[CANCER RESEARCH 39, 1525–1528, May 1979]

Mediated Mutagenesis of Dimethylnitrosamine in *Neurospora crassa* by Various Metabolic Activation Systems

Wen-Zong Whong[1] and Tong-man Ong[2]

National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

Four metabolic activation systems (growth mediated, mycelium extract mediated, host mediated, and organ homogenate mediated) were used to study the mutagenic activity of dimethylnitrosamine (DMN) in both forward and reverse mutation systems in the *ad-3* (adenine-3) region of *Neurospora crassa*. DMN was not mutagenic in *Neurospora* if conidia alone were treated. It was highly mutagenic, however, if conidia were treated with this compound under any of the four activation systems. Quantitative differences in DMN-induced mutation frequencies were observed between *in vivo* (growth and host mediated) and *in vitro* (mycelium extract and organ homogenate mediated) activations. The efficiency of the conversion of DMN to a mutagenic metabolite by the organs of rats and mice appeared to be in a reversed order between the host-mediated (liver > kidney > lung) and the *in vitro* organ homogenate-mediated (lung > kidney > liver) assays. Inductions of reverse mutations in strain N23 indicated that DMN induces base-pair substitution in *N. crassa*.

INTRODUCTION

DMN[3] is highly carcinogenic in some species of rodents (6, 22). DMN mutagenesis in bacteria has been shown to be dependent upon metabolic activation (10, 15, 23). It has been suggested that products of the dealkylation of DMN by mixed-function oxidases (11, 12, 16) are responsible for the mutagenic and carcinogenic activities of DMN.

DMN was not mutagenic in *Neurospora* if the resting cells (conidia) were treated (13). It was mutagenic, however, if conidia were treated with DMN in the presence of S-9 of mouse liver homogenate and a NADPH-generating system (20). The studies reported here were conducted to determine whether DMN can be converted to metabolites mutagenic in *Neurospora* under different *in vivo* (growth- and host-mediated) and *in vitro* (mycelial extract- and organ homogenate-mediated) activation systems. The activation of DMN to mutagenic metabolites by liver, lung, and kidney of mouse and rat was also compared by *in vivo* and *in vitro* assays.

In these studies, both *ad-3* forward mutation and reverse mutation systems were used. In the forward mutation system, mutants resulting from point mutations as well as recessive chromosomal deletions can all be recovered at the *ad-3* region. Whether DMN induces any particular type of genetic alterations can be determined by the analysis of mutants isolated in the forward mutation experiments and by reverse mutation assays with specific tester strains.

MATERIALS AND METHODS

Strains of *Neurospora crassa*. A 2-component heterokaryon (H59) of *N. crassa* was used for forward mutation experiments. This strain was constructed by incorporating a *uvs-2* marker (21) into heterokaryon 12 (74-OR60-29A + 74-OR31-16A) of *N. crassa* by de Serres.[4] A homokaryotic *ad-3A* mutant (N23) was used for reversion assay. Strain N23, developed by Ong, is a presumptive base-pair substitution mutant. The genetic markers of heterokaryon 12 (H12) and homokaryon N23 have been described elsewhere (7, 18).

Chemicals. DMN was obtained from Aldrich Chemical Co., Inc., Milwaukee, Wis. NADP and glucose 6-phosphate were purchased from Calbiochem, San Diego, Calif. Stock solutions of DMN were prepared with sterile distilled water.

Animals. Six- to 7-week-old male Sprague-Dawley rats and 10-to 12-week-old male Swiss outbred albino mice were used for both the host-mediated and the organ homogenate-mediated activations. Both rats and mice were from Charles River Breeding Inc., Wilmington, Mass.

Vegetative Growth-mediated Activation. Various concentrations of DMN were added into each of 10 ml of melted complete agar media (4), and slants were then made. Approximately 10^6 conidia of mutant strain N23 of *N. crassa* were inoculated onto each slant and then incubated at 25° for 10 days. After incubation, conidia from vegetative cultures were harvested and plated. Methods used in the determination of the reversion frequency from adenine dependent to adenine independent and the survival of conidia were described previously (4).

