Immunoprophylaxis of Methylcholanthrene-induced Tumors in Mice with Bacillus Calmette-Guérin and Methanol-extracted Residue

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SUMMARY

The immunoprophylactic effects of i.p. administration of Bacillus Calmette-Guérin (BCG) and methanol-extracted residue of BCG (MER) on the production of tumors by 3-methylcholanthrene (MCA) in C57BL/6 mice were investigated. At the dose level of MCA used (500 μg i.m.), almost all mice developed tumors. Pretreatment of the mice with BCG either 2 weeks before or 2 weeks after carcinogen administration provided a significant temporary protection, demonstrated by a decreased rate of tumor appearance. Administration of MER at a 0.5-mg dose level conferred relatively little protection against tumor production. In those mice receiving 2.5 mg MER, either on the day of carcinogen administration or both at that time and when the first experimental tumor was found, significant temporary protection against MCA carcinogenesis was obtained. This was demonstrated by a lengthened latent period before tumor production, although subsequently the rate of tumor production was similar to that of the controls.

It is concluded that BCG and MER are capable of affecting the induction of MCA-induced tumors.

INTRODUCTION

There have been repeated demonstrations of depression of the immune response in animals given chemical carcinogens (22, 24) and also in both animal (19, 27) and human tumor-bearing hosts (20, 21). A number of agents capable of nonspecific stimulation of the immune response have been investigated for their capacity to reverse this immune depression (28). The most prominent of these agents has been BCG,3 which has been shown to reverse the immunodepressive effect of MCA (23) and to be capable of protecting animals against syngeneic tumor challenge (12, 13, 28).

BCG has also been used to suppress the formation of tumors. Old et al. (12) were able to demonstrate a transient suppression of the formation of MCA tumors and also of virus-induced mammary tumors and leukemias using systemic BCG administration. Nilsson et al. (11) demonstrated a similar mild protection by BCG against 90Sr-induced bone tumors. Using MER, Weiss was able to demonstrate protection against a number of tumor isografts (25) and obtained a considerable reduction in the incidence of spontaneous mammary carcinomas and precancerous hyperplastic alveolar nodules in C3H mice (26). Clinically, it was noted in a retrospective study in Canada that children immunized with BCG had a lower leukemia incidence than did nontreated children (6), although this observation has not been confirmed (5).

This communication reports the results of experiments on the use of BCG and MER in the immunoprophylaxis of MCA-induced tumor formation.

MATERIALS AND METHODS

C57BL/6 mice were obtained through the Mammalian Genetics and Animal Production Section of the Chemotherapy Division of the National Cancer Institute, Bethesda, Md. (Those used in Experiment 1 were obtained from the Laboratory Supply Co., Indianapolis, Ind.; those in Experiment 2 were obtained from Simonsen Laboratories, Gilroy, Calif.) Young adult male mice, ages 7 to 10 weeks, were fed Wayne Lab Blox and water ad libitum. They were distributed randomly into the respective experimental groups prior to use.

MCA (Eastman Kodak Co., Rochester, N. Y.) was administered to each mouse at 0.5 mg in 0.05 ml of triocanoin by i.m. inoculation into the right thigh. Regular palpation and tumor measurement were carried out. The date of appearance of all tumors was recorded, and the diameter of all tumors was measured weekly to determine tumor growth rates.

BCG (Trudeau Institute, Saranac Lake, N. Y., TMC No. 1029, Lot 9A714) was administered i.p., 1 ml (5 X 107 organisms/ml) per mouse. Control mice were inoculated with the same amount of Dubos medium.

MER (Lot 675738-0-7) was obtained from Merck, Sharpe, and Dohme, Rahway, N. J., where it was prepared under contract to the National Cancer Institute. The activity of this...
material was not assayed, but the optimal dose was anticipated to be approximately 0.25 to 0.5 mg/mouse (D. W. Weiss, personal communication). The MER, which is somewhat hydrophobic, was prepared by making a paste of the accurately weighed MER with PBS in a small mortar and pestle. The paste was transferred to a VirTis homogenizer, where it was homogenized at medium speed for 5 min in 75% of the final volume of PBS. It was then transferred to serum bottles where it was made up to a final volume of PBS such that 0.2 ml contained either 0.2 or 2.5 mg of MER. The MER suspension was shaken well. For prevention of settling and inequalities of the dose administered, only amounts of the suspension sufficient to treat 1 animal at a time were taken into the syringe.

