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STUDIES ON THE HETEROLOGOUS IMMUNOGENICITY OF A METHANOL-INSOLUBLE FRACTION OF ATTENUATED TUBERCLE BACILLI (BCG)

II. Protection against Tumor Isografts*

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Tubercle bacilli and a variety of crude fractions derived from them have long been known to be capable of activating the immunological reactivity of higher animals. The homologous and heterologous antimicrobial immunogenicity of one such fraction, a methanol-insoluble residue of phenol-killed, acetone-washed bacilli of the BCG strain, has been described in previous communications (1, 2), and it was pointed out (2) that this material (which will be referred to as MER in this and succeeding papers) possesses a number of distinct advantages over living BCG and other hitherto described fractions as a heterologous immunogen: MER is nonliving, very stable to chemical and physical manipulations, and predictably effective under defined experimental conditions; its homologous and heterologous immunogenicity is at least as great as, and in many instances greater than, that of living BCG; it is not toxic systemically in quantities several times larger than the optimally immunogenic amounts, and it is well tolerated by tissues locally; and it induces only a low degree of tuberculin hypersensitivity.

The mode of action of the MER remains to be determined, but it has already been shown to exert profound effects on the anatomy and physiology of the reticuloendothelial system, and on immunological responsiveness to specific antigenic stimuli (3). These broad immunological propensities of the fraction encouraged an investigation of its possible efficacy in heightening the resistance of animals to neoplastic cells. This investigation was based on the following premise:

The morphology and physiology of neoplastic cells differ, sometimes markedly, from those of corresponding normal tissue. Such differences in appearance and behavior are not unlikely to reflect changes in the structure and arrangement of macro-

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molecular cell components, and it is well recognized that even relatively minor changes in macromolecular composition or organization can result in new antigenic properties. It is not unreasonable to expect, therefore, that neoplastic cells may carry antigenic determinants acquired in the course of the neoplastic transformation, and not possessed by the normal cells of the individual.

Support for this expectation comes from the repeated demonstrations in recent years that many of the neoplasms studied in the laboratory do, indeed, have antigenic characteristics specific to the neoplastic condition or specifically associated with the presence of oncogenic viruses. This has been shown for tumors induced experimentally by chemical carcinogens (4–9), developing at the site of implantation of cellophane films (10), or initiated by the polyoma (11, 12), Rous sarcoma (13), and certain murine leukemogenic viruses (14–19). The tumor-associated antigenicity of spontaneous mammary carcinomas of mice, first indicated by Hirsch et al. (20), has now also been established (21–29).

The failure of an animal to reject its own, but antigenically foreign, tumor cells would, therefore, seem to demand an immunological explanation. Such an explanation has been advanced by several investigators (e.g. reference 30) who suggest that a major function of the homograft reaction may be the eradication of neoplastic mutants, and that progressive neoplasia occurs, accordingly, only in animals suffering a preexisting immunological disability. This hypothesis is supported by observations which point to an inverse association between susceptibility to neoplastic disease and immunological ability as manifested by antibody synthetic activity (31, 32), allergic disease (33), homograft reactivity (34), and reticuloendothelial phagocytic function (35, 36).

It would follow from this consideration that agents which act as powerful stimulators of immunological reactivity might also enable an animal to cope more sucessfully, by immunological means, with its indigenous neoplastic cells.

When the present investigation was begun, it had already been reported by Old et al. that infection with living BCG increased the resistance of mice to transplants of several nonisogenic, laboratory-adapted cultures of neoplastic cells (37). This activity of BCG was subsequently confirmed in isogenic host-tumor systems by Old et al. (38) and Weiss et al. (39). Preliminary studies with the MER fraction revealed it to be generally more effective than living BCG in evoking a degree of heightened resistance to isogenic tumors (39). The present communication describes the results of further studies of the ability of the MER fraction to heighten host resistance against isografts of spontaneous and induced cancers of mice.

Materials and Methods

Methanol Extraction Residue and BCG.—The preparation of cultures of living BCG and of the MER fraction has been described previously (2).

Animals.—The experiments were carried out with young adult (10- to 15-wk-old) inbred mice of the BALB/c, C3H, C3Hf, RIII, A, DBA/2, and C57Bl strains, derived from the breeding colony of the Cancer Research Genetics Laboratory of the University of California

in Berkeley. Animals were distributed randomly among the several experimental groups in each experiment and were maintained on a diet of standard mouse pellets and water ad lib.

It is crucial that animals employed in experiments designed to detect immunological reactions specifically directed at tumor-associated antigens be isogenic with the tumor donors. The operative isogenicity[1] of the mouse strains used in this study is evident from two categories of observation: intrastrain transplants of ovarian, pituitary, adrenal, and mammary tissues are performed continually at the Cancer Research Genetics Laboratory, and these tissues are well accepted upon second as well as upon first exposure; and, reciprocal first and second set skin grafts exchanged between randomly selected pairs of control animals of the same sex from each strain throughout the course of this study provided no indication of isoantigenic contamination of the strains.

Tumors.—The tumors used had arisen spontaneously in isogenic animals at the Cancer Research Genetics Laboratory or were induced by exposure to chemical carcinogens. The tumors were identified by histological examination, carried out by Dr. K. B. DeOme of the Laboratory, and were maintained by serial passage in isogenic tumor bank hosts.

