PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 162, 410–415 (1979)

The Methanol Extraction Residue of *Bacillus Calmette–Guerin* Protects against 7,12-Dimethylbenz(α)anthracene-Induced Rat Mammary Carcinoma (40693)[1]

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The carcinogen, 7,12-dimethylbenz(α)-anthracene (DMBA), induces mammary adenocarcinoma in Sprague–Dawley female rats when administered by stomach tube. Tumor incidence depended on the quantity and type of dietary fat (1–4). Rats maintained on diets containing high levels of polyunsaturated fat were more susceptible to tumor development than rats maintained on diets containing similar levels of saturated fat. Rats maintained on low-fat diets were least susceptible as measured by tumor incidence, latent period, number of tumors per tumor-bearing rat, and rate of tumor growth.

Other studies have indicated that tumor incidence can be reduced by treating rats either before or after exposure to DMBA. For example, Takeda *et al.* (5) observed an increase in the latent period and a reduction in tumor incidence if diets fed after DMBA were supplemented with arginine. Similar results were observed when antioxidants were added to diets (6–8).

More recently, Weislow *et al.* (9) reported that rats were protected against DMBA-induced mammary carcinoma by immunization with a mouse xenotropic type C virus, before exposure to DMBA. The mouse virus used in their studies acted as a nonspecific immunostimulant. Spleen lymphocytes were much more responsive to PHA when taken from immunized rats compared to nonimmunized controls. Furthermore, DMBA-induced lymphocyte anergy was prevented by immunization.

The methanol extraction residue (MER) of *Bacillus* Calmette–Guerin is a nonspecific immunostimulant which has been used in both prophylactic (10–12) and therapeutic (13, 14) animal tumor models. Since MER potentiates the immune response to microbial pathogens, antigens, and tumor cells, experiments were designed to determine if MER provided prophylactic or therapeutic benefit in Sprague–Dawley female rats exposed to DMBA. Rats maintained on different fat diets were used to determine if protection was influenced by dietary fat.

Materials and methods. Animals. Sprague–Dawley female, albino, outbred rats were used for these studies. Weanlings (21 days old) were obtained from the Charles Rivers Colony (Willmington, Mass.) or from Charles Rivers Breeders maintained at the University of Oklahoma Health Sciences Center (Oklahoma City, Okla.). Rats were maintained (*ad libitum*) on one of three diets (ICN Pharmaceutical, Inc., Life Sciences Group, Cleveland, Ohio) outlined in Table I. Based on calories per gram of diet and the average amount of food consumed per day, it was estimated that the daily caloric intake was similar for all groups. Growth curves indicated no differences between rats on the high- and low-fat diets.

Exposure to DMBA. Rats which were exposed to the carcinogen were given 10 mg of DMBA (Sigma Chemical Co., St. Louis, Mo.) in 1 ml of stripped corn oil via stomach tube at 50 days of age. Animals were weighed and examined for tumors on a weekly basis. In experiments where rats were immunized with MER (National Cancer Institute, Silver Springs, Md.) before exposure to DMBA, rats were killed when the experiment was terminated (32 to 34 weeks after DMBA). Tumors were removed and certified histologically as

[1] This project was supported in part by BRSG Grant S07 RR05538 awarded by the Biomedical Research Support Grant Program, Division of Research Resources, National Institutes of Health.

0037-9727/79/110410-06$01.00/0

TABLE I. CONTENT OF VARIOUS DIETS

	Polyunsaturated fat diet (g)	Saturated fat diet (g)	Low-fat diet (g)
Casein	23	23	23
Fat	20[a]	20[b]	2[c]
Sucrose	46	46	64
Salt mixture[d]	4	4	4
Alphacel (nonnutrient bulk)	6	6	6
Vitamin mixture[e]	1	1	1

[a] Stripped corn oil.
[b] Stripped, hydrogenated coconut oil, 18% + linoleic acid, 2%.
[c] Linoleic acid.
[d] Salt mixture (15).
[e] Vitamin mixture (vitamin fortification mixture of ICN Life Sciences Co., Cleveland, Ohio 44128).

mammary adenocarcinoma. Mammary adenocarcinoma did not appear in rats which were not exposed to DMBA. Experiments done to determine the therapeutic value of MER are still in progress. Observations have been made for 28 weeks after exposure to DMBA.

