Bacillus Calmette-Guérin Vaccination and Skin Tumor Promotion with Croton Oil in Mice

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SUMMARY

Charles River mice were painted once with dimethylbenzanthracene (tumor initiation), followed by repeated applications of croton oil (tumor promotion). The i.v. injection of Bacillus Calmette-Guérin (BCG) 3 weeks prior to the initiation of croton oil treatment reduced the incidence and rate of appearance of both benign and malignant skin tumors. After 50 applications of croton oil, it was possible to demonstrate that the BCG-treated mice without tumors had a smaller spleen index and showed a greater cell-mediated immunological responsiveness to purified protein derivative than did the BCG-treated mice with the greatest number of tumors in the same group. Tubercle organisms were isolated from spleen cultures of the BCG-treated mice, regardless of their capacity to respond to in vivo and in vitro tests with purified protein derivative as antigen.

INTRODUCTION

Stimulation of the reticuloendothelial system by the BCG has been experimentally performed with varying degrees of success during attempts to prevent or treat malignant disease (8, 12, 15, 17, 24). It has been proposed that BCG vaccination exerts its effect on neoplasms through nonspecific enhancement (8, 12, 15, 17, 24). It has been proposed that BCG vaccination exerts its effect on neoplasms through nonspecific enhancement (8, 12, 15, 17, 24). It has been proposed that BCG vaccination exerts its effect on neoplasms through nonspecific enhancement (8, 12, 15, 17, 24). It has been proposed that BCG vaccination exerts its effect on neoplasms through nonspecific enhancement (8, 12, 15, 17, 24).

MATERIALS AND METHODS

Three groups of Charles River mice (Charles River Mouse Farm, North Wilmington, Mass.), each consisting of 30 females, 6 to 8 weeks of age, were used for this study. All animals were shaved, and the dorsal surface of each was painted once with 125 μg DMBA (3) (Eastman Organic Chemicals, Rochester, N. Y.), low doses of which initiate skin cancer in mice (6). Animals in Group 1 received no further treatment and served as controls. Beginning 3 weeks after their exposure to DMBA, Group 2 mice were treated biweekly for 25 weeks with topical applications of 0.6 mg croton oil (S. B. Penick & Co., New York, N. Y.), a powerful promoter of mouse skin cancer (4). DMBA and croton oil were applied in 0.2 ml acetone with a Biopet (Schwarz/Mann, Orangeburg, N. Y.). Animals in Groups 2 and 3 received identical treatment; however, 2 days after DMBA application, Group 3 mice received 1.0 mg of BCG in 0.1 ml 0.09% NaCl solution i.v. Six weeks after the 1st injection of BCG, an i.v. booster injection of 0.25 mg of BCG was given.

Tuberculin hypersensitivity was measured in vivo by footpad tests and in vitro by a macrophage migration inhibition assay, with the use of PPD as antigen. Footpad tests were performed by the injection of 2 μg of PPD in 0.025 ml diluent into 1 hind footpad and of an equal volume of diluent into the other footpad as a control. Footpad thickness was measured at 4, 24, 48, and 72 hr by means of a Schnelltester, as previously described (1). Assays for the release of macrophage MIF by specifically sensitized lymphocytes were performed by the methods described by Yamamoto and Anacker (23), except that peritoneal exudates, induced by 5 ml of mineral oil, injected i.p., were used as the source of cells. PPD, 10 μg/ml, was added to the Sykes-Moore tissue culture chamber for an assay of lymphocyte sensitivity to mycobacteria. We prepared an antigenic extract of tumor by homogenizing DMBA- and croton oil-induced cutaneous tumors from Group 2 mice in an equal volume of phosphate-buffered saline. After homogenization and centrifugation at 1500 × g for 30 min, the supernatant fluid was sterilized by filtration, and the necessary volume adjustments were made for a final protein concentration of 2.5 mg/ml. One hundred μg of this protein extract were used as antigen in the Sykes-Moore migration chambers as an assay to determine the sensitivity of lymphocytes to soluble tumor antigens. The degree of macrophage migration was measured after 36 hr of incubation. MIF results are expressed as a percentage of migration inhibition determined, as previously described (9), by the use of the following formula: percentage inhibition of migration = [1 - (area of migration in presence of antigen/mean area of migration without antigen)] × 100]. All migration measurements were carried out in quadruplicate.
Squamous cell carcinomas were differentiated from papillomas by gross morphological appearance and were verified by random histological examinations. Spleens were aseptically removed, weighed, finely minced, and cultured in oleic acid-albumin agar for the presence of BCG. Spleen indices were calculated according to the method of Simonsen (16). Throughout this study, data are either individually presented for each animal or are recorded as mean ± S.E. of the group responses. Wherever indicated, group means were compared by Student’s t test. All mice were examined at weekly intervals over a 25-week period.