Tumor Implantation.—Tumors were removed surgically from the tumor bank hosts immediately before they were to be used. Necrotic and sinusoidal tissue was trimmed away, and the remaining neoplastic tissue was cut with a razor blade into fragments of either 0.5 to 1.0 or 0.05 to 0.1 mm^3. In order to prevent drying of the tumor tissue during manipulation, the tissue was flooded with tissue culture medium 199. The smaller pieces were selected for appropriate size by floating them over an ocular micrometer and making the selection under a dissecting microscope. Although considerable care was thus taken to assure that all the animals in any one experiment were challenged with tumor implants of nearly equal size, it is not certain that the number of living cells introduced at each challenge site was roughly equal. This is because only a small proportion of the cells in a tumor fragment or, for that matter, in a suspension of tumor cells, is likely to survive the implantation procedure, and it is probable that this proportion varies from aliquot to aliquot and from one animal to the next. On the other hand, such differences could be expected to be distributed equally among animals of the various treatment groups in any one experiment. It has also been shown (40) that variation in measured tumor (mammary carcinoma) implant volume over a 2-log range, 0.06 to 6.0 mm^3, had no significant effect on the times of appearance of palpable tumors or on their subsequent growth rates.

Implantation was either with the larger tumor pieces inserted subcutaneously along the flanks of the animals by means of a trocar, or with the smaller pieces placed surgically into the inguinal mammary fat pads. The latter technique has been described elsewhere (22).

The animals were observed periodically after tumor implantation for development of palpable tumors. The growth of the tumors was followed by periodic caliper measurements of the two diameters evenly bisecting the neoplasms at right angles to each other. The same observer always read the tumors in any one experiment, to avoid individual variations in assessment of tumor size.

Growth of the tumors is expressed in terms of increments of the calculated tumor volumes. Tumor volumes were calculated from the formula $V = (0.4)\ (ab^2)$, where V = volume; a and

[1] Complete homogeneity of a multichromosomal species is probably not attainable. The term "isogenicity" has only a limited, operative meaning: a sufficient degree of isoantigenic similarity, developed by repeated brother-sister matings, to permit the permanent acceptance of second set tissue grafts (skin is the tissue usually considered to be the most sensitive in detecting slight manifestations of homograft activity). Such isogenicity is adequate for experiments whose object it is to detect immunological reactions directed at antigens not possessed by the *normal* tissues of the animals.

b are the major and minor tumor diameters, respectively, as measured in the intact animal; and 0.4 is a constant derived from the relationship of values *a* and *b* of 121 spontaneous mammary carcinomas to their actual saline displacement volumes (41). As pointed our previously (41), expression of tumor growth as increments in tumor *volumes* provides a more accurate estimate of actual tumor mass increase than is obtained by a consideration of increments of tumor *diameters*.

RESULTS

Effect of Pretreatment with MER on Acceptance of Skin Isografts.—Although animals of the inbred mouse strains employed in this study were shown to accept skin isografts permanently and with no apparent evidence of even a transient homograft reaction, a slight residual heterozygosis of isoantigenic

TABLE I

Effect of Pretreatment of C3H and A Mice with MER on the Acceptance of First Skin Isografts

Pretreatment with	No. of animals	Isografts		Autografts	
		No. of technical failures	No. of rejections	No. of technical failures	No. of rejections
Saline	19	1	1 (?)*	0	1 (?)*
MER‡	19§	1	0	2	1 (?)*

* Graft rejection may have resulted from technical failure.

‡ 0.5 mg injected intraperitoneally 2 to 6 wk prior to grafting.

§ Reciprocal isografts of body skin were exchanged between 10 pairs of young (3 to 6 months old) C3H and 10 pairs of young A females. Autografts were also performed on each animal to provide procedure controls. The animals were observed for 4 to 12 months. 1 C3H animal was lost in each group immediately after the grafts were performed.

characteristics could have remained undetected. The possibility that reactivity to such heterozygosis might be enlarged by treatment of the animals with MER had to be ruled out in order to assure that any effects of MER on the fate of tumor implants could not be ascribed to this artifact.[2]

To this end, half of all the animals on which routine skin grafts were performed as isogenicity checks throughout the course of this study were pretreated with 0.5 mg of the MER 2 to 6 wk before reciprocal skin grafts were exchanged. The animals were examined 3 to 5 times weekly following the removal of the bandages 1 wk after grafting.

The effects of MER pretreatment on the acceptance of first set reciprocal body skin grafts exchanged between 10 pairs of strain A and 10 pairs of C3H mice are shown in Table I. Half the pairs were pretreated with MER, the other half with saline. Autografts were performed on every animal as well, to control for technical failure.

[2] It has already been found that pretreatment with MER will increase homograft reactivity against tissue of relatively weak antigenicity, male skin transplanted to isogenic females (42).

It is seen from Table I that MER pretreatment did not affect skin graft acceptance. The few failures to take could be ascribed to technical difficulty in every instance—loss of bandage, infection, removal of the graft as a result of scratching—and they occurred no more often among isografts than among the autografts. The appearance of the surviving isografts throughout the observation periods of 4 to 12 months was indistinguishable from that of the autografts, regardless of the animals' pretreatment.