Immunization with MER. MER, suspended in vehicle (0.5 mg/0.2 ml) was injected intraperitoneally (0.5 mg/injection, all immunized rats were given two injections). Control animals were given 0.2 ml of vehicle, via the same route, which contained the following per milliliter of water: 9 mg sodium chloride, 5 mg sodium carboxymethylcellulose, 0.004 ml of polysorbate, and 0.009 ml of benzyl alcohol. Rats given MER before exposure to DMBA were immunized at 28 and 35 days of age. In therapy studies, rats were placed on the polyunsaturated fat diet at time of weaning and maintained on these diets for either 3 or 4 weeks after DMBA. At each of these times, rats were assigned to one of four groups. One group was maintained on the polyunsaturated fat diet and not treated. A second group was maintained on the polyunsaturated fat diet and treated with MER. MER was given either at 3 and 5 weeks after DMBA or at 4 and 6 weeks after DMBA. A third group was placed on the low-fat diet. A fourth group was placed on the low-fat diet and treated with MER either at 3 and 5 weeks after DMBA or at 4 and 6 weeks after DMBA.

Statistical analysis. The χ^2 statistics were used to compare final tumor incidence between immunized and nonimmunized rats in each dietary group, and 28-week incidence between therapy groups. A median test was performed to compare size of tumor in tumor-bearing rats, between immunized and nonimmunized rats.

Results. Tumor incidence in rats immunized with MER or treated with vehicle before exposure to DMBA is shown in Fig. 1. Figure 1A shows the protective effect of MER in rats maintained on the polyunsaturated fat diet. Rats treated with MER had a longer latent period and a significant reduction in tumor incidence ($P \leq 0.01$) compared to vehicle-treated controls. Figure 1B illustrates similar data for rats maintained on the saturated fat diet. Immunized rats had a significantly lower tumor incidence ($P \leq 0.05$) compared to nonimmunized rats. Data for rats maintained on the low-fat diet is shown in Fig. 1C. MER-treated rats had a longer latent period and a significantly lower tumor incidence ($P \leq 0.01$) compared to the nonimmunized group.

Since MER-treated rats had longer latent periods compared to nonimmunized controls, it appeared that MER provided benefit to all rats, although some rats were protected only temporarily. Further evidence of the prophylactic benefit observed with MER was obtained by evaluating tumor mass in rats with tumor. The median tumor mass of all rats, independent of diet and treatment was 40 g. The proportion of rats with small tumor masses (less than 40 g) was significantly higher in MER-treated rats compared to vehicle-treated controls.

Results from therapy studies are shown in Fig. 2. Since results were similar when therapy was initiated at 3 or at 4 weeks, these data were combined. Results indicated that MER provided no protection when given to rats on the polyunsaturated fat diet. Rats placed on the low-fat diet had a significantly lower tumor incidence ($P \leq 0.01$) compared to untreated rats maintained on the polyunsaturated fat diet. Further therapeutic benefit was observed when rats on the low-fat diet were treated with MER. This group had significantly fewer tumors ($P \leq 0.01$) compared to rats placed on the low-fat diet. Preliminary data indicate that these groups may also be different based on tumor mass and number of tumors.

