Protection of Mice against 7,12-Dimethylbenz(a)anthracene-induced Skin Tumors by Immunization with a Fluorinated Analog of the Carcinogen

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ABSTRACT

5-Fluoro-12-methylbenzanthryl-7-acetic acid (5-FMBAAA) is an analog of the carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) with little or no carcinogenic activity. CD-1 mice immunized with 5-FMBAAA conjugated to bovine serum albumin (BSA) developed serum antibodies capable of binding DMBA. As a means of testing whether this immunization protected against DMBA-induced tumors, a low-dose carcinogenesis model system was developed, entailing the repeated skin application of 25 ng DMBA in dodecane alternating with applications of the tumor promoter, phorbol myristate acetate. Mice immunized with the 5-FMBAAA:BSA conjugate and subsequently exposed to this low-dose regimen for 40 weeks developed significantly fewer skin tumors (0.23 papilloma/mouse) than did unimmunized mice, mice immunized with BSA, or mice immunized with an unconjugated mixture of BSA and 5-FMBAAA (0.47 to 0.54 papilloma/mouse). Immunization did not reduce tumor incidence in mice treated with phorbol myristate acetate alone. The results suggest that, when mice are exposed to a carcinogen at doses low enough to approach environmental levels, immunization against the carcinogen can provide specific protection.

INTRODUCTION

Most of us are exposed daily to small quantities of PAHs in the environment, and it is therefore likely that these compounds contribute to the incidence of human cancer. The present study is part of an effort to investigate immunization as one possible approach to protection against carcinogenic PAHs. Pioneering studies by Creech et al. (4-6) demonstrated that PAH carcinogens, when rendered antigenic by conjugation to protein carriers, could elicit antibodies in experimental animals. These studies failed to demonstrate significant protection when immunized mice were exposed to PAH carcinogens (3, 10), but the use of high doses of PAHs, which was standard practice in experimental carcinogenesis, until the later discovery of tumor promoters, may have overwhelmed the neutralizing capacity of any antibodies that the mice produced (15).

Our current investigations, which focus on the protective potential of immunization, have utilized a 2 step approach: (a) to seek an analog of PAH carcinogens that is itself noncarcinogenic but which can induce animals to produce antibodies capable of binding carcinogenic PAHs; (b) to determine whether immunization with this analog can protect animals against the carcinogenicity of PAH's administered in low doses. 5-FMBAAA, an analog of the potent carcinogen DMBA, was chosen for study because of evidence that fluorination at position 5 abrogates the carcinogenicity of benzanthracene derivatives (16). 5-FMBAAA failed to induce tumors in mice after s.c. injection; when conjugated to the protein carrier BSA, it elicited broadly reactive antibodies that could bind several PAH carcinogens, neutralize the in vitro cytotoxicity of DMBA, and reduce the body burden of benzo(a)pyrene in immunized mice exposed to this carcinogen in vivo (14, 15). The present study was undertaken to determine whether the immune response that 5-FMBAAA:BSA conjugates elicit is protective; i.e., can it prevent tumors in animals exposed to PAH carcinogens? To avoid exceeding the neutralizing capacity of the immune response, the study required a regimen capable of inducing tumors at very low doses of carcinogens, doses not greatly exceeding environmental levels of PAH's. We found that repeated cutaneous applications of ng quantities of DMBA dissolved in dodecane, alternating with applications of the potent tumor promoter PMA, constitute a satisfactory low-dose carcinogenesis model and that, when this model is used, immunization with 5-FMBAAA:BSA protects mice against DMBA-induced tumors.