At the end of the observation period, the surviving animals received a second body skin graft, from the same donors where these were available, and from a randomly selected isogenic female where the other member of a pair had been lost. These second set grafts were accepted as well as the first ones, and, again, no evidence of an MER effect could be discerned. It seems safe to assume, therefore, that any effects displayed by the MER against neoplastic tissue implants are directed at tumor-associated components of such tissue.

Effect of Pretreatment with MER and Other BCG Preparations on Acceptance of Isografts of a Spontaneous Uterine Sarcoma in BALB/c Mice.—

BALB/c females received single intraperitoneal injections of one of the following in 0.5 cc: living BCG; intact phenol-killed BCG; a methanol extract of phenolized BCG; MER; or saline. Different quantities of the tubercle bacillus preparations were given. The animals were challenged 2, 4, or 12 wk later with two subcutaneous implants each of a spontaneous uterine sarcoma. This tumor had arisen 2 yr previously, and was in the 15th transplant generations when employed in the present study. The animals were observed for the development of progressively growing tumors and were retained in the experiment until several weeks after all control mice in each challenge interval group had developed at least one tumor (only 1 control animal remained tumor-free 4 months after challenge). The results are shown in Table II.

It is seen from Table II that only 1 of 27 saline-treated controls failed to support at least one progressively growing tumor. In contrast, many of the animals treated with the BCG preparation remained free of tumors. Pretreatment with living BCG did not appear to be as effective as pretreatment with the bacillary fractions. 0.5 mg of MER protected 4 of 5 animals challenged 4 wk after vaccination, but larger quantities were ineffective in preventing growth of tumors from transplants implanted at any of the three intervals. The longer vaccination-challenge intervals appeared to be more favorable for the manifestation of increased resistance. Thus, treatment with 1.5 mg of intact phenolized bacilli evoked effective resistance in only 1 of 10 animals challenged 2 wk later, but protected 7 of 9 animals implanted with the tumor 12 wk after treatment. When all the treatment groups are considered together, it is seen that 21/87 animals resisted tumor challenge, but that, of the 28 challenged after only 2 wk, all but one developed tumors.

It has been observed in this laboratory that periods of time as long as 11 months sometimes elapse before palpable tumors appear at the site of implan-

tation with small numbers of tumor cells (43). In the present instance, mice were held for only several weeks after tumors had appeared in the control animals. In order to ascertain whether failure of tumor appearance in the apparently protected animals reflected complete destruction of the implants, or only an increased lag period of their growth, many of the animals grossly free of tumors at the termination of the experiment were autopsied and examined. In most instances, no evidence of growing neoplastic tissue was seen.

TABLE II

Effect of Pretreatment of BALB/c Mice with Various BCG Preparations on the Acceptance of Isografts of a Spontaneous Uterine Sarcoma

Pretreatment in 0.5 cc, i.p. with:	Proportion of animals remaining tumor-free* when treatment-tumor challenge interval was:		
	2 wk	4 wk	12 wk
Living BCG, 1:10 dilution‡	—	2/7	—
Living BCG, 1:50 dilution	0/9	—	1/8
Phenolized BCG,§ 1.0 mg	—	3/6	—
Phenolized BCG, 1.5 mg	1/10	—	7/9
Methanol extract,§ 1.0 mg	—	3/7	—
MER,‖ 0.5 mg	—	4/5	—
MER, 1.0 mg	—	0/7	—
MER, 1.5 mg	0/9	—	0/10
All treatment groups combined	1/28	12/32	8/27
Saline (controls)	1/10	0/7	0/10

* The animals were observed for 2 to 4 months after challenge. Animals dying from extraneous causes before the termination of the experiment are not included in the table.

‡ Living 7-day-old cultures of BCG in Dubos Tween broth were diluted 1:10 and 1:50 in sterile saline.

§ The preparation of intact phenol-killed BCG and of a methanolic extract derived from them has been described previously (1).

‖ MER, methanol extraction residue.

In some animals, no trace of the initial implants could be found; in others, the tumor implants could be detected, but these had usually grown only a little, were not vascularized, and did not appear to be composed of viable tissue. In only a few mice scored as tumor-free upon palpation was there indication that significant growth of the implants had occurred.

In order to ascertain whether treated animals resistant to a first tumor challenge would display similar resistance upon a second, more stringent challenge, 6 of the 7 mice which had been given 1.5 mg of phenolized BCG and which had failed to develop tumors when challenged 12 wk later were rechallenged with the same tumor (1 animal had escaped). The second

challenge took place 4 months after the first (i.e. 7 months after treatment), with one subcutaneous implant each of larger fragments of the tumor, 1 to 2 mm^3. A control group of 15 BALB/c males was similarly challenged. The animals were observed for 80 days after challenge. The results are shown in Table III.

As seen from Table III, none of the 6 rechallenged animals developed a tumor, while all of the 15 controls did.

It should be noted that some of the animals treated with MER which nonetheless permitted growth of the cancer implants still exhibited a delayed onset of this growth, or a retardation of its rate, or lived significantly longer despite their tumors than did the control animals. It was not possible in all experiments to bestow complete protection against the uterine sarcoma on a significant proportion of treated animals, but some evidence of the protective efficacy of pretreatment with the MER and with other BCG preparations was always clearly discernible against this tumor.