Mycelium Extract-mediated Activation. Mycelia from a 6-day-old culture of mutant strain N23 of *N. crassa* in liquid-complete medium (4) were collected by filtration through cheese cloth. The mycelium mass (10 g) was homogenized in 10 ml of 0.1 M phosphate buffer (pH 7.4) with a glass bead shaker homogenizer (B. Braun, Melsungen, West Germany). The mycelial homogenates were centrifuged at 9000 × *g* for 20 min at 4°. The supernatants were saved as mycelial extract and used for activation. For the experiment, 2.2 ml of conidial suspension containing 1.5×10^8 conidia and 3.8 ml of the mycelium extract were mixed and treated with or without DMN in the presence of cofactors (NADP, 2.34 mg/ml; glucose 6-phosphate, 2.24 mg/ml; $MgCl_2 \cdot 6H_2O$, 1.59 mg/ml; KCl, 2.34 mg/ml); and O_2 at 35° for 4 hr. At the end of the treatment, conidia were washed twice with water by centrifugation. The

[1] Present address: Cancer Research Center, Thermo Electron Corporation, Waltham, Mass.

[2] Present address: National Institute for Occupational Safety and Health, Morgantown, W. Va. 26505. To whom requests for reprints should be addressed.

[3] The abbreviations used are: DMN, dimethylnitrosamine; S-9, 9000 × *g* supernatant; *ad-3*, adenine-3.

Received September 13, 1978; accepted February 8, 1979.

[4] F. J. de Serres, personal communication.

treated and untreated conidia were plated, and the reversion frequencies as well as survivals were determined.

Host-mediated Activation. A 0.3-ml conidial suspension containing 3×10^8 conidia and a 0.2-ml conidial suspension containing 10^8 conidia of H59 were injected into the tail vein of each rat and mouse, respectively. Immediately after injection, animals received 0.15 to 0.25 ml of DMN stock solutions i.m. to give a final concentration of 100 mg DMN per kg. In the control animals, similar amounts of sterile distilled water were injected. After 16 hr, organs (liver, lung, and kidney) from animals were removed and homogenized (at 0–4°) separately with a Teflon homogenizer. Conidia in each organ homogenate were recovered as a conidial pellet after 4 to 5 washes with ice-cold water by centrifugation. The recovered conidia were further treated with penicillin (200 units/ml) and streptomycin (0.2 mg/ml) for 30 min to eliminate possible bacterial contamination. The *ad-3* mutation frequency and survival of conidia were measured by the direct method (7, 8).

Organ Homogenate-mediated Activation. Organs (liver, lung, and kidney) isolated from several animals (3 for rats and 10 for mice) were rinsed with ice-cold 0.1 M phosphate buffer (pH 7.4) and then homogenized in 2 ml of phosphate buffer per g of organ with a Teflon homogenizer. Each homogenate was centrifuged at 9000 × *g* for 20 min. The supernatants (S-9) thus produced were used for activation. In each treatment tube, conidia (1.5×10^8) of strain N23, cofactors (same as used for mycelial extract-mediated activation), and 2 ml organ homogenate were incubated with or without DMN in a total volume of 6 ml. The incubation was carried out in the presence of O_2 at 37° for 3 hr. At the end of incubation, conidia were washed and plated. The reversion frequency and survival of conidia were determined by the methods described previously (4).

RESULTS

As shown in Table 1, the reversion frequency induced by DMN at the lowest concentration (6.25 mM) with the vegetative growth-mediated activation system was increased by about 300-fold over the spontaneous reversion frequency. With this activation system, the DMN-induced reversion frequency increased linearly as the concentrations of DMN increased. However, the decreases in survivals of conidia treated with increasing doses of DMN by this system were not prominent.

With mycelial extract-mediated activation, high reversion frequencies of conidia were observed following treatment with various concentrations of DMN (Table 2). There was no significant DMN-induced reversion when conidia were treated with a high concentration of DMN in the absence of mycelial extract. Only a slight decrease in survival was noted in DMN-treated conidia in the presence of the mycelial extract.

Table 3 shows the results of the mutagenic and lethal activities of DMN with a host-mediated assay using rats and mice as hosts. In mouse experiments, no increases in the mutation frequency over the *in vitro* spontaneous mutation frequency were found in conidia recovered from liver, kidney, or lung of the nontreated mice. In rats, however, the *ad-3* mutation fre-

Table 1

Mutagenic and killing activities of DMN in N23 of N. crassa with the growth-mediated activation system

Concentration of mutagen (mM)	% survival	No. of revertants	Revertants/10^7 survivors
0	100	5	0.6
6.25	86.7	1,222	183.1
12.5	76.9	4,545	814.5
25	52.3	11,130	3,044.8

Table 2

Mutagenic and killing activities of DMN in N23 of N. crassa with the mycelial extract-mediated activation system

Concentration of DMN (mM)	% survival	No. of revertants	Revertants/10^7 survivors
0	100	3	0.4
25	97.5	1,071	176.8
50	92.0	1,997	355.8
100	91.2	3,224	566.3
100[a]	101.5	5	0.7

[a] Without mycelial extract.

Table 3

Mutagenicity and cytotoxicity of DMN in H59 of N. crassa with the host-mediated assay

DMN (mg/kg)	Host	Organ	No. of viable conidia assayed (× 10^5)	No. of colonies (× 10^5)	% survival	No. of *ad-3* mutants	*ad-3* mutants/10^6 survivors
0			3.7	3.7	100	0	0
100[a]			7.5	7.4	98.1	0	0
0	Mouse	Liver[b]	16.9	16.9	100	0	0
		Lung					
		Kidney					
100	Mouse	Liver	39.7	5.4	13.6	444	822.2
100	Mouse	Lung	9.4	3.7	39.4	43	116.2
100	Mouse	Kidney	6.8	1.8	26.5	124	688.9
0	Rat	Liver[b]	14.6	14.6	100	14	9.6
		Lung					
		Kidney					
100	Rat	Liver	23.7	8.7	36.7	556	639.0
100	Rat	Lung	13.1	7.8	59.5	136	174.4
100	Rat	Kidney	2.3	1.3	57.0	48	369.2

[a] 100 μg/ml treatment solution.

[b] Conidia were pooled from 3 different organs. No difference in the mutation frequencies was found among the conidia recovered from different organs.

quency increased from 0 in the *in vitro* control to an average of $9.6/10^6$ surviving conidia recovered from liver, kidney, and lung of the nontreated rats. The increases are similar in conidia recovered from 3 different organs.

The *ad-3* mutation frequencies of conidia recovered from the livers of DMN-treated mice or rats were the highest, whereas those recovered from the lungs of either host were the lowest. The same relative efficiencies of the killing activity of DMN for conidia recovered from these 3 organs were also observed. In the dose-response study, DMN-induced mutation and killing of conidia recovered from mouse liver increased as the concentrations of DMN increased (Table 4).

Data on the DMN-induced reversion and killing of strain N23 with an *in vitro* activation system using S-9 of organ homogenates from rats and mice are presented in Table 5. With mouse S-9, the reversion induced by DMN was higher with lung than with kidney; however, it was higher with kidney than with liver. A similar pattern of activation was also found when S-9 of rat organ homogenates were used in the *in vitro* activation experiments. No obvious DMN-induced lethality in strain N23 was observed with S-9 from either rats or mice. With rat S-9, the induction of *ad-3* forward mutation by DMN in H59 (Table 6) displayed the same pattern as shown in the reverse mutation analysis (Table 5).