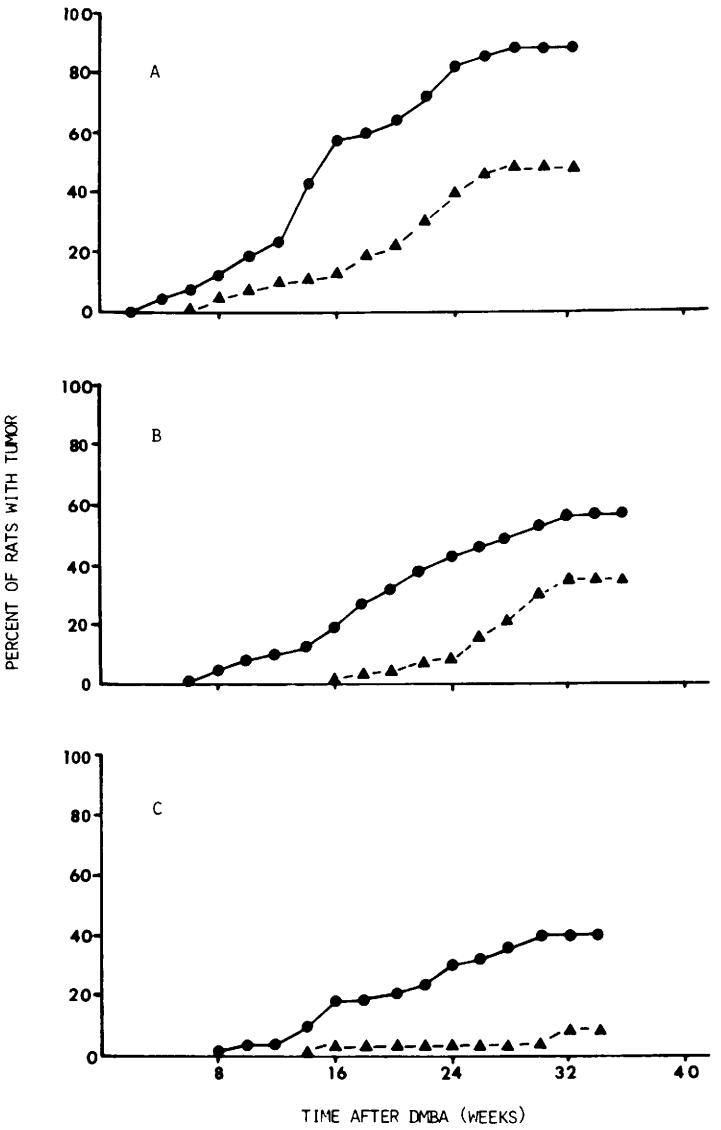

FIG. 1. Rats were maintained on (A) polyunsaturated fat diet, (B) saturated fat diet, or (C) low-fat diet and immunized with MER or treated with vehicle at 28 and 35 days of age. DMBA was given on Day 50. Tumor incidence is shown as a function of time after DMBA. MER-treated rats (▲) had a significantly low tumor incidence compared to rats treated with vehicle (●). Groups consisted of 26 to 30 rats.

Discussion. Results from these studies indicated that MER provided both prophylactic and therapeutic benefit in this tumor–host system. Prophylactic benefit was measured by a reduction in tumor incidence, an increase in the latent period, and a reduction in tumor mass. Further protection may depend on the dose of MER, route of injection, or schedule and frequency of immunizations.

A recent report by Weislow *et al.* (9) indicates that Sprague–Dawley female rats were protected against DMBA-induced mammary adenocarcinoma when immunized with a mouse xenotropic type C virus 7 days before DMBA. Immunization was not effective when given 20 or 30 days before DMBA. It is not possible to determine from their data if this difference in response was due to age

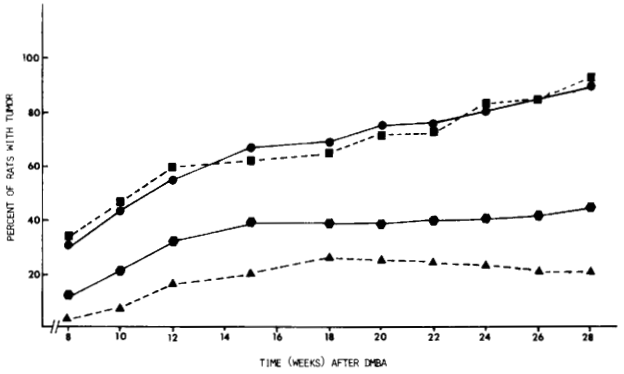

FIG. 2. Rats were maintained on the polyunsaturated fat diet from time of weaning, exposed to DMBA on Day 50, and maintained on the polyunsaturated fat diet for either 3 or 4 weeks. At those times, some rats were placed on low-fat diet or placed on low-fat diet and treated with MER. Other rats were maintained on the polyunsaturated fat diet or maintained on this diet and treated with MER. Rats placed on low-fat diet (●) had a significant reduction in tumor incidence compared to rats maintained on the polyunsaturated fat diet (●). Rats placed on low-fat diet and treated with MER (▲) had a significant reduction in tumor incidence compared to rats placed on low-fat diet (●). MER provided no benefit when given to rats on the polyunsaturated fat diet (■). Groups consisted of 26 to 58 rats.

of rats at the time of immunization or the interval between immunization and exposure to DMBA.