TABLE III

Effect of Pretreatment of BALB/c Mice with 1.5 mg Phenol-Killed BCG on the Acceptance of a Second Isograft of a Spontaneous Uterine Sarcoma 7 Months Later

Pretreatment in 0.5 cc, i.p. with:	Proportion of animals remaining tumor-free*
Untreated controls	0/15
Phenolized BCG, 1.5 mg‡	6/6§

* The animals were observed for 80 days after challenge.

‡ Vaccination was 7 months previously; first tumor challenge was 12 wk after vaccination.

§ The animals were autopsied at termination of the experiment; in none was tumor tissue found.

Effect of Treatment with MER and Other BCG Preparations on the Growth of Established Implants of the Uterine Sarcoma in BALB/c Mice.—Two experiments were conducted to ascertain the efficacy of tubercle bacillus substances against transplants of the uterine sarcoma established simultaneously with, or prior to, treatment.

Experiment 1:

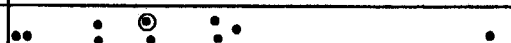
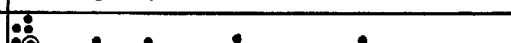
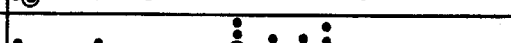
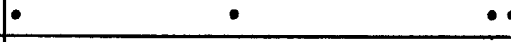
BALB/c females were given two intraperitoneal injections of one of the following: saline; a 1:10 dilution of a 7-day-old BCG culture; or, 0.25 or 0.5 mg of the MER. Each injection was in 0.5 cc of saline. 9 or 10 animals were employed in each group. The first injection was administered on the day of implantation of the uterine sarcoma, the second injection 6 days later. Tumor challenge was by a single implant, placed subcutaneously. The animals were observed for 40 days.

The support given by the differently treated animals to growth of the tumor isografts is shown diagrammatically in Table IV.

It is common to find a wide range of variation in the rapidity of development of tumors growing from implants of recently arisen neoplasms, and this was

TABLE IV

*Effect of Treatment of BALB/c Mice with Various BCG Preparations on the Development of Isografts of a Spontaneous Uterine Sarcoma (Treatment on Days 1 + 7 of Implantation)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors,‡ mm^3	Significance of comparison of each group with saline control§
days 20	Saline		*P* —
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
30	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
40	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg	 (1 animal dead)	>0.05

10^1 10^2 10^3

* First treatment on day of implantation; second, identical treatment 6 days later. See text and footnotes to preceding tables for details.

‡ The circled dots indicate the median value of each series.

§ Statistical analysis by Mann-Whitney *u* test.

the case in the present experiment. The data are therefore presented by giving the size of each tumor in each animal at several intervals after challenge, thus showing the different patterns of tumor size distribution. The significance of these differences relative to the saline-treated controls was analyzed by the nonparametric Mann-Whitney distribution-free test (44).

It is seen from Table IV that almost all the tumor implants developed progressively, regardless of the treatment of the animals. However, there was some retardation of the rate of tumor development in the animals given 0.25 mg of MER. The degree of retardation was significant at the 0.1 but not quite at the 0.05 level.

Experiment 2:

The plan of this experiment was identical to the preceding one, except that treatment of the animals was administered 6 and 13 days after implantation of the tumors. The results are depicted in Table V.

It is seen from Table V that most of the tumor implants again developed, and that treatment of the animals with 0.5 mg of MER, but not with 0.25 mg, retarded their growth significantly. The requirement for the larger quantity of MER to effect retardation of tumor growth in this experiment may have been due to the fact that treatment was not initiated until 6 days after tumor implantation, and that a stronger stimulation of the immunological apparatus of the animals was, therefore, required to bring about a degree of tumor inhibition.

The failure of the tubercle bacillus substances to prevent tumor growth entirely when administered simultaneously with, or after, the tumor implantation could well reflect the necessity of stimulating immunological responsiveness some time *before* challenge in order to obtain complete protection. It could also be due to the fact that administration by multiple injections constitutes a supra-optimal stimulation. The narrowness of the optimum dosage of MER in antimicrobial protection experiments has already been indicated (2); the similar importance of dosage in antitumor systems will be further documented in the following experiments.

Effect of Pretreatment with MER and Living BCG on the Growth of Isografts of a Hepatoma Arising in C3H Mice Treated with Carbon Tetrachloride.—

C3H males were given single intraperitoneal injections of one of the following in 0.5 cc of saline: living BCG; 0.25 or 0.5 mg of MER; or saline only. The animals were challenged 2 or 4 wk later with a single subcutaneous implant of a hepatoma which had arisen 5 yr previously, and had been transplanted every 2 to 3 wk. 8 to 10 animals were included in each group. The animals were observed for 2 to 3 months after implantation of the tumors. The results are presented in Tables VI *A* and VI *B*.

It is seen from Tables VI *A* and VI *B* that pretreatment of the animals with

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TABLE V

*Effect of Treatment of BALB/c Mice with Various BCG Preparations on the Development of Isografts of a Spontaneous Uterine Sarcoma (Treatment on Days 7 + 14 of Implantation)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			
20	Saline		*P*
			—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.025
30	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.05
40	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.05

10^1 10^2 10^3

* First treatment 6 days after implantation; second, identical treatment 7 days later. See text and footnotes to preceding tables for details.

TABLE VI *A*

*Effect of Pretreatment of C3H Mice with MER and Living BCG on the Development of Isografts of a Hepatoma (Treatment-Challenge Interval: 2 Wk)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days'			*P*
19	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
26	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
33	Saline	(2 animals dead)	—
	Living BCG, 1:10 dilution	(1 animal dead)	>0.05
	MER, 0.25 mg		<0.025
	MER, 0.5 mg		<0.05

10^2 10^3

* See text for experimental details.

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TABLE VI *B*

*Effect of Pretreatment of C3H Mice with MER and Living BCG on the Development of Isografts of a Hepatoma (Treatment-Challenge Interval: 4 Wk)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			*P*
19	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.025
26	Saline		—
	Living BCG, 1:10 dilution		>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.025
35	Saline		—
	Living BCG, 1:10 dilution	(3 animals dead)	>0.05
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.025

10^2 10^3

* See text for experimental details.

the smaller quantity of MER, 0.25 mg, sufficed to retard development of hepatomas implanted 2 wk after administration, and that 0.5 mg of MER was necessary to elicit such an effect when 4 wk elapsed between treatment and tumor challenge. The significance of dosage of MER in relation to treatment-challenge interval is thus indicated in this as well as in the preceding experiment.

The effect of pretreatment with MER was also manifested by a prolonged survival of the animals. This is illustrated in Table VI *C*.

TABLE VI *C*

Survival of C3H Mice Following Implantation of an Isogenic Hepatoma

Treatment of animals	No. of animals	Cumulative No. of animals dead at following intervals after tumor challenge, *wk**								
		5	6	7	8	9	10	11	12	13
Treatment-challenge interval: 2 wk‡										
Saline	9	2	6	9	—	—	—	—	—	—
Living BCG	9	1	3	6	7	7	8	9	—	—
MER, 0.25 mg	8	0	0	0	2	2	3	6	7	8
MER, 0.5 mg	8	1	2	3	5	5	5	5	6	7§
Treatment-challenge interval: 4 wk‖										
Saline	10	0	0	1	3	6	4 animals alive, with tumors			
Living BCG	10	3	3	4	7	7	3 animals alive, with tumors			
MER, 0.25 mg	10	0	1	1	3	6	4 animals alive, with tumors			
MER, 0.5 mg	10	0	0	0	2	2	8 animals alive, with tumors			

* All animals had large tumors at death.

‡ Experiment terminated at 13 wk.

§ 1 animal alive, with massive tumor, at termination of experiment.

‖ Experiment terminated at 9 wk.

Pretreatment with living BCG exerted no appreciable effect on the course of development of the hepatoma isografts.

Effect of Pretreatment with MER on the Growth of Isografts of a Spontaneous Mammary Carcinoma in RIII Mice.—

RIII females were given single intraperitoneal injections of 0.5 mg of MER or saline only. There were 15 animals in each group. 4 months later, the animals were challenged with two intramammary implants each of a first generation transplant of a mammary adenocarcinoma which had arisen spontaneously in a multiparous RIII breeding female. The animals were observed for 17 wk after implantation. The results are presented in Table VII.

It is evident from Table VII that administration of the MER fraction delayed considerably the development of the mammary tumor isografts.

Effect of Pretreatment with MER on the Growth of Isografts of a Spontaneous Osteogenic Sarcoma.—

C3H males and females were given single intraperitoneal injections of either 0.25 or 0.5 mg of MER in 0.5 cc of saline, or of saline only. Each group consisted of 20 animals. Half the animals in each group were challenged 2 wk later with a single subcutaneous implant of an osteogenic sarcoma which had arisen spontaneously in a C3H female 18 yr previously, and had been transplanted every 6 wk. The remaining animals were similarly challenged 4 wk after treatment. The development of the tumors was observed for 50 days after challenge. The results are shown in Tables VIII *A* and VIII *B*.

TABLE VII

*Effect of Pretreatment with MER on the Development of Isografts of a Spontaneous Mammary Carcinoma in RIII Mice**

Treatment of animals in 0.5 cc, i.p.	No. of animals	Cumulative No. of animals with palpable tumors at following intervals after implantation, *wk*			
		5	9	13	17
Saline	15	5	7	7	7
MER, 0.5 mg	15	0	0	0	2

* See text for experiment details.

It is seen from Tables VIII *A* and VIII *B* that pretreatment with MER succeeded in retarding significantly the development of the challenge tumors implanted after both time intervals.

Effect of Treatment with MER on the Growth of Established Implants of the Osteogenic Sarcoma.—

C3Hf males were given single subcutaneous implants of the osteogenic sarcoma described above. 2 and 7 days after implantation, the animals were treated with either 0.3 or 0.6 mg of MER in 0.5 cc of saline, or with saline alone. 20 animals were included in each group, half the animals receiving treatment by the intraperitoneal route, the other half by the subcutaneous one. All animals were observed for 51 days after implantation. The results are shown in Tables IX *A* and IX *B*.

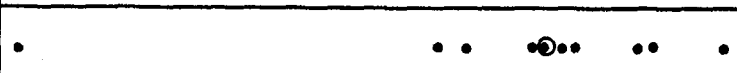
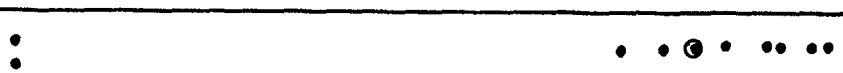
As is seen from Tables IX *A* and IX *B*, initiation of treatment with MER by either the intraperitoneal or the subcutaneous routes *after* implantation of isografts of the osteogenic sarcoma was still effective in bringing about some retardation of the development of the tumors.