MATERIALS AND METHODS

Chemicals and Antisera. DMBA was obtained from Eastman Organic Chemicals, Rochester, N. Y.; DMBA solutions were prepared weekly in the dark, stored in the dark at 4°C in dodecane at a concentration of 0.125 µg/ml, and handled under subdued light. n-Dodecane was obtained from Sigma Chemical Co., St. Louis, Mo. PMA was obtained from Consolidated Midland Corp., Brewster, N. Y., and was stored at −20°C in acetone. [14C]DMBA (115 mCi/mmol) was obtained from New England Nuclear, Boston, Mass. Freund's complete and incomplete adjuvant and rabbit IgG antibodies against mouse γ-globulin were obtained from Cappel Laboratories, Inc., Cochranville, Pa. 5-FMBAAA:BSA conjugates (12% 5-FMBAAA by weight) were prepared as described previously (15).

Immunization of Mice. Male CD-1 mice were purchased from Charles River Mouse Farms, North Wilmington, Mass.; they were housed in stainless steel cages with pine chip bedding and fed Purina laboratory chow and water ad libitum. For immunization, a PBS solution containing 5-FMBAAA:BSA conjugate, 7 mg/ml, was emulsified with 2 volumes of Freund's complete adjuvant; the emulsion was injected i.p. into 9-week-old mice, and the injection was repeated 2 and 4 weeks later.

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3 The abbreviations used are: PAH, polycyclic aromatic hydrocarbon; 5-FMBAAA, 5-fluoro-12-methylbenzanthryl-7-acetic acid; DMBA, 7,12-dimethylbenz(a)anthracene; BSA, bovine serum albumin; PMA, phorbol 12-myristate 13-acetate, also known as 12-O-tetradecanoylphorbol-13-acetate; PBS, phosphate-buffered saline (0.01 M sodium phosphate in 0.15 M NaCl, pH 7.4).

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In preliminary experiments, 0.1- and 0.3-ml doses were compared; antibody responses were slightly (but not significantly) higher with the 0.3-ml dose, but this dose at times caused significant mortality. In contrast, the 0.1-ml dose was well tolerated, causing no deaths immediately and permitting greater than 90% survival during a subsequent 7-month observation period. The protection experiments were therefore performed with the latter dose. Thirty-two days after the last injection of antigen emulsion, the mice were reimmunized with 0.53 mg of antigen in 0.15 ml of PBS without adjuvant. Mice in control groups remained unimmunized, or they were immunized with BSA alone or with a mixture of unconjugated 5-FMBAAA and unconjugated BSA. Unconjugated 5-FMBAAA is not water soluble but could be dissolved in the Freund’s adjuvant used to prepare the antigen emulsion. For the final injection of antigen, which did not utilize adjuvant, 5-FMBAAA dissolved in dimethyl sulfoxide was mixed with an equal volume of a PBS solution of BSA. Mice immunized in preliminary experiments (but not those actually used in the protection experiments) were bled periodically by orbital puncture to determine serum levels of DMBA-binding antibody. The assay was a modification of a double-antibody precipitation method described previously (14). Briefly, 20 μl of mouse serum, 400 μl of PBS, and 150 μl of dioxane:water (1:1) containing 0.015 μg of 14C]DMBA were mixed and allowed to stand for 40 min at room temperature. Rabbit IgG antibodies against mouse γ-globulin (1.6 mg specific antibodies in 0.4 ml water) were then added, and the precipitates which formed after overnight refrigeration were washed once with PBS:dioxane (20:1), washed twice with PBS, and then dissolved in NCS solubilizer (Amersham Corp., Arlington Heights, I11.) for measurement of antibody-bound radioactivity by liquid scintillation spectrometry.

**Tumor Experiments.** Mice were housed 10/cage (except for one cage of 6). To avoid order effects, the cages for each experimental group were distributed evenly throughout the cage rack, and the order in which the cages were pulled from the rack for treatment was reversed weekly. Two days after the third immunization (29 days after initial immunization), the backs of the mice were shaved with electric clippers. All mice were shaved periodically thereafter whenever some of them exhibited substantial regrowth of hair (no differences in hair growth were observed among any of the experimental groups).