In our study the prophylactic benefit of MER was observed in all dietary groups, but therapeutic effect was limited to rats on the low-fat diet. While these experiments are still in progress, preliminary data indicate that MER may increase tumor mass when given to rats on the polyunsaturated diet. Alternatively, rats on the low-fat diet, treated with MER, has smaller tumor masses compared to rats on the low-fat diet only.

The observation that tumors could be prevented, but could not be treated in rats on the polyunsaturated fat diet, may be due to inherent differences in the experimental designs. Rats treated prophylactically were 4 to 5 weeks old when immunized, while rats given therapy were 10 to 11 weeks old when immunized. The former group had been on the polyunsaturated fat diet for 7 to 14 days at time of treatment, while the latter group had been on this diet for 50 to 57 days. In addition, prophylaxis was given before DMBA, while therapy was given after DMBA when early tumors were probably present in all rats. DMBA, like other carcinogens, suppresses the immune response (16, 17).

Observations by Carroll and Khor (3) indicate that all rats maintained on the polyunsaturated fat diet had tumors within 3 to 4 weeks after exposure to DMBA. Prolonged feeding of the polyunsaturated fat diet may alter functional properties of lymphoid cells. For example, Stuart *et al.* (18) reported that the functional capacity of the reticuloendothelial system was depressed by simple lipid complexes. Berken and Benacerraf (19) also reported that lipids depressed phagocytosis by the reticuloendothelial system. Since macrophages probably play a major role in regulation of antigen-induced proliferation (20), depression of other lymphoid cells may be mediated through macrophages.

If function of lymphoid cells was inhibited in rats on the polyunsaturated diet, it may have been restored when rats were placed on the low fat. However, the therapeutic benefit associated with the low-fat diet does not necessarily implicate immunological mechanisms. Tumors which developed in rats on the polyunsaturated fat diet may have been selected for their ability to grow in the microenvironment furnished by this diet. Changing to the low-fat diet may select for tumors with different nutritional requirements, analogous to surgical ablation of hormone-producing glands in patients with hormone-dependent breast tumors (21, 22).

However, the observation that MER pro-

duced additional therapeutic benefit when given to rats on the low-fat diet, suggests that these tumors were susceptible to immunological control. Other studies have shown that MER increases the number and distribution of lymphoid cells (23, 24). The uptake of collodial substrates is markedly potentiated in mice pretreated with MER (24). The uptake, degradation, and elimination of foreign protein is also enhanced by MER treatment.

Both intraperitoneal and intravenous injection of MER elicited pronounced elevation in hydrolytic lysozomal enzyme activity of mouse peritoneal macrophages for at least 30 days after treatment (25). Such macrophages also exhibited a greatly amplified ability to phagocytize pathogenic bacteria *in vitro*, notably *staphylococci*, and to inhibit their intracellular replication (26).

If macrophages play a pivotal role in directing lymphoid responses against tumor cells, inhibition of macrophage function via serum constituents (27), which are determined by diets, may jeopardize host immune responses or minimize their benefit.

Summary. Sprague–Dawley female rats were placed on a low-fat diet, a high saturated fat diet, or a high polyunsaturated fat diet at time of weaning (21 days of age). Half of the rats in each dietary group were given an intraperitoneal injection of MER (0.5 mg) on Day 28 and again on Day 35. On Day 50, all rats were given 10 mg of DMBA in 1 ml of stripped corn oil via stomach tube. Tumor incidence was determined weekly by palpation and confirmed by histological analysis upon completion of the experiment. In each dietary group, rats immunized with MER had a lower tumor incidence, a longer latent period, and smaller tumors compared to nonimmunized rats. In other experiments, rats were placed on the high polyunsaturated fat diet at time of weaning, exposed to DMBA on Day 50, and maintained on this diet for either 3 or 4 weeks after DMBA. At those times, some rats were placed on the low-fat diet while other rats were maintained on the high polyunsaturated fat diet. Half of the rats from each group were treated with MER. Results indicate that the low-fat diet provided significant therapeutic benefit. Further benefit was observed if these rats had been treated with MER. However, MER was not beneficial if given to rats maintained on the high polyunsaturated fat diet.