Effect of Pretreatment with MER and Other BCG Preparations on the Growth of Isografts of a Methylcholanthrene-Induced Fibrosarcoma in BALB/c Mice.—

BALB/c females were given single intraperitoneal injections of one of the following in 0.5 cc of saline: living BCG; phenol-killed BCG, 0.5, 1.0, or 1.5 mg; MER, 0.5 or 1.0 mg; or saline only. Each group contained 8 to 10 animals. 3 wk later, the animals were challenged with a single subcutaneous implant of a fibrosarcoma induced in a young adult BALB/c female by

TABLE VIII *A*

*Effect of Pretreatment of C3H Mice with MER on the Development of Isografts of a Spontaneous Osteogenic Sarcoma (Treatment-Challenge Interval: 2 Wk)**

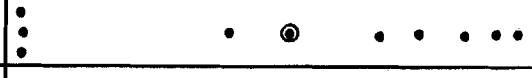
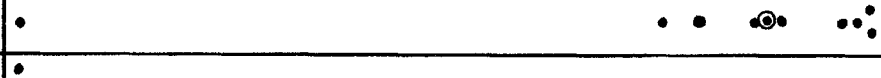
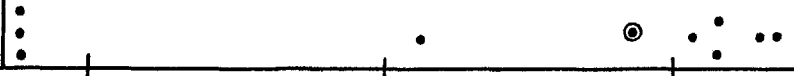
Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			*P*
21	Saline		—
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		<0.05
35	Saline		—
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
49	Saline		—
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05

10^1 10^2 10^3

* See text for experimental details.

TABLE VIII *B*

*Effect of Pretreatment of C3H Mice with MER on the Development of Isografts of a Spontaneous Osteogenic Sarcoma (Treatment-Challenge Interval: 4 Wk)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			
22	Saline		*P* —
	MER, 0.25 mg		<0.025
	MER, 0.5 mg		>0.05
37	Saline		—
	MER, 0.25 mg		>0.05
	MER, 0.5 mg		>0.05
50	Saline		—
	MER, 0.25 mg		<0.05
	MER, 0.5 mg		<0.025

10^1 10^2 10^3

* See text for experimental details.

TABLE IX *A*

*Effect of Treatment of C3H Mice with MER on the Development of Previously Implanted Isografts of a Spontaneous Osteogenic Sarcoma (Treatment Intraperitoneally)**

Time after challenge implantation	Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			*P*
21	Saline		—
	MER, 0.3 mg		<0.05
	MER, 0.6 mg		>0.05
36	Saline		—
	MER, 0.3 mg		>0.05
	MER, 0.6 mg		>0.05
51	Saline	(1 animal dead)	—
	MER, 0.3 mg		>0.05
	MER, 0.6 mg		>0.05

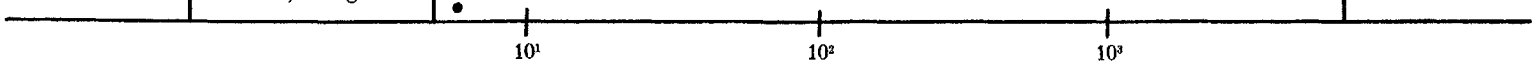
10^1 10^2 10^3

* Treatment 2 and 7 days after implantation; see text for experimental details.

TABLE IX *B*

*Effect of Treatment of C3H Mice with MER on the Development of Previously Implanted Isografts of a Spontaneous Osteogenic Sarcoma (Treatment Subcutaneously)**

Time after challenge implantation	Treatment of animals in 0.5 cc, s.c.	Calculated volumes of individual tumors, mm^3	Significance of comparison of each group with saline control
days			*P*
21	Saline		—
	MER, 0.3 mg		>0.05
	MER, 0.6 mg		>0.05
36	Saline		—
	MER, 0.3 mg		>0.05
	MER, 0.6 mg		>0.05
51	Saline		—
	MER, 0.3 mg		>0.05
	MER, 0.6 mg		<0.05



* Treatment 2 and 7 days after implantation; see text for experimental details.

intramuscular injection of 3-methylcholanthrene. The tumor was in its first transplant generation. The tumor developed very rapidly, and the animals were maintained until death. Table X shows the calculated volumes of the tumors 13 days after implantation (tumor volumes at later intervals are not shown because many of the animals died in the 3rd and 4th wk), and the mean and median survival times.

It is evident from Table X that the tumor isografts developed more *rapidly* in the groups treated with *any* of the BCG preparations. It has already been observed that *direct* immunization against isografts of spontaneous mammary carcinomas, i.e. by means of tumor cell preparations, can result in *either* heightened resistance *or* enhancement of tumor growth, and evidence has been brought forward to indicate that both effects may be based on immunological mechanisms (22, 26, 41): Heightened resistance appears to result when the increment in cellular reactivity is more pronounced, heightened susceptibility when the humoral response is more significantly enlarged. The present observation that tubercle bacillus substances can enhance the rate of tumor development might, accordingly, also have an immunological explanation. It is of interest that living BCG, which usually proved inferior to MER and phenol-killed tubercle bacilli in eliciting heightened tumor resistance was here less effective than the killed preparations in enlarging tumor susceptibility. It is also to be noted that more rapid growth of the tumors was directly correlated with a decreased survival time of the animals after tumor challenge. In the experiments described above, a slowing of tumor development was always accompanied by an *increase* in survival times. Longevity after tumor implantation can probably be viewed, therefore, as a function of tumor growth rate; and the effect of MER and other BCG preparations in prolonging life after challenge with most of the tumors here studied probably cannot be taken to reflect merely a heightened resistance against secondary microbial disease.