Statistical Analysis. The statistical analysis in this paper used 2 distinct approaches: a nonparametric one based on an actuarial or life table method of analysis [e.g., see Pike and Roe (17)] and a censored rank test (3); and a parametric approach based on a model in which a Weibull distribution is used to fit the data (16).

In the life table procedure, the probability that an animal will remain tumor free is estimated with the losses (e.g., deaths of tumor-free animals) taken into account. The censored rank test provides the statistical basis for comparing tumor development of the treatment groups.

In the parametric approach, the Weibull distribution relates the development of tumors to the time since carcinogen administration. The probability of being tumor free at time t is assumed to have the form:

\[
\exp \left[ -b(t - w)^k \right] t > w
\]

where \( b \) is a measure of the carcinogenic effect, \( w \) is a measure of the latent period, and \( k \) is a constant related to the animal tumor type. The curve is fitted to the data by obtaining maximum likelihood estimates of the 3 parameters. The distributions of the treatment groups are then compared via likelihood ratio tests.

The 2 approaches gave similar results both in representing the data and in the tests comparing the different groups. In particular, the Weibull curves fit the data very well. This will be seen in Charts 1 and 2 where life table graphs and fitted curves are plotted together.

RESULTS

Experiment 1. Study of the Effects of BCG at Various Times on the Incidence of MCA-induced Tumors. In this experiment, we wished to determine the effects of BCG, administered before or after MCA, on the latent period and rate of tumor formation. Also, in order to simulate BCG administration to a population at immediate risk, and because the results of Old et al. (12) had indicated that maximum tumor protection might be found in such groups, we also administered BCG to animals when the 1st tumor was detected in the experiment. MCA was administered to 4 randomized groups (Groups III to VI) of 102 mice each and to a 5th control group (Group II) containing 204 mice. A 6th group (Group I) of 50 mice was maintained, treatment free, as a control of survival under the colony conditions.

Groups I and II received no BCG, Group III received BCG 13 days prior to MCA injection, and Group IV received BCG 14 days following the carcinogen. In Group V, BCG was administered both 13 days prior to MCA and when the 1st tumor was detected (which was 50 days after MCA), in order to determine whether prior sensitization to BCG had any potentiating effect on the results of administering BCG at the time when the 1st tumor could be palpated. In Group VI, BCG

Chart 1. Estimated percentage of tumors in MCA-treated mice following various dosage schedules of BCG. Step plots, life table estimates of percentage of tumor incidences; associated smooth plots, Weibull distributions of percentage of tumor development at different times following MCA administration; II, no BCG; III + IV + V, 3 groups combined (BCG on Day -14, Day +13, and Days -14 and +50, respectively); VI, BCG on Day +50.

Chart 2. Estimated percentage of tumors in MCA-treated mice following administration of 2.5 mg of MER. Step plots, life table estimates of percentage of tumor incidences; associated smooth plots, Weibull distributions of tumor development at different times following MCA administration; II, no MER; VII + VIII, 2 groups combined (MER on Day 0 and on Days 0 and +61, respectively).

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was given only at 50 days. The cumulative tumor incidences and cumulative number of mice dying without tumors are shown in Table 1. Only animals surviving to the time of appearance of the 1st tumor, which was Day 50 were considered in the statistical analysis of results. In addition, the estimated percentage of tumor incidence, arrived at with life table statistics, is given for each group.

The probability that animals were tumor free was virtually zero for all MCA-treated groups by the 196th day (Table 1). There was, however, a clear, albeit temporary, protection (i.e., a decrease in the rate of tumors appearing) in the groups that received BCG either before or shortly after MCA administration (Groups III, IV, and V). By contrast, in animals given BCG only at 50 days after MCA injection (Group VI), the production of tumors was significantly more rapid than in the non-BCG-treated controls.