1. Carroll, K. K., and Khor, H. T., Cancer Res. **30,** 2260 (1970).
2. Carroll, K. K., and Khor, H. T., Lipids **6,** 415 (1971).
3. Carroll, K. K., and Khor, H. T., *in* "Progress in Biochemical Pharmacology" (K. K. Carroll, ed.), Vol. 10, p. 308. Karger Press, Basel (1975).
4. Gammal, E. B., Carroll, K. K., and Plunkett, E. R., Cancer Res. **27,** 1737 (1967).
5. Takenda, Y., Tominaga, T., Tei, N., Kitamura, M., Taga, S., Murase, J., Taquchi, T., and Miwatani, T., Cancer Res. **35,** 2390 (1975).
6. King, M. M., Bailey, D. M., Gibson, D. D., Pitha, J. V., and McCay, P. B., J. Nat. Cancer Inst., in press.
7. Wattenberg, L. W., Digest. Dis. **19,** 947 (1974).
8. Wattenberg, L. W., *in* "Advances in Cancer Research" (G. Klein and S. Weinhouse, eds.), Vol. 26, p. 197. Academic Press, New York (1978).
9. Weislow, O. S., Allen, P. T., Shepherd, R. E., Twardzik, D. R., Fowler, A. K., and Hellman, A., J. Nat. Cancer Inst. **61,** 123 (1978).
10. Wainberg, M. A., Deutsch, V., and Weiss, D. W., Brit. J. Cancer **34,** 500 (1976).
11. Weiss, D. W., Bonhag, R. S., and DeOme, K. B., Nature (London) **190,** 889 (1961).
12. Weiss, D. W., Bonhag, R. S., and Leslie, P., J. Exp. Med. **124,** 1039 (1966).
13. Haran-Gera, N., and Weiss, D., J. Nat. Cancer Inst. **50,** 229 (1973).
14. Weiss, D. W., Faulkin, L. J., Jr., and DeOme, K. B., Cancer Res. **24,** 732 (1964).
15. Hayward, J., *in* "Recent Studies in Cancer Research" (J. Hayward, ed.), Vol. 24, p. 140. Springer-Verlag, New York (1970).
16. Szakal, A. K., and Hanna, M. G., *in* "N.C.I. Monograph, Conference on Immunology of Carcinogenesis" (John L. Gilbert, ed.), Vol. 35, p. 173. NCI Press, Bethesda, Md. (1972).
17. Szakal, A. K., And Hanna, M. G., Cancer Res. **34,** 138 (1974).
18. Stuart, A. E., Biozzi, G., Stiffel, C., Halpern, B. N., and Mouton, D., Brit. J. Exp. Pathol. **41,** 599 (1960).
19. Berken, A., and Benacerraf, B., Proc. Soc. Exp. Biol. Med. **128,** 793 (1968).
20. Levy, M. H., and Wheelock, E. F., Advan. Cancer Res. **20,** 131 (1974).
21. McGuire, W. L., Chamness, G. C., Costlow, M. E., and Shepherd, R. E., Metabolism **23,** 75 (1974).
22. Mobbs, B. G., J. Endocrinol. **44,** 463 (1969).
23. Gery, I., Baer, A., Stupp, Y., and Weiss, D. W., *in* "Immunological Parameters of Host–Tumor Relationships" (D. W. Weiss, ed.), Vol. 3, p. 170. Academic Press, New York (1974).
24. Steinkuller, C. B., Krigbaum, L. G., and Weiss, D. W., Immunology **16,** 255 (1969).
25. Yagel, S., Gallily, R., and Weiss, D. W., Cell. Im-

munol. **19,** 381 (1975).
26. Gallily, R., Yagel, S., and Weiss, D. W., *in* "Advances in Experimental Medicine and Biology" (S. M. Reichard, M. R. Escobar, and H. Friedman, eds.), Vol. 73, Part A, Reticuloendothelial System in Health and Disease, p. 351. Plenum Press, New York (1976).
27. Rhodes, J., Bishop, M., and Benfield, J., Science **203,** 179 (1979).

Received May 31, 1979. P.S.E.B.M. 1979, Vol. 162.