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Germ-Free Status and Colon Tumor Induction by *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine[1] (38700)

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In recent years, a number of efforts were made to study the effect of the intestinal microflora in tumor development in order to understand the many aspects bearing on the mechanisms of carcinogenesis. Several studies imply that a lower yield of cancers was induced with certain chemicals in germfree animals compared to conventional controls (1–3). The carcinogens used in these tests were of a type necessitating enzymic metabolic activation (4, 5).

It is well documented that host factors such as immune system, both humoral and cellular, play a role in the ultimate expression of neoplasia (6). The immunological status may be altered by the microbial environment, and thus affect the incidence of tumors resulting from exposure to carcinogens (7).

N-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) is a direct-acting carcinogen not requiring enzymic activation, which induces a high yield of colon cancers in rats upon intrarectal instillation (8). This appeared a good model to evaluate the possible involvement of immune status in the causation of such neoplasm by comparing the response of germfree and conventional rats.

Materials and Methods. Weanling female CD Fischer germfree and conventional rats were obtained from Charles River Laboratories, Wilmington, Massachusetts, and fed *ad libitum* a steam-sterilized Purina Lab Chow, 5010 C. Germfree rats were maintained in the Trexler flexible plastic isolators and the conventional rats in a temperature and humidity controlled clean room (9). The microbial status of the germfree animals was checked at biweekly intervals and also at the termination of the experiment by routine procedures (10). All cultures from germfree animals and isolators were negative.

At 50 days of age, the germfree and conventional animals, except controls, were given weekly intrarectal injections of MNNG at a dose level of 1–3 mg/rat/week (1 mg first 3 wk, 2 mg next 6 wk, then 3 mg/wk) for 20 wk for a total dose of 48 mg/rat. Just before injection, MNNG was dissolved in 0.9% NaCl solution and sterilized by Millipore filtration. Controls were given an equal volume of 0.9% NaCl solution.

Both germfree and conventional rats were autopsied 30 wk after last injection. All organs, including the intestine were examined grossly and histologically for the number and type of tumors. Histopathologic evaluations were made on tissues fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin.

Results and Discussion. Table I summarizes the tumor incidence in germfree and conventional rats treated intrarectally with MNNG. There were no tumors in vehicle-treated germfree and conventional rats. MNNG induced multiple colonic tumors in all germfree and conventional rats.

Examination of other organs, including small intestine and cecum, of MNNG-treated rats showed no tumors. The terminal body weights of MNNG-treated germfree rats were significantly lower than conventional controls, perhaps an indication of the extent of tumor formation. There was no difference in body weights between vehicle-treated germfree and conventional rats. Frequently, there was passage of bloody stools in both germfree and conventional rats given MNNG. The diameter of tumors was 0.1–1.5 cm in germfree and conventional

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TABLE I. COLON TUMOR INDUCTION BY MNNG IN GERMFREE AND CONVENTIONAL RATS.

Status	Body weight	Animals with colon tumors	Colon tumors/animal	Adenocarcinoma/ animal	Adenoma/animal
Germfree	204 ± 3[a, b]	24/24	5.80 ± 0.58[b]	1.91 ± 0.30	3.90 ± 0.48[b]
Conventional	245 ± 4	23/23	3.00 ± 0.31	1.26 ± 0.17	1.80 ± 0.30

[a] Averages ± SEM, 23–24 animals per group.
[b] Significantly different from conventional control by Student's t test, $P < 0.01$.

rats, and metastases were not seen. The number of colon adenomas per rat induced by MNNG was nearly doubled in germfree rats compared to their conventional counterparts. Just as in the case of lung tumors in mice (11), the multiplicity of tumors in the large bowel is a good indicator of carcinogenicity (12). There was a slight, but not significant, increase in the adenocarcinomas in the germfree rats. It is possible that, provided experiments were prolonged, the incidence of adenocarcinomas would have markedly increased in germfree rats. Our findings with conventional rats were similar to those reported by Narisawa *et al.* (13).

In both germfree and conventional rats, the adenocarcinomas were dish-like tumors or sessile-polyp types, and were well differentiated, invading the serosa and muscular layer. Some tumors in both groups showed infiltration by lymphocytes and inflammatory cells.

It has been mentioned that the host factors such as immune competence (increased or decreased) are important to the ultimate expression of neoplastic growth (1, 6, 7). The germfree animal is immunologically as competent as the conventional animal (14). Although the latent period following exposure to an antigen is a little longer in germfree animals, the ultimate production of antibody is the same (14). The findings reported here indicate that the immune status may play a lesser role than heretofore assumed, inasmuch as the overall tumor yield was higher in the germfree group.

Lasting immunosuppression with antilymphocytic serum had only slight effects in carcinogenesis in liver, small and large intestine, although it did lead to the induction of a kind of tumor absent in control rats (15).[3] Immunosuppression and carcinogenesis was the subject of a recent review (16).

Perhaps the microflora in the conventional rats acted by absorption and/or chemical reaction with the carcinogen and thus led to lower effective levels at the target. Reductive elimination of the nitroso group with consequent deactivation has been documented (17). Also, the absorption of a variety of compounds was found to be increased in the germfree animals (18). It is probable that pharmacodynamics and metabolism account for major effects in the action of carcinogens such as MNNG. Many aspects of this problem require further study.

Summary. Female germfree and conventional rats of 50 days of age were injected intrarectally with MNNG for 20 wk (total dose, 48 mg/rat) and autopsied 30 wk after last injection. The colon adenomas induced by MNNG were doubled in germfree rats compared to conventional animals. However, germfree status had no effect on the incidence of adenocarcinomas. It is concluded that pharmacodynamics and metabolism of carcinogen play a role greater than the immune status of the animal in the action of carcinogens such as MNNG.

1. Roe, F. J. C., and Grant, G. A., Int. J. Cancer **6**, 133 (1970).
2. Burnstein, N. A., McIntire, K. R., and Allison, A. C., J. Nat. Cancer Inst. **44**, 212 (1970).
3. Reddy, B. S., Weisburger, J. H., Narisawa, T., and Wynder, E. L., Cancer Res., **34**, 2368 (1974).
4. Miller, J. A., and Miller, E. C., J. Nat. Cancer Inst. **47**, 5 (1971).
5. Weisburger, J. H., *in* "Cancer Medicine" (J. F.

[3] Kroes, R., Berkvens, J. M., and Weisburger, J. H. Unpublished observations, 1974.

Holland and E. Frei, eds.) p. 45, Lea and Febiger, Philadelphia (1973).
6. National Cancer Institute, Monograph 35, Conference on Immunology of Carcinogenesis, DHEW Publication No. (NIH) 72-334, National Cancer Institute, Bethesda, MD (1972).
7. Walburg, H. E., Jr., *in* "Germfree Research" (J. Heneghan, ed.) p. 115, Academic Press, New York (1973).
8. Narisawa, T., Magadia, N. E., Weisburger, J. H., and Wynder, E. L. J. Nat. Cancer Inst. **53**, 1093 (1974).
9. Reddy, B. S., and Wostmann, B. S., Arch. Biochem. Biophys. **113**, 609 (1966).
10. Wagner, M., Ann. N.Y. Acad. Sci. **78**, 89 (1959).
11. Shimkin, M. B., Weisburger, J. H., Weisburger, E. K., Gubareff, N., and Suntzeff, V., J. Nat. Cancer Inst. **36**, 915 (1966).
12. Ward, J. M., Yamamoto, R. S., Benjamin, T., Brown, C. A., and Weisburger, J. H., J. Amer. Vet. Med. Ass. **164**, 729 (1974).
13. Narisawa, T., Sato, T., Hayakawa, M., Sakuma, A., and Nakaro, H., Gann **62**, 231 (1971).
14. Pollard, M., *in* "Environment and Cancer", p. 394, The Williams and Wilkins Co., Baltimore, MD (1972).
15. Weisburger, J. H., Madison, R. M., Ward, J. M., Viguera, C., and Weisburger, J. H., J. Natl. Cancer Inst., in press (1975).
16. Kripke, M. L., and Borsos, T., Israel J. Med. Sci. **10**, 888 (1974).
17. Kawachi, T., Kogure, K., Kamijo, Y., and Sugimura, T., Biochim. Biophys. Acta **222**, 409 (1970).
18. Reddy, B. S., Fed. Proc. **30**, 1815 (1971).

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