Moreover, the statistical analysis established that Groups III, IV, and V (those that received BCG "early") fell into a common population. There were then 3 distinct "groups": Group VI, with an enhanced tumor development relative to the control ($p < 0.001$); Group II, the control; and Group "III + IV + V," with a protection over both the control ($p < 0.001$) and Group VI ($p < 0.001$). The life table graphs and the fitted Weibull curves, which approximate the data well, are shown for the 3 groups in Chart 1. From these plots and the likelihood ratio tests, it appears that, although tumors start to appear in all experimental groups at about the same time after MCA injection, the subsequent rate of tumor appearance is slower in Group III + IV + V than in the control and faster in Group VI (Chart 1).

Whereas few deaths occurred in the non-BCG-, non-MCA-treated controls (Group I) and the non-BCG-treated controls (Group II), there were considerably more nontumor deaths in the BCG-treated groups (Table 1). The reason for these deaths was not established, but it may possibly be due to overwhelming BCG infection, to an increased susceptibility to other infectious agents, or to unusual hypersensitivity reactions to the BCG. The statistical analysis used methods that adjusted for such nontumor deaths and their differences in the treatment groups.

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of mice on Day 50</th>
<th>Days following MCA administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>None</td>
<td>47</td>
<td>Tumors(^a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Death no tumor(^b)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estimated tumor %</td>
</tr>
<tr>
<td>II</td>
<td>No BCG</td>
<td>198</td>
<td>Tumors</td>
</tr>
<tr>
<td>MCA, Day 0</td>
<td></td>
<td></td>
<td>Death no tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estimated tumor %</td>
</tr>
<tr>
<td>III</td>
<td>BCG, Day -14</td>
<td>100</td>
<td>Tumors</td>
</tr>
<tr>
<td>MCA, Day 0</td>
<td></td>
<td></td>
<td>Death no tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estimated tumor %</td>
</tr>
<tr>
<td>IV</td>
<td>BCG, Day +13</td>
<td>84</td>
<td>Tumors</td>
</tr>
<tr>
<td>MCA, Day 0</td>
<td></td>
<td></td>
<td>Death no tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estimated tumor %</td>
</tr>
<tr>
<td>V</td>
<td>BCG, Day -14 +50</td>
<td>93</td>
<td>Tumors</td>
</tr>
<tr>
<td>MCA, Day 0</td>
<td></td>
<td></td>
<td>Death no tumor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Estimated tumor %</td>
</tr>
<tr>
<td>III + IV</td>
<td>Combined</td>
<td>277</td>
<td>Tumors</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Death no tumor</td>
</tr>
<tr>
<td>VI</td>
<td>BCG, Day +50</td>
<td>94</td>
<td>Tumors</td>
</tr>
</tbody>
</table>

\(^a\) Cumulative numbers of animals developing tumors and dying without detectable tumors.

\(^b\) The estimated tumor percentage determined statistically by life table analysis, as mentioned in text.
Experiment 2. Study of the Effect of MER at Various Times and Doses in the Incidence of MCA-induced Tumors.
This experiment was essentially similar in design to the preceding one. Group I (24 mice) was a double control with no treatment, and Group II (152 mice) was given MCA only, as was described for Experiment 1. MER, at the 0.5-mg dose, was administered to Groups III and IV (76 mice each), respectively, 14 days prior to and 14 days following MCA injection. In Group V (76 mice), 0.5 mg MER was given at -14, +14, and +42 days and at 1st tumor (61 days). In Group VI (76 mice), MER was given only at 61 days. In addition, since 2.5 mg MER caused enhanced tumor formation in the murine sarcoma virus-tumor system (D. Lavrin, unpublished results) and enhanced growth in transplanted MCA-induced fibrosarcoma (25), the effects of 2.5 mg MER were also examined in this experiment. In 2 groups of 25 mice each (Groups VII and VIII), 2.5 mg MER were administered only on the day of MCA administration (Day 0) and at Day 0 and +61 days, respectively. The results are shown in Table 2.

The probability that the animals would be tumor free by the 137th day was almost zero for all the treatment groups (Groups II to VIII). The control group, receiving MCA but not MER (Group II), generally demonstrated the highest tumor incidence throughout the experiment and the two 2.5-mg MER dose groups (Groups VII and VIII) had the lowest tumor incidence.

### Table 2
**Effect of treatment with MER on MCA-induced tumorigenesis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>No. of mice on Day 60</th>
<th>Days following MCA administration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>60</td>
</tr>
<tr>
<td>I</td>
<td>None</td>
<td>25</td>
<td>Tumors&lt;sup&gt;a&lt;/sup&gt; 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
</tr>
<tr>
<td>II</td>
<td>No MER</td>
<td>147</td>
<td>Tumors 0 6 14 39 55 82 102 116 123 125 127 128</td>
</tr>
<tr>
<td></td>
<td>MCA, Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>MER, 0.5 mg, Day -14</td>
<td>70</td>
<td>Tumors 0 6 14 17 19 26 34 41 46 49 51 51</td>
</tr>
<tr>
<td></td>
<td>MCA, Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV</td>
<td>MER, 0.5 mg, Day +14</td>
<td>68</td>
<td>Tumors 0 3 8 14 23 34 43 51 56 58 58 61</td>
</tr>
<tr>
<td></td>
<td>MCA, Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>MER, 0.5 mg, Days -14, +14</td>
<td>70</td>
<td>Tumors 0 4 7 10 18 27 33 37 41 42 43 46</td>
</tr>
<tr>
<td>VI</td>
<td>MER, 0.5 mg, +16</td>
<td>73</td>
<td>Tumors 0 0 5 10 18 24 30 40 46 46 50 50</td>
</tr>
<tr>
<td></td>
<td>MCA, Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII</td>
<td>MER, 2.5 mg, Day 0</td>
<td>24</td>
<td>Tumors 0 0 0 0 5 7 9 12 15 16 16 19</td>
</tr>
<tr>
<td></td>
<td>MCA, Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIII</td>
<td>MER, 2.5 mg, Days 0 and +61</td>
<td>23</td>
<td>Tumors 0 0 0 1 2 4 8 13 13 13 14 14 15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VII + VIII Combined</td>
<td>47</td>
<td>Estimated tumor % 0 0 0 3 20 32 50 73 82 88 88 100</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cumulative numbers of animals developing tumors and dying without detectable tumors.

<sup>b</sup> The estimated tumor percentage determined statistically by life table analysis, as mentioned in text.
The protective effect of MER at the 0.5-mg dose level varied with the times of administration. Compared to the control, little protection was seen in Groups III or IV (p ~ 0.2 and p < 0.09, respectively); some protection was seen for Group VI (p ~ 0.03) and a significant protection was seen for Group V (p < 0.01).

The two 2.5-mg dose groups (Groups VII and VIII) could be combined as a common "2.5-mg dose group" which gave statistically significant protection relative to the control (p < 0.005). The life table graphs and the fitted curves are shown for the control group and the "2.5-mg dose group" in Chart 2. From these plots and the likelihood ratio tests, it appears that the difference in these groups is primarily due to a lengthened latent period of the 2.5-mg dose group before tumor appearance but that following the lengthened latent period the rates of tumor occurrence were similar in both groups.

As was true for the BCG-treated mice, there were many deaths among animals not bearing tumors. The cause for this was not established. Since, unlike BCG, MER is not infectious, the cause could not be BCG bacteremia. Many of the deaths occurred in cages where there had recently been other deaths, making it seem possible that other bacterial or viral infections were the cause, possibly potentiated in MER-treated hosts. Consistent with this possibility is the higher proportion of deaths in the "2.5-mg dose group."

DISCUSSION

Immunodepression following chemical carcinogen or oncogenic virus administration has been well demonstrated (19, 22-24). It extends both to the cellular (14, 22) and humoral (24) immune systems. The immune suppression induced by carcinogens or by the presence of tumors can be reversed by nonspecific stimulation of the immune system (28) with a number of bacterial materials, such as BCG (23), and other mycobacteria and their derivatives, such as MER (25). Gram-negative endotoxin-producing bacteria, a number of gram-positive bacteria (such as staphylococci, streptococci, corynebacteria, and Listeria) and macromolecules (such as nucleic acids and synthetic polynucleotides) have also been used (see Ref. 28).

These agents have been less extensivly studied for their capacity to prevent or decrease the induction of new tumors. In the course of a comprehensive examination of the immune activation properties of BCG, Old et al. (12) demonstrated a slight, transient protection of mice both against chemical carcinogen-induced tumors and against spontaneous mammary tumors and leukemias. Lemonde and Clode-Hyde (8) demonstrated a reduction in polyoma virus-induced tumors using BCG. Nilsson et al. (11) obtained a significant decrease in the incidence of 90Sr-induced osteogenic tumors in CBA mice given BCG. Similarly, Piessens et al. used BCG to obtain transient protection against the induction of dimethylbenzanthracene-produced mammary tumors in rats (14) although, when BCG was given after the appearance of mammary tumors, enhanced growth was obtained (15). Bekierkunst et al. (1) have reported a significant decrease in urethan-induced lung adenomas in mice by the prior i.v. injection either of living BCG or of a chemically defined glycolipid from tubercle bacilli, cord factor (trehalose 6,6-dimycolate). A strong protection against production of both spontaneous mammary tumors and of precancerous hyperplastic alveolar nodules was obtained in C3H mice that received MER (26).

Using polynucleotides (4) or polycarboxylate (2), a number of investigators have demonstrated a similar protection against virus-induced mammary tumor production. This protection may be mediated at least in part by interferon action on the virus involved. However, such is less likely to be the case in the temporary abrogation of skin carcinogenesis by MCA obtained by the use of polyinosinic-polycytidylic acid by Degre and Elgio (7) (although the mediation of endogenous viruses in chemical carcinogenesis cannot be ruled out).

In the present study, protection against MCA-induced tumor formation was obtained with BCG and MER. The patterns of protection were different with these 2 agents. In the BCG experiment (Experiment 1), although tumors began appearing at the same rate after MCA injection in all of the experimental groups, some groups showed a subsequent decrease (Group III + IV + V) or increase (Group VI) in the rate of tumor appearance compared to the controls (Chart 1). In contrast, in the MER experiment (Experiment 2) the latent period of the appearance of 1st tumors was lengthened in Group VII + VIII, compared to the controls, but the subsequent rate of tumor appearance was the same in both groups (Chart 2). These differences may be due to different susceptibilities to the immunoprophylactic effects of BCG in subpopulations of mice, contrasted to a more uniform susceptibility to the effects of MER.

This protection by BCG and MER as in similar studies (7, 12, 14) was only a transient one, all groups tending to 100% incidence of tumors. There was no detectable difference in the typical growth rates of tumors in the various groups once they had become palpable. Whether there was any difference in the immunogenicity of the tumors arising in these groups (i.e., whether in BCG- or MER-treated hosts there was a selection for less immunogenic tumors) was not determined. It is possible that the effects of BCG and MER may be different if lower doses of MCA or weaker carcinogens are used or if different dosage schedules of the immunostimulants are administered.

In clinical studies, injection of BCG into melanoma lesions has been demonstrated to produce regression, not only of injection-treated lesions but also of those not given injections (10, 18). Administration of large amounts of BCG as a sequel to drug-induced remission in a number of leukemia cases has resulted in complete remission for up to 5 years (9).

In the animal studies reported here, however, an enhanced rate of appearance of new tumors was obtained by administration of BCG to mice at the time when they were just beginning to produce palpable tumors (Experiment 1, Group VI). Since such enhancement was not obtained in those animals treated with BCG both 14 days prior to MCA and at the time of appearance of the 1st tumor (Group VI), it is possible that prior sensitization to BCG may be an important prerequisite for such an immunotherapeutic use of systemic BCG. Piessens et al. (15) also have reported enhanced growth of dimethylbenzanthracene-induced rat mammary tumors.
when BCG was administered after the appearance of tumors. On the basis of those observations, caution is indicated in the systemic administration of BCG to humans bearing tumors, particularly if they have not previously been immunized to BCG.

The immunoprophylactic effect of MER appeared to be dependent on the schedule of administration at the 0.5-mg dose. This did not seem to be because the effect of the MER was lost over the latent period of the tumor induction, since in Group V the animals were given 0.5 mg MER at monthly intervals up to the time of tumor induction with no increased protection. At the 2.5-mg dose level, a fairly striking although still temporary protection was noted. Whether the significant activity only at the higher level is due to low activity of the MER preparation used must still be determined.

From the experiments reported here, it is felt that, while administration of either BCG or MER seems to be capable of exerting an immunoprophylactic effect against tumor induction, this effect is a small one. It is possible that these effects might be heightened by alterations in the dosage of MCA or in the dosage schedules of BCG or MER.

The enhanced tumor growth that we have seen with one particular regimen of BCG administration (Experiment 1, Group VI) emphasizes the need for caution in the clinical use of systemic BCG.

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