It seemed possible that the phenomenon of enhancement of tumor growth brought about in this experiment by pretreatment with BCG preparations might occur only vis-à-vis tumors induced by a hydrocarbon carcinogen. Further investigation revealed, however, that enhancement of tumor growth by MER is also seen occasionally with spontaneous tumors. This is illustrated in the following experiment.

Effect of Pretreatment with MER on the Growth of Isografts of a Spontaneous Fibrosarcoma in DBA/2 Mice.—

DBA/2 females were given a single intraperitoneal injection of 0.5 mg of MER or of 0.5 cc of saline; 15 animals were treated with the MER and 13 with saline only. 25 days later, the animals were challenged with a single subcutaneous implant of a first generation transplant of a fibrosarcoma which had arisen spontaneously in a multiparous DBA/2 breeding female. This tumor developed very slowly; the animals were maintained for 320 days after challenge.

Tumor development was considerable more rapid in the mice treated with MER. In Table XI there are shown the calculated tumor volumes 2 months after implantation, and the cumulative numbers of animals dead with large tumors at intervals after tumor challenge.

TABLE X

*Effect of Pretreatment of BALB/c Mice with MER and Other BCG Preparations on the Development of Isografts of a Methylcholanthrene-Induced Sarcoma (Treatment-Challenge Interval: 3 Wk)**

Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors 13 days after implantation, mm^3	Significance of comparison of each group with saline control	Survival times	
			Mean	Median
		P	*days*	*days*
Saline		—	94	107
Living BCG, 1:10 dilution		<0.025	57	50
Phenol-killed BCG, 0.5 mg		<0.025	35	28
Phenol-killed BCG, 1.0 mg		<0.025	34	22
Phenol-killed BCG, 1.5 mg		<0.025	29	32
MER, 0.5 mg		<0.025	44	30
MER, 1.0 mg		<0.025	40	40

10^1 10^2 10^3

* See text for experimental details.

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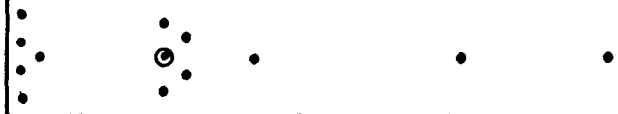
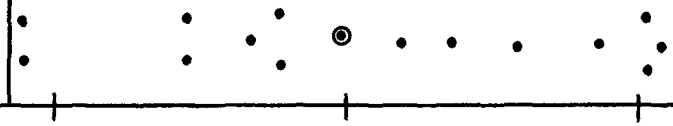
TABLE XI

*Effect of Pretreatment of DBA/2 Mice with MER on the Development of Isografts of a Spontaneous Fibrosarcoma (Treatment-Challenge Interval: 25 Days)**

Treatment of animals in 0.5 cc, i.p.	Calculated volumes of individual tumors 2 months after implantation, mm^3	No. of animals	Significance of comparison with saline control	Cumulative No. of animals dead with large tumors at following intervals after implantation, *days*					
				100	120	140	200	260	320
Saline		13	*P* —	0	0	2	5	7	9‡
MER, 0.5 mg		15	<0.025	2	5	9	9	13	13§

* See text for experimental details.
‡ 4 animals survived without tumors.
§ 2 animals survived without tumors.

The results show that enhancement of tumor growth may be elicited by treatment with MER prior to implantation of a spontaneous as well as of a carcinogen-induced neoplasm.

Effect of Pretreatment with MER and Living BCG on the Growth of Isografts of a Spontaneous Myeloid Leukemia in C57Bl Mice.—

Although MER appeared to be more effective than living BCG in raising the resistance of mice against isografts of most of the tumors tested, living BCG proved capable of eliciting resistance against one tumor, a spontaneous myeloid leukemia in C57Bl mice, which proved

TABLE XII

*Survival of C57Bl Mice Following Implantation of an Isogenic Myeloid Leukemia**

Treatment of animals in 0.5 cc	Cumulative No. dead at following intervals after tumor challenge,‡ *wk*					
	6	7	8	9	10	13
Route of treatment: intraperitoneal						
Saline	3	10	15	17	17	20
Living BCG, 1:25 dilution	0	1	3	6	11	17
MER, 0.3 mg	2	3	7	13	18	20
MER, 0.6 mg	3	7	10	16	19	20
Route of treatment: subcutaneous						
Saline	1	6	14	17	19	20
Living BCG, 1:25 dilution	0	1	3	5	10	16
MER, 0.3 mg	0	5	8	13	15	19
MER, 0.6 mg	2	6	8	10	16	20

* See text for experimental details.

‡ 20 animals per group.

refractory to pretreatment with MER. This observation was made in the following experiment:

C57Bl males were given single intraperitoneal or subcutaneous injections of living BCG, 0.3 or 0.6 mg of MER, or saline only. Each treatment group contained 20 mice. 40 days after the injections, the animals were challenged subcutaneously with a fragment of a myeloid leukemia which had arisen in a female animal 5 yr previously, and was now in the 47th transplant generation. The animals were maintained for 13 wk after challenge. Table XII shows the mean and median survival times of the animals in each group.

It is seen from Table XII that pretreatment with living BCG by either the intraperitoneal or the subcutaneous route prolonged significantly the survival times of C57Bl mice challenged with a myeloid leukemia, and that pretreatment with MER exerted no, or only a very slight, protective effect.

DISCUSSION

The findings here presented show clearly that the heterologous immunogenicity of MER is not limited to effects manifested towards pathogenic microorganisms, but is also exerted vis-à-vis isografts of neoplastic tissue.

The results which were obtained with new or recently arisen tumors are more suggestive of immunological reactions directed at the *neoplastic* condition of the implanted tissues than are findings derived from experiments in which the challenge tumors, albeit of isogenic origin, have been transplanted for many generations. Isogenic drift within inbred strains of animals occurs not infrequently over long periods of time, and the genetic characteristics of a group of test animals may differ considerably from that of a tumor which had arisen in the strain many generations previously. Moreover, the loss of isoantigens upon repeated passage of tumor cells has been well documented (e.g. reference 45). The resulting changes of the cell surface may affect acceptability of the cells by "isogenic" animals for nonimmunological reasons (46), and also for immunological ones not associated directly with the neoplastic nature of the tissue: The loss of some transplantation antigens from the surface of cells may well result in the exposure of other, weak transplantation antigens whose nonisogenicity within a supposedly isogenic population may have gone undetected. The phenomenon of "antigenic diversion" in transplanted tumors (47), including the appearance of "fetal" antigens (48), has also been shown. Moreover, it is not unlikely that clones of cells passaged repeatedly in animals or in tissue culture may acquire new antigenic characteristics as a manifestation of the physiological consequences of a very artificial existence, and not necessarily as a manifestation of the neoplastic state as such. Continued passage of cells must also result in the selection of clones possessing physiologic and morphologic characteristics favored by the conditions of the passages, and these are likely to be dissimilar to those prevailing when the cells are finally tested for ability to grow in a test host; it cannot be assumed, therefore, that long-passaged cells bear a close biological resemblance to populations of neoplastic cells newly arisen in the autochthonous host. It is thus significant that MER could evoke heightened resistance against first transplant generation tumors as well as against neoplasms carried for many generations in isogenic hosts.

The MER fraction was usually, but not always, more effective in eliciting increased resistance against tumors than were living BCG and the other tubercle bacillus fractions, thereby confirming the high heterologous immunogenicity displayed by this moiety against pathogenic bacteria (2). It was clear from this, and from similar, unpublished studies, that treatment with MER, as well as with the other BCG substances, produces different effects vis-à-vis the different tumors tested, eliciting various degrees of resistance against

many,[3] producing enhanced susceptibility towards a few, and occasionally exerting no effect. The importance of the experimental conditions—dosage and route of administration of the tubercle bacillus substance, treatment-challenge interval, and others—was again (2) indicated in these studies.

It will be shown in subsequent communications that quantities of MER much in excess of the optimal protective dose sometimes elicit enhancement rather than heightened resistance towards spontaneous mouse mammary tumors *in situ*. It is possible, therefore, that the enhanced growth of tumor implants which is occasionally elicited by pretreatment with MER similarly results from administration of too large a quantity, and that resistance would be evoked against the tumors if smaller amounts of the activator were employed. If this can be shown to be the case, MER could be considered as a general stimulator of resistance against the development of tumor isografts, provided the appropriate dosage is administered.

SUMMARY

A methanol-insoluble residue (MER) of phenol-killed attenuated tubercle bacilli (BCG), which has been reported previously to be capable of evoking heightened resistance to infection with antigenically unrelated microorganisms, was found to affect as well the resistance of highly inbred mice against tumor isografts.

In most instances, the MER evoked heightened resistance against the tumor implants, but heightened susceptibility was the effect induced against two of the tumors tested, and no effect was elicited against one neoplasm. It is suggested that the heightened susceptibility occasionally produced by pretreatment with MER may also be of immunological nature, i.e. immunological enhancement.

Treatment with MER was more effective when administered some time before tumor challenge than when given simultaneously with, or after, tumor implantation. The protective effects manifested against some tumors were of a high order, a significant number of animals rejecting the neoplastic implants, and were displayed even when several months elapsed between treatment and challenge.

Living BCG and intact phenol-killed bacilli also evoked heightened resistance against some of the tumors tested, and in one experiment living BCG proved effective whereas MER did not. On the whole, however, MER was the most active (and least toxic, as shown previously) of the several tubercle bacillus preparations tested.

MER elicited heightened reactivity against first transplant generation tu-

[3] The protective effect of MER has also been observed recently by Old against a murine leukemia (49).

mors as well as against tumors maintained for considerable periods of time by repeated animal passage, and against spontaneously arising as well as against induced neoplasms. The experimental parameters necessary to demonstrate maximal effects varied somewhat from tumor to tumor. In general, however, single intraperitoneal injections of small quantities of MER, of the order of 0.25 to 1.0 mg, afforded the best protection.

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