DISCUSSION

The growth-mediated activation system is a unique tool in *N. crassa* for the detection of the genetic effects of promutagens which are not mutagenic in the resting cells of *N. crassa*. This system has been used to demonstrate the mediated mutagenicity of a number of promutagens (17, 19). The high sensitivity of this method in displaying DMN mutagenesis in this study (Table 1) is further evidence that this activation is useful for screening environmental promutagens with *N. crassa*.

Brozdowicz and Huang (3) reported that the mycelia of *N. crassa* show no detectable microsomal activation of vinyl chloride in an *in vitro* assay. In this study, however, the activation of DMN was clearly shown both in the growth-mediated (Table 1) and with the mycelial extract-mediated (Table 2) activation systems in *N. crassa*. It is possible, yet unlikely, that the activation process for DMN (and possibly other compounds) occurring in the growing cells of *N. crassa* is by a metabolic activation system other than the mixed-function oxidase system.

The increase in the *ad-3* mutation frequency of conidia recovered from untreated rats is in agreement with observations made by Malling (14), who reported that a significant increase in the *ad-3* mutation frequency was found in conidia injected into the peritoneal cavity of rats for more than 24 hr. The increased mutation frequency at a shorter incubation time (16 hr), as observed in this report, is probably due to differences in the strains of *Neurospora* used. Strain H59, used in this study, is a repair deficient strain which appears to be more sensitive to some mutagenic agents than is strain H12 (2), the strain used by Malling (14). Increases in the mutation frequency were also found by Malling in conidia incubated for more than 24 hr in the peritoneal cavity of nontreated mice. The rate of increase in mutation frequency of conidia in mice, however, is much slower than that of conidia in rats. These data do not directly provide an explanation for our observed lack of an increase in mutation frequency in mice. Different mouse strains

Table 4

Mutagenic and killing activities of different DMN concentrations in H59 of N. crassa with the host-mediated activation system

Conidia were recovered from the liver of mice.

Concentration of DMN (mM)	No. of viable conidia assayed ($\times 10^5$)	No. of colonies ($\times 10^5$)	% survival	No. of *ad-3* mutants	*ad-3* mutants/10^6 survivors
0	67.1	67.1	100	3	0.4
50	33.9	18.6	54.8	427	229.6
100	39.8	7.6	19.1	600	789.5
200	64.2	7.9	12.3	1,046	1,324.1

Table 5

Mutagenic and killing activities of DMN in H59 of N. crassa with activation by rat S-9

S-9 from	Concentration of DMN (mM)	No. of viable conidia assayed ($\times 10^6$)	No. of colonies ($\times 10^5$)	% survival	No. of *ad-3* mutants	*ad-3* mutants/10^6 survivors
Liver[a]	0	23.8	23.8	100	0	0
Lung						
Kidney						
Liver	100	35.7	30.9	86.5	493	159.5
Lung	100	23.4	12.9	54.0	716	555.0
Kidney	100	24.2	16.4	67.8	432	263.4

[a] No difference in the spontaneous mutation frequencies of the conidia treated with different organ S-9 was found.

Table 6

Mutagenic and killing activities of DMN in N23 of N. crassa with the S-9-mediated activation system

Animal	S-9 from	Concentration of DMN (mM)	% survival	No. of revertants	Revertants/10^7 survivors
		100	100	5	0.7
Mouse	Liver	0	100	4	0.5
		25	101.4	21	4.2
		50	88.5	68	18.1
		100	94.1	119	26.1
	Lung	0	100	2	0.2
		25	97.0	154	29.4
		50	89.4	336	73.4
		100	96.6	1,001	192.2
	Kidney	0	100	1	0.1
		25	99.2	76	15.2
		50	98.6	200	39.5
		100	94.6	411	85.7
Rat	Liver	0	100	1	0.1
		25	89.4	95	12.9
		50	92.8	202	26.5
		100	87.4	501	70.1
	Lung	0	100	1	0.1
		25	84.7	4,179	592.3
		50	78.4	6,908	1,075.6
		100	82.5	8,426	1,255.8
	Kidney	0	100	2	0.2
		25	97.0	271	34.2
		50	99.0	766	93.6
		100	88.5	1,540	227.2

used in 2 separate studies might result in different responses.

With rat liver, the DMN-induced mutation frequency of conidia was higher with the *in vivo* host-mediated assay (Table 3) than with the *in vitro* homogenate activation system (Table 5). A comparison of the efficiency of DMN activation by the 3 organs (liver, lung, and kidney) with the host-mediated assay in both rats and mice showed that the liver was most efficient, followed by the kidney, with the lung having the lowest activating potency. Similar results were obtained by Pueyo[5] using *Salmonella* as indication organism. The highest DMN-activating potency of the liver is expected, inasmuch as the liver is the major organ for the metabolism of DMN in these animals. However, activation efficiencies were in reverse order among these organs of rats and mice with the *in vitro* S-9 activation system. The results obtained in the *in vitro* S-9-mediated assay are very similar in both forward (Table 5) and reverse (Table 6) mutation analyses. The nature of the difference between *in vivo* and *in vitro* activations is not understood. It might be due to differences in activation and detoxification mechanisms between *in vivo* and *in vitro* systems. It should be noted that the concentrations of DMN used in the *in vitro* test are much higher than those used in the *in vivo* test. No response was found in the *in vitro* studies when low concentration of this compound was used. A difference in the activation of promutagens between whole liver cells and the microsomal system has also been reported (9). It is reasonable to believe that the host-mediated assay may better reflect the organ specificity than does the S-9 (microsomal) activation system.

The relative DMN-activating potencies of S-9 from 3 different organs of mice as determined with *N. crassa* were completely opposite to those determined with a prokaryote (*Salmonella typhimurium*)[5] (23). The reason for this discrepancy is not known. However, differences in the responses of the prokaryotes and the eukaryotes to the mutagenicity of other mutagens (*e.g.*, ICR-170 and ICR-191) have been described previously (1, 5).

With these activation systems, DMN appeared to be a potent mutagen in *N. crassa* in both forward and reverse mutation analyses. N23 is an *ad-3A* presumptive base-pair substitution mutant. Induction of DMN-induced reverse mutations in N23 suggested that DMN induces base-pair substitution mutations in *N. crassa*. Genetic analysis of DMN-induced *ad-3* mutants, to determine whether DMN causes any other type of genetic alterations, is in progress.

REFERENCES

1. Ames, B. N., and Whitfield, H. J. Jr. Frameshift mutagenesis in salmonella. Cold Spring Harbor Symp. Quant. Biol., *31:* 221–225, 1966.
2. Brockman, H. E., Hung, C. Y., Ong, T. M., and de Serres, F. J. Mutagenic and killing activities of two alkylating agents and two nonalkylating agents in heterokaryons of *Neurospora crassa* homokaryotic for *UVS-2*$^+$ or *UVS-2*. Mutat. Res., *53:* 81, 1978.
3. Brozdowicz, B. Z., and Huang, P. C. Lack of mutagenicity of vinyl chloride in two strains of *Neurospora crassa*. Mutat. Res., *48:* 43–50, 1977.
4. Brusick, D. J. Reversion of acridine mustard-induced *ad-3* mutants of *Neurospora crassa*. Mutat. Res., *8:* 247–254, 1969.
5. Brusick, D. J., and Zeiger, E. A comparison of chemically induced reversion patterns of *Salmonella typhimurium* and *Saccharomyces cerevisiae* mutants, using *in vitro* plate tests. Mutat. Res., *14:* 271–275, 1972.
6. Clapp, N. K., Tyndall, R. L., and Otten, T. A. Differences in tumor types and organ susceptibility in BALB/c and RF mice following dimethylnitrosamine and diethylnitrosamine. Cancer Res., *31:* 196–198, 1971.
7. de Serres, F. J., and Malling, H. V. Measurement of recessive lethal damage over the entire genome and at two specific loci in the *ad-3* region of a two-component heterokaryon of *Neurospora crassa*, *In* A. Hollaender (ed.), Chemical Mutagens: Principles and Methods for Their Detection, Vol. 2, pp. 311–342. New York: Plenum Press, 1971.
8. de Serres, F. J., and Osterbind, R. S. Estimation of the relative frequencies of X-ray-induced viable and recessive lethal mutations in the *ad-3* region of *Neurospora crassa*. Genetics, *47:* 793–796, 1962.
9. Green, M. H. L., Bridges, B. A., Rogers, A. M., Horspool, G., Muriel, W. J., Bridges, J. W., and Fry, J. R. Mutagen screening by a simplified bacterial fluctuation test: use of microsomal preparations and whole liver cells for metabolic activation. Mutat. Res., *48:* 287–294, 1977.
10. Legator, M. S., and Malling, H. V. The host-mediated assay, a practical procedure for evaluating potential mutagenic agents in mammals. *In*: A. Hollaender (ed.), Chemical Mutagens: Principles and Methods for Their Detection. Vol. 2, pp. 569–589, New York: Plenum Press, 1971.
11. Magee, P. N., and Barnes, J. M. Carcinogenic nitroso compounds. Adv. Cancer Res., *10:* 163–246, 1967.
12. Magee, P. N., and Farber, E. Toxic liver injury and carcinogenesis, methylation of rat-liver nucleic acids by dimethylnitrosamine *in vivo*. Biochem. J., *83:* 114–124, 1962.
13. Malling, H. V. Mutagenicity of two potent carcinogens, dimethylnitrosamine and diethylnitrosamine in *Neurospora crassa*. Mutat. Res., *3:* 537–540, 1966.
14. Malling, H. V. Mutation induction in *Neurospora crassa* incubated in mice and rats. Mol. Gen. Genet., 116: 211–222, 1972.
15. Malling, H. V., and Frantz, C. N. *In vitro versus in vivo* metabolic activation of mutagens. Environ. Health, *6:* 71–82, 1973.
16. Miller, E. C., and Miller, J. A. The mutagenicity of chemical carcinogens: correlation, problems and interpretations. *In*: A. Hollaender (ed.), Chemical Mutagens, Principles and Methods for Their Detection, Vol. 1, pp. 83–119, New York: Plenum Press, 1971.
17. Ong, T. Mutagenic activities of aflatoxin B_1 and G_1 in *Neurospora crassa*. Mol. Gen. Genet., *111:* 159–170, 1971.
18. Ong, T. Use of the spot, plate and suspension test systems for the detection of the mutagenicity of environmental agents and chemical carcinogens in *Neurospora crassa*. Mutat. Res., *53:* 297–308, 1978.
19. Ong, T., and de Serres, F. J. Mutagenicity of chemical carcinogens in *Neurospora crassa*. Cancer Res., *32:* 1890–1893, 1972.
20. Ong, T., and Malling, H. V. Microsomal activation of dimethylnitrosamine to metabolites mutagenic in *Neurospora crassa*. Mutat. Res., *31:* 195–196, 1975.
21. Stadler, D. R., and Smith, D. A. A new mutation in neurospora for sensitivity of ultraviolet. Can. J. Genet. Cytol., *10:* 916–919, 1968.
22. Swann, P. E., and Magee, P. N. Nitrosamine-induced carcinogenesis. Biochem. J., *10:* 39–47, 1968.
23. Weekes, U., and Brusick, D. *In vitro* metabolic activation of chemical mutagens, II. The relationships among mutagen formation, metabolism, and carcinogenicity for dimethylnitrosamine and diethylnitrosamine in the livers, kidneys, and lungs of BALB/cJ, C_{57}BL/6J and RF/J mice. Mutat. Res., *31:* 175–183, 1975.

[5] C. Pueyo, personal communication.

Mediated Mutagenesis of Dimethylnitrosamine in *Neurospora crassa* by Various Metabolic Activation Systems

Wen-Zong Whong and Tong-man Ong

Cancer Res 1979;39:1525-1528.