The day after they were first shaved, the mice received their first skin treatment, consisting of 12 μg PMA in 0.2 ml acetone, and 4 days after this they were treated with either 0.2 ml dodecane alone or 0.2 ml dodecane containing 25 ng DMBA. This was the first of regular Monday, Wednesday, and Friday applications of DMBA or dodecane, which alternated with Tuesday and Thursday applications of PMA throughout the remainder of the experiment. Skin treatments were omitted on days when the mice were reshelved. Also, after their final immunization, which was given 1 month after skin treatments were started, the mice received no PMA or DMBA for 5 and 6 days, respectively. The use of 12 μg of PMA, which is slightly higher than the 10- to 10.5-μg doses more typically used in CD-1 mice, and the administration of an anticipatory dose of PMA before any DMBA was administered were based on the possibility that the inflammatory actions of this promoter might cause exudation of antibody molecules into the skin and thus increase the opportunities for these antibodies to intercept DMBA molecules that were applied subsequently. Whether this potential advantage was actually realized is unknown; on gross inspection, the skin of these mice did not differ perceptibly from other mice in this laboratory that have been exposed to 10-μg PMA doses. The development of skin tumors was recorded weekly, and papillomas greater than 2 mm in diameter which persisted for at least 2 weeks were included in the cumulative total. Occasional tumors were removed at random for histological verification; in one additional case, progression of a papilloma to a carcinoma was suspected, but histological examination failed to confirm this diagnosis. The tumor incidence was expressed as the average number of papillomas per mouse; this was almost identical to the fraction of mice with papillomas, since except for 2 mice which developed 2 tumors each (both in the group immunized with BSA and treated with DMBA plus PMA), no mouse developed more than a single tumor. During the experiment, moribund mice were killed and autopsied at random. At the end of the experiment, all surviving mice were killed and subjected to gross autopsy. The autopsies were performed by Dr. A. Handler, Findley Research, Inc., Assonet, Mass.

**Experimental Design and Statistical Analysis.** Because of the low DMBA dose that we planned to use, we anticipated that none of the treatment groups would exhibit more than a low rate of tumor development and that therefore the experiment must be designed to permit statistically meaningful comparisons between groups even under these circumstances. Treatment groups of conventional size (e.g., 30 mice) appeared inadequate for this purpose; accordingly, most of the treatment groups that we used were assigned more mice than this. In addition, analysis of the data by the commonly used method of comparing tumor incidences at the end of a predetermined interval, while statistically valid, promised to be unsatisfactory, since a short interval might prove insufficient for significant differences between groups to have yet emerged, whereas a long interval might permit too few mice to survive for useful comparisons, particularly since long-term promotion with high doses of PMA is known to cause significant mortality (2). This dilemma was avoided by using the log-rank test of Peto et al. (17) to compare cumulative tumor incidences in the different groups. This test derives its power from the fact that it compares groups not at a single time but throughout an experiment, and the differences between groups to have yet emerged, whereas a long interval might permit too few mice to survive for useful comparisons, particularly since long-term promotion with high doses of PMA is known to cause significant mortality (2). This dilemma was avoided by using the log-rank test of Peto et al. (17) to compare cumulative tumor incidences in the different groups. This test derives its power from the fact that it compares groups not at a single time but throughout an experiment, in increments between groups even under these circumstances. Treatment groups of conventional size (e.g., 30 mice) appeared inadequate for this purpose; accordingly, most of the treatment groups that we used were assigned more mice than this. In addition, analysis of the data by the commonly used method of comparing tumor incidences at the end of a predetermined interval, while statistically valid, promised to be unsatisfactory, since a short interval might prove insufficient for significant differences between groups to have yet emerged, whereas a long interval might permit too few mice to survive for useful comparisons, particularly since long-term promotion with high doses of PMA is known to cause significant mortality (2). This dilemma was avoided by using the log-rank test of Peto et al. (17) to compare cumulative tumor incidences in the different groups. This test derives its power from the fact that it compares groups not at a single time but throughout an experiment, in increments.
so that every tumor-bearing mouse, and the time it first exhibits a tumor enters into the analysis. This property permits experiments to be extended until most or all animals have died, since data gathered toward the end of the experiment supplement rather than replace data gathered earlier. We chose to continue our study until none of the groups contained more than 15% of its initial number. At this point (40 weeks), the paucity of remaining mice made any further impact on the significance of the results highly unlikely, and the experiment was terminated. The composition of the experimental groups is presented in Table 1, which shows their initial size, their size at 40 weeks, and their median survival duration.