RESULTS

At no time did Group 1 mice develop tumors (Table 1). After seven weeks of tumor promotion, 34% of the mice in Group 2 (DMBA + croton oil) developed 5.2 ± 0.5 tumors/tumor-bearing animal. Although the latency period (7 weeks) was the same in Groups 2 and 3, at all weekly examinations Group 2 mice had a significantly greater number (p < 0.001) of papillomas per mouse than did Group 3 mice (Chart 1). By 25 weeks, all Group 2 mice had developed 16.3 ± 1.8 tumors/mouse. Similar observations of mice in Group 3 (DMBA + croton oil + BCG) revealed that 23% of the animals were tumor bearing, with a mean of 1.3 ± 0.2 tumors/tumor-bearing mouse at 7 weeks. At 25 weeks, 77% were tumor positive, with a mean of 6.0 ± 1.9 tumors/tumor-bearing mouse (Table 1; Chart 1).

Five mice from both Groups 1 and 2, 5 BCG-treated mice without tumors (Group 3A), and 5 of the BCG-treated mice with the maximum number (9 to 46 papillomas/mouse) of tumors (Group 3B) were footpad tested with PPD. Although the animals in Group 3B showed some reactivity to PPD, these levels were significantly less (p < 0.001) than those observed for Group 3A animals (Chart 3). These data, in addition to other in vitro measurements for each animal, are summarized in Table 2 and Chart 2.

Animals in Group 1 (DMBA) showed no significant macrophage migration inhibition, with PPD as antigen. On the other hand, Groups 3A and 3B were able to produce MIF in response to PPD stimulation in vitro. However, the levels of macrophage inhibition recorded for Group 3A tumor-negative animals were at least qualitatively greater than those of Group 3B (Table 2). The degree of tumor-specific, antigen-induced macrophage inhibition for Animal 4, Group 3A, was comparable to that induced with PPD (67 and 75%, respectively). For Animal 3, (Group 3B), which was similarly tested, the levels of macrophage inhibition effected with tumor antigen and PPD were 8 and 28%, respectively.

The spleen index of the Group 3B mice exceeded that of mice in Group 3A; Group 3 mice had spleen indices greater than 1.30 [upper limits of normal as defined by Simonsen (16)]. However, the spleen index of Group 2 mice (no BCG, tumor positive) was 1.19.

Tubercle bacilli were cultured from all spleens of animals given BCG injections, regardless of the presence or absence of skin tumors. The spleens of the BCG-treated tumor-bearing mice could not be histologically differentiated from those of the BCG-treated tumor-free mice. There was no evidence of septic splenitis in either group. Both sets of spleens histologically showed marked hypertrophy and hyperplasia of lymphoid follicles (Table 2).

DISCUSSION

It has been suggested that the establishment and proliferation of malignant neoplasms may result from the inability of the host to recognize the tumor immunologically as foreign and destroy it (5, 8). Experiments have shown that substances that by themselves can cause cancer (complete carcinogens) (21) and some substances that can act only to promote cancer, such as croton oil (2, 20), are immunosuppressive agents. Evidence indicates that both animals receiving carcinogens (21) and tumor-bearing humans (11, 18) are at least partially immunoincompetent. It was further demonstrated that an immunostimulant such as BCG can act as both an immunoprophylactic and an immunotherapeutic agent against various experimental and clinical cancers (10). Working with several strains of mice and various tumors, Old et al. (13, 14) theorized that the success of BCG as an immunoprophylactic agent was directly related to the fact that the immunological surveillance mechanism of the animals is enhanced by BCG. The results of our experiments suggest that vaccination with BCG or even the presence of tubercle bacilli in a host

<table>
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<tr>
<th>Group</th>
<th>Treatment</th>
<th>Response after 25 wk of promotion</th>
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<tr>
<td></td>
<td>DMBA initiation at 0 time (μg)</td>
<td>BCG (mg)</td>
</tr>
<tr>
<td>1</td>
<td>125</td>
<td>None 0 time</td>
</tr>
<tr>
<td>2</td>
<td>125</td>
<td>None 0 time</td>
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<tr>
<td>3</td>
<td>125</td>
<td>1 (i.v.) 0.25 (i.v.)</td>
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a Mean ± S.E.; numbers in parentheses, group range.
b p < 0.001.
Chart 1. The incidence of papillomas (mean ± S.E.) in mice as a function of time during croton oil promotion. Group treatments consisted of DMBA + croton oil + BCG (●) (30 mice) or DMBA + croton oil (▲) (29 mice).

Chart 2. Mean footpad reactivity ± S.E. to 2 µg of PPD of tumor-negative (●) and tumor-positive (▲) mice (Table 2, Group 3) at 22 weeks after the 2nd BCG injection. The DMBA mice (■) were also tested with PPD and served as negative controls.

following vaccination with BCG does not guarantee protection against further cancer. It is only when the host is able to demonstrate strong, cell-mediated immunological reactivity to the tubercle organisms that some degree of protection against cancer can be anticipated.

The higher spleen index in the BCG-treated tumor-bearing mice (Group 3B) may be due to the inability of these mice to destroy the tumors, although the tumors are recognized as foreign. Therefore, the reticuloendothelial systems of tumor-bearing animals were continually stimulated by the foreign antigens (tumors?). An immunological defect may exist in these animals at the efferent level of responsiveness.

In these studies, croton oil in addition to promoting the development of papillomas and carcinomas could have prevented, by its immunosuppressive effect, the immunostimulatory activity of BCG in mice with an intrinsically greater susceptibility to croton oil-induced immunosuppression. Conversely, those mice able to resist tumor establishment despite the immunosuppressive effect of croton oil were also able to retain their reactivity to BCG, as demonstrated by the footpad tests and MIF production in response to PPD.

Vaage et al. (22) reported macrophage migration inhibition when peritoneal exudate cells from mice previously sensitized to fibrosarcoma were incubated with soluble tumor antigen extracts from syngeneic mice with methylcholanthrene-induced fibrosarcomas. These results correlate with ours, which showed that sensitized Mouse 4 in Group 3A had far greater macrophage migration inhibition and reactivity to PPD and tumor antigen extract than did Mouse 3 in Group 3B, which responded poorly to both PPD and tumor antigen extract.

Although the presence of BCG organisms was demonstrable within hypertrophied spleens of all BCG-treated tumor-bearing mice, it was possible to evoke only a relatively weak
immunological response to PPD in Group 3B mice. The results of our experiments suggest that, in those animals showing strong PPD sensitivity, BCG may have prevented tumor proliferation through enhancement of the immunological surveillance mechanism.

Conflicting clinical reports (3, 6, 7, 19) concerning the efficacy of BCG vaccination for cancer immunoprophylaxis and immunotherapy in humans may be resolved if, as our experiments suggest, BCG is an effective immunoprophylactic agent against cancer only as long as the host is able to mount a strong, cell-mediated response to PPD and the tumor is immunogenic.

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REFERENCES

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