 500 mg/kg Mo and $NaNO_2$ was around LD_{50}. The animals were given standard laboratory chow and water ad libitum for 24 h and the embryos were then excised. The entire fetuses were finely chopped up and the tissues were then digested with 0.25% trypsin (Difco Lab., Detroit, U.S.A.) at room temperature for 40 min. The primary culture of the trypsinized cells was initiated by seeding 5 to 10 x 10^6 cells into 10 ml of culture medium in Falcon plastic flasks (Falcon Plastic, Oxnad, Calif., U.S.A.). Cells were grown in Eagle's MEM medium supplement with 10% fetal calf serum (GIBCO, Grand Island, N.Y., U.S.A.) at 37°C under 5% CO_2 in air. For induction of 8 azaguanine (8AG)-resistant mutation, cells that had been cultured in standard MEM medium for 72 h from initiation of primary culture were gently trypsinized with 0.125% trypsin and then inoculated into medium containing 10 and 20 µg/ml of 8AG. The medium containing 8AG was changed every day for first 3 days, and then every 3 days. After a total cultivation period of 15 to 20 days, the dishes were fixed and stained with Giemsa (Merk, Darmastat, West Germany) and number of 8AG-resistant-mutant colonies was scored. Embryos from mothers that received Mo or $NaNO_2$, and those that were not treated served as controls. The embryos from the mothers that received N-Mo were also used a positive control. These were cultured and selected in the same way as those in the experimental group. To detect directly the production of N-Mo in stomach from $NaNO_2$ and Mo, hamsters were operated on under ether anesthesia. The sto-

Table 1. Induction of 8AG-resistant mutations and other biological data in Syrian golden hamster cells by concurrent application of $NaNO_2$ and Mo.

Chemicals & doses	Selection doses	Survival	Mutant colonies: Mutant observed /10^7 cells	Mutant colonies: Adjusted cells*	Induced colony	Induced ratio**	Revertant /10^7 cells	Transformation***
Control	AG10	100	9.05±2.75†	9.05	-	-	N.D.††	(-)
	AG20		1.96±1.91	1.96	-	-	N.D.	
Mo (500 mg)	AG10	84.5	19.52±3.66	23.03	13.98	x 2.5	0,2	(-)
	AG20		1.75±0.58	2.06	0.1	x 1.1	0,0,0	
$NaNO_2$ (500 mg)	AG10	85.5	59.64±20.65	69.78	60.73	x 7.7	2,1,2	(+)
	AG20		5.90±2.97	6.90	4.94	x 3.5	3,1,0	
$NaNO_2$ + Mo (500mg) (500 mg)	AG10	59.8	107.70±16.00	179.85	170.80	x18.9	0,3,1	(++)
	AG20		34.27±4.68	57.27	55.27	x29.2	3,3,0	
N-Mo (100 mg)	AG10	61.8	28.13±6.51	45.57	36.52	x 5.0	2,0,3	(+)
	AG20		5.98±1.51	9.68	7.72	x 4.9	0,0,0	
N-Mo (200 mg)	AG10	65.2	96.2±28.10	173.0	163.95	x18.1	N.D.	(+++)
	AG20		11.0±4.98	14.57	12.61	x 6.4	N.D.	

* The value is correct for mean survival rate of control.
** The ratio means the ratio between induced mutation frequency and spontaneous mutation frequency.
*** Morphological transformation.
† Results from four repeated experiments.
†† Not determined.

mach-duodenum junction was bound lightly with a thread and chemicals were applied by means of a stomach tube. Immediately after the administration of these chemicals, the lower part of the oesophagus was also tied, and 30 min or 1 h after treatment, the stomach contents were removed for detection of nitrosamines. Chemical analysis of nitrosamine was carried out by procedure of Scen et al. (1969) (16) with slight modification using thin layer and gas-liquid chromatography.

RESULTS AND DISCUSSION

The experimental results are shown in Table 1. Under the experimental condition of concurrently applying $NaNO_2$ and Mo, some toxicity to the cells (59.8% survival) was shown, but in contrast with a single administration of $NaNO_2$ or Mo no toxicity was shown (over 80% survival). Concurrent treatment with $NaNO_2$ and Mo caused a marked increase in 8AG-resistant mutants. Oral transplacental application of 500 mg/kg of each of these chemicals induced, respectively, almost 18- to 30-fold increase in mutants

Table 2. Nitrosamine formation in hamster stomach received $NaNO_2$ and Mo.

Chemicals	Hours	Product (mg/head)	Mean
$NaNO_2$ + Mo	30 min	2.3, 3.5, 2.7, 3.7*	3.05 ± 0.57
	1 h	2.6, 1.1, 1.2*	1.63 ± 0.68
$NaNO_2$	30 min	0.5, 0.2, 0.7**	0.46 ± 0.20
Mo	30 min	0, 0, 0	0
Diet		0, 0, 0, 0	0

* N-nitrosomorpholine
** Some nitrosocompounds

selected with 8AG at 10 and 20 μg/ml in comparison with that among control cells. Furthermore a single treatment with 200 mg/kg N-Mo also induced an increase of almost 6.4 to 18.1 times of resistant mutants. A few mutant colonies were observed on cells with single application of 500 mg/kg $NaNO_2$. No induced colonies was observed on the cells from mothers treated with Mo or those not treated at all. Few or no revertant cells were obtained in this study after 8AG-resistant cells were transferred into the HAT medium. These results clearly indicated that the product from $NaNO_2$ and Mo *in vivo* is transplacentally mutagenic. As shown in Table 2, N-Mo was only produced in stomach of animals treated concurrently with $NaNO_2$ and Mo. A small amount of nitrosamine (0.2-0.7 mg/animal), which is different from N-Mo in RF value, was detected in the case of treatment with a single application of $NaNO_2$ but no nitrosocompounds were detected in stomach treated with Mo or the diet (Clea Japan Inc., Tokyo) itself. The present

results provide the first evidence that concurrent transplacental application of $NaNO_2$ and amine (morpholine) can cause gene mutation in cultured embryonic cells from mothers that received these chemicals. This is also the first report of a very simple, sensitive, and rapid method for detection of the mutagens or carcinogens produced from two or more chemicals *in vivo*.

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