RESULTS

Antibody Responses. Mice immunized with 5-FMBAAA:BSA conjugates developed antibodies capable of binding DMBA (Chart 1). Antibody concentrations reached a peak about 2 months after immunization was begun, declined, and then persisted at lower levels for an additional 4 to 9 months in the absence of further immunization. Although persistence of antibodies could reasonably be attributed to the initial immunizations, an alternative possibility was that environmental PAH carcinogens that the mice subsequently encountered combined in vivo with tissue proteins to form complete antigens and that these antigens were responsible for maintaining immunity. To determine whether DMBA could serve as this type of antigenic stimulus in mice previously immunized with 5-FMBAAA:BSA, we gave 4 such mice injections of DMBA at Day 159 [0.5 mg i.p. in 0.1 ml of water:dimethyl sulfoxide (1:1)]. None of the mice exhibited a rise in antibody concentration when their sera were assayed 12 days later, the levels before and after DMBA exposure averaging 33 ± 8 (S.E.) and 25 ± 4 ng DMBA bound per ml, respectively. Two mice reimmunized on Day 159 with 5-FMBAAA:BSA exhibited rises in antibody levels from 25 ± 1 to 40 ± 15 ng DMBA bound per ml. While the negative results obtained with DMBA do not exclude the possibility that exposure to environmental PAH's may have helped to maintain immunity, they suggest that the major factor responsible for the long-term persistence of anti-DMBA antibodies in mice immunized with 5-FMBAAA:BSA conjugates was the efficacy of the initial immunization regimen with the conjugates.

Carcinogenesis Studies. Two major findings emerged from this experiment: (a) 25 ng of DMBA in dodecane, administered repeatedly in alternation with PMA, induced skin papillomas in mice over the course of 40 weeks; (b) mice immunized with 5-FMBAAA:BSA conjugates developed significantly fewer tumors than did unimmunized mice or mice subjected to any of the control immunization regimens. These results are depicted in Chart 2A, which shows that the tumor incidence curves for all DMBA-treated mice begin to rise progressively after about 17 weeks of the DMBA-PMA treatment. The rises are slow initially for all groups, but differences gradually become apparent, the curves rising more steeply for the control groups than for the mice immunized with 5-FMBAAA:BSA. Among the controls, tumor incidence rates were higher in mice immunized with BSA or with the BSA:5-FMBAAA mixture than in unimmunized mice, suggesting a possible, slight cocarcinogenic effect of these immunizations. The differences were small, however, and not statistically significant.

Chart 1. DMBA-binding antibodies in sera of 13 mice immunized with 5-FMBAAA:BSA or 9 mice immunized with BSA alone. In general, 3 mice were randomly selected from each group for each bleeding, each serum was assayed in duplicate, and points indicating mean values were plotted; bars, S.E. For some early bleedings, only a single mouse was bled; these values are plotted without bars. Arrows, days on which antigen was injected. The mean value for ng DMBA bound per ml for the sera of 7 unimmunized mice was 18 ± 4.5. The immunized and unimmunized mice used in this phase of the study received no subsequent skin treatments, and none developed skin tumors.

Chart 2. The effect of immunization on skin tumor induction by DMBA. Tumors per mouse were calculated for each treatment group by dividing the number of tumors that appeared each week by the number of mice at risk during that week and adding these values cumulatively. A, cumulative tumor incidence in mice treated with DMBA plus PMA. The tumor incidence in mice immunized with 5-FMBAAA:BSA was significantly lower than the incidence in each of the other groups (p < 0.05). These incidence curves represent the following total number of tumors that appeared in each of the treatment groups (original size of the groups in parentheses): 5-FMBAAA:BSA-immunized mice, 11(60); unimmunized mice, 31(96); BSA-immunized mice, 26(80); and mice immunized with the mixture of BSA and 5-FMBAAA, 9(30). B, "excess" tumor incidence in mice receiving DMBA. These values were derived from the values in A by subtracting from them the tumor incidence in comparably immunized mice that received PMA alone.
In contrast to its effects in DMBA-treated mice, immunization with 5-FMBAAA:BSA did not reduce the tumor incidence in mice treated with PMA alone. After 40 weeks of PMA treatment, mice immunized with 5-FMBAAA:BSA exhibited a cumulative tumor incidence of 0.20 tumor/mouse, while unimmunized mice exhibited 0.13 tumor/mouse and BSA-immunized mice had 0.10 tumor/mouse. The total numbers of tumors that this represents (with the original size of the treatment group in parentheses) are 6(40), 5(50), and 3(40), respectively; these results do not differ significantly. Thus, the protective effect of immunization observed in DMBA-treated mice was not seen when skin tumors were elicited without this carcinogen. Since PMA can elicit a small number of tumors even in the absence of DMBA, it is likely that tumors of this type comprised part of the total observed in mice that received DMBA plus PMA. To determine the magnitude of the protection that immunization afforded against tumors initiated by DMBA, it would be necessary to exclude tumors not initiated by DMBA from the total. Chart 2B represents an attempt to do this; for each immunization regimen, it shows the tumor incidence curve derived by subtracting tumor incidence in the PMA-treated group from tumor incidence in the corresponding group treated with DMBA plus PMA. If an assumption is made that repeated exposure to 25-ng doses of DMBA did not alter the incidence of tumors that were not initiated by DMBA, the data in Chart 2B indicate that 5-FMBAAA:BSA immunization provided approximately 100% protection against DMBA-initiated tumors. Even without this assumption, the data indicate that the 5-FMBAAA:BSA-immunized mice behaved empirically as though they had not been exposed to DMBA.

At the end of the experiment, the surviving mice were killed and autopsied to determine whether any mouse had developed widespread neoplasia or whether any treatment group consistently exhibited neoplasia in particular organs other than skin. Neither of these results was observed.

**DISCUSSION**

Environmental carcinogenesis and the means to prevent it are areas of investigation that may benefit from experimental model systems that utilize carcinogens in doses low enough to approach environmental levels. In the present study, we found that repeated skin applications of 25 ng of DMBA were capable of inducing skin tumors in CD-1 mice. Although the use of the potent tumor promoter PMA undoubtedly contributed to this result, a factor of equal or possibly greater importance may have been the use of dodecane as a vehicle for the DMBA. Bingham and Falk (1) have reported that, when dodecane was used as a solvent, repeated miniscule doses of benzo(a)pyrene induced skin tumors in C3H mice in the absence of other cocarcinogens or promoters. In their study, doses as low as 10 ng of benzo(a)pyrene were tumorigenic, 21% of mice developing skin tumors with an average latency of 80 weeks. In the present study, a slightly higher dose of DMBA induced tumors in about 45% of mice within 40 weeks. It is likely that even smaller doses might be effective, particularly if highly sensitive mouse strains, such as the Sencar strain (9), are used.

Utilizing 25-ng doses of DMBA, we found that prior immunization of mice with 5-FMBAAA:BSA prevented tumor induction by this carcinogen. That the observed protection was the result of DMBA-specific immunity is suggested by the presence of DMBA-binding antibody and its persistence long enough to account for the long-term protection observed, the absence of protection by immunization with BSA alone, the absence of protection by 5-FMBAAA when this compound was not rendered antigenic by conjugation to a macromolecular carrier, and the absence of protection by 5-FMBAAA:BSA against skin tumors not induced by DMBA. These data do not exclude the possibility that the injections of 5-FMBAAA:BSA also induced metabolic changes which might have operated independently or synergistically with the antibody to affect the skin response to DMBA administered 6 days to many months afterward. Such changes, if they contributed to the long-term protection observed, would be of exceptional interest. To date, assays performed at a few of the shorter intervals after immunization have failed to reveal an effect of immunization on the ability of epidermal homogenates to catalyze the binding of [$^{14}$C]DMBA to DNA or any evidence of induction by 5-FMBAAA:BSA of aryl hydrocarbon hydroxylase activity. Additional intervals, however, and additional facets of DMBA metabolism must be investigated before a metabolic contribution to the observed results can be adequately evaluated; these experiments are in progress.

We have shown previously that appropriate immunization regimens can induce mucosal antibodies capable of binding PAH carcinogens in the respiratory (14) and gastrointestinal (15) tracts. Unfortunately, experiments to determine whether immunization can prevent tumor induction in these organs must probably await the development of a low-dose carcinogenesis model analogous to that used for the skin. How low this dose need be will depend on the quantity of mucosal antibodies achievable and the fraction of administered carcinogen that reaches susceptible mucosal cells. Evidence recently reported by Curtis et al. (8, 18, 19) indicates that, when PAH carcinogens are administered in doses that greatly exceed the binding capacity of an immune response, immunity may not merely fail to prevent tumors but may actually increase their incidence. This evidence suggests that what is protective in an immune response is its ability to intercept PAH molecules and that the inflammatory components of the response are probably not beneficial and may in fact have cocarcinogenic or promoter effects. As a corollary, such evidence also tends to emphasize the protective role of antibody-mediated immunity as opposed to cell-mediated immunity (which was not measured in the present study), since the latter is a much less efficient mechanism for clearing diffusible antigens. Nevertheless, the role of cell-mediated responses deserves further investigation, particularly since cell-mediated reactions against a variety of antigens have been shown to be capable of killing tumor cells in their vicinity (12).

DMBA failed to boost immune responses under the conditions tested in our study; in contrast, Curtis et al. have reported that PAH carcinogens such as benzo(a)pyrene can induce antibody responses in experimental animals (8, 19) and possibly in humans who inhale this carcinogen in cigarette smoke (7). If inadequate immunity is more hazardous than no immunity, it is possible that individuals with low concentrations of antibodies to PAH carcinogens as a result of natural exposure might be the ones to derive the most protection from stimulation of immunity with antigens such as 5-FMBAAA:BSA. If immunity to PAH carcinogens could be induced in humans at a level...
equivalent to the immunity that protected 30-g mice against 25 ng of DMBA in the present study, it should protect against most environmental exposures to the carcinogens.

Whether 5-FMBAAA is the most suitable agent for this purpose is uncertain. Although it has failed to exhibit carcinogenicity in preliminary testing, recent studies with DMBA indicate that fluorine substitution at position 5 might be more likely than substitution at position 2 to abrogate carcinogenicity completely (11); whether 2-fluoro-12-methylbenzanthryl-7-acetic acid and 5-FMBAAA would differ in antigenic efficacy is unknown. Finally, recent technological advances imply the future feasibility of passive administration of antibodies (obviating the need to administer any antigen) as an alternative to active immunization. Thus, for example, protection of the gastrointestinal tract might be achievable by daily ingestion of antibodies of the IgA class if the latter were available in large quantities. "Hybridomas," murine myeloma cells fused to spleen cells from immunized mice, are a current source of large quantities of pure antibodies to a variety of antigens (13), and recombinant DNA techniques should provide additional sources of such antibodies in the future.

REFERENCES

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