Inhibition by Cysteamine-HCl of Oncogenesis Induced by 7,12-Dimethylbenz(a)anthracene without Affecting Toxicity

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SUMMARY

The effects of cysteamine-HCl, a radical scavenger, on 7,12-dimethylbenz(a)anthracene-induced toxicity and oncogenesis were studied in vitro and in vivo. While the addition of cysteamine-HCl to mouse fibroblasts (M2 line) prior to and after the addition of 7,12-dimethylbenz(a)anthracene did not affect the carcinogen-induced toxicity (reduced plating efficiency), the number of transformed foci was markedly reduced. In vivo, the i.p. administration of cysteamine-HCl to Sprague-Dawley rats, prior to and following the injection of 7,12-dimethylbenz(a)anthracene i.v., did not affect the carcinogen-induced adrenal necrosis and lesions of the small intestinal epithelium. Similar treatment did, however, markedly reduce the number of mammary tumors formed. These results suggest that the toxic and oncogenic changes induced by 7,12-dimethylbenz(a)anthracene are due to two different metabolites, and support the concept that the latter effect may be mediated by a radical.

INTRODUCTION

It is now generally believed that most chemical carcinogens must be metabolically converted into chemically reactive "ultimate carcinogens" (14). However, the chemistry of the activation of polycyclic hydrocarbons is not entirely clear. The frequency of transformation and toxicity induced by polycyclic hydrocarbons such as benz(a)anthracene, dibenz(a,h)anthracene, and 3-methylcholanthrene is markedly influenced by alterations of microsomal mixed-function oxidase activity (11) and, in vitro, the microsomal oxidase-derived K-region epoxides of these compounds are more transforming and more toxic than the parent hydrocarbons (8,12). These data suggest that epoxides may be "proximate carcinogens." In contrast, the K-region epoxide of DMBA, although more toxic than DMBA, is less active in transforming mouse fibroblasts (13). Moreover, alterations of microsomal enzyme activity affect DMBA-induced toxicity without influencing transforming activity (11,13). Such results suggest the possibility that there are 2 active metabolites of DMBA; that derived from microsomal enzyme activity may be responsible for the toxic effects of DMBA, while a 2nd metabolite, possibly formed by enzymes not influenced by the commonly used inducers and inhibitors of microsomal enzyme activity, may cause malignant transformation.

Since radical cations (22) and free radicals (15, 16, 18, 19) can be formed from polycyclic hydrocarbons under conditions similar to those found in vivo (15,16), it is conceivable that the postulated 2nd metabolite of DMBA is of this nature. To test this possibility we have studied the effects of cysteamine-HCl, a radical scavenger used effectively to protect animals against damage by ionizing irradiation (3), on the toxic and oncogenic effects of DMBA in vitro and in vivo.

MATERIALS AND METHODS

DMBA was obtained from Eastman Kodak Co., Rochester, N. Y.; cysteamine-HCl was from Calbiochem, Los Angeles, Calif. and Schwarz/Mann, Orangeburg, N. Y.; and thymidine-methyl-3H (2 Ci/mmole) was from New England Nuclear, Boston, Mass. The K-region epoxide of DMBA, DMBA-5,6-oxide, was kindly provided by Dr. P. Sims and Dr. P. L. Grover, Chester Beatty Research Institute, London, England. Female Sprague-Dawley rats (CD line) were obtained from Charles River Breeding Laboratories, Brookline, Mass., and were given food and water ad libitum. The animals were treated when 49 days old (150 to 200 g).

The effects of cysteamine-HCl on DMBA-induced lethality and malignant transformation in mouse fibroblasts (M2 clone) were investigated. The methods used have been described in detail (13). Briefly, cells (10^5, to determine rates of transformation; and 10^6 to determine plating efficiency) were plated in 60-mm dishes. Twenty-four hr later, the cells were treated with DMBA (10 μg/ml) dissolved in acetone (final medium concentration of acetone, 0.5%). Cysteamine-HCl, 1 μg/ml of nutrient medium, was added to the cultures at 20 min before, at the time of, and at 5 and 24 hr after DMBA addition. At 25 hr after addition of DMBA, the fluid was removed and replaced with fresh medium; thereafter, medium was changed twice weekly. At 7 to 14 days, cultures plated with 10^5 cells were fixed with methanol and stained with Giemsa; the colonies were counted to determine plating.

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2 Performed in partial fulfillment of the requirements for the degree of Doctor of Philosophy from the Graduate School of Medical Sciences, Cornell University.
3 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.

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efficiency. At 56 days, dishes plated with $10^3$ cells were examined for transformed foci.

Adrenal necrosis was induced by giving rats i.v. injections of DMBA, 25 mg/kg (9). The animals were sacrificed 72 hr later and the adrenals were examined both grossly and microscopically. To induce mammary tumors, rats were given DMBA, 15 mg/kg, i.v. (10). The tumor incidence was determined 4 and 11 months later. DNA synthesis in rat small intestine was measured 6, 25, and 48 hr after an i.v. injection of DMBA, 25 mg/kg. TdR-methyl-$^3$H (50 µCi/2 µmoles/kg) was given i.v., and 10 min later the intestines were removed and analyzed for thymidine incorporation into DNA as previously described (23). In all of these studies, DMBA was given as an aqueous emulsion as previously described (17). DMBA, 250 mg for adrenal necrosis studies and 150 mg for mammary tumor studies, was dissolved in 15 ml of cotton-seed oil. To this was added 1.2 ml of purified soybean lecithin and 300 mg of Pluronic-F68, obtained from the Upjohn Company, Kalamazoo, Mich. Sterile 0.9% NaCl solution was added to a final volume of 100 ml, and the mixture was then homogenized for 5 min in a Waring Blendor. Cysteamine-HC1 dissolved in 0.9% NaCl solution was given i.p. at a dose of 150 mg/kg at 20 min prior to and 5 and 24 hr after the administration of DMBA. All solutions were given in a volume of 10 ml/kg.

RESULTS

In Vitro Studies. The results in Table 1 show that cysteamine-HC1, while not affecting DMBA-induced lethality (plating efficiency), markedly reduced the number of DMBA-induced transformed foci. The concentration of cysteamine-HC1 used in these studies (1 µg/ml) was the maximum that could be used without any reduction in plating efficiency. The fact that cysteamine-HC1 did not influence DMBA-induced toxicity suggests that the radical scavenger does not interact directly with the microsomal metabolite of DMBA, DMBA-5,6-oxide. In support of this, additional studies showed that cysteamine-HC1 did not affect the toxicity induced by DMBA-5,6-oxide. Plating efficiencies of cells treated with DMBA-5,6-oxide, 0.1 and 0.2 µg/ml, were, respectively, 15 and 9%. The addition of cysteamine-HC1, as described above, to cells treated with DMBA-5,6-oxide, 0.1 and 0.2 µg/ml, resulted in plating efficiencies of 14 and 8.5%, respectively.

In Vivo Studies. The effect of cysteamine-HC1 on DMBA-induced mammary tumors in female Sprague-Dawley rats is shown in Table 2. The results represent a summary of 2 separate experiments and were recorded at both 4 and 11 months after treatment. No tumors were found in control groups receiving either emulsion or cysteamine-HC1. At 4 months, whereas 12 of 18 rats treated with DMBA, 15 mg/kg, developed a total of 17 mammary tumors, only 4 of 17 rats receiving DMBA and cysteamine-HC1 developed a total of 7 tumors. Between 4 and 11 months, an increase in tumor incidence was observed in both groups of animals (Table 2). At 11 months, the number of rats with tumors and the total number of tumors in rats treated with cysteamine-HC1 and DMBA was equivalent to the tumor incidence observed at 4 months in those rats given only DMBA. The data show that cysteamine-HC1 prolonged the latent period for tumor development. Huggins et al. (10) have shown that lower doses of DMBA induce fewer tumors and require longer latent periods. The results, therefore, are consistent with the suggestion that cysteamine-HC1 effectively reduced the concentration of the oncogenic derivative of DMBA.

The effects of cysteamine-HC1 on DMBA-induced toxicity in vivo were also studied. The results (Table 2) show that cysteamine-HC1 did not influence the incidence or severity of the DMBA-induced adrenal necrosis. All adrenals were examined both grossly and microscopically. Except for 1 animal receiving DMBA and cysteamine-HC1 (Table 2), at autopsy all animals had enlarged and hemorrhagic adrenals. These gross observations were confirmed by histopathological examination. Since polycyclic hydrocarbons are preferentially toxic to cell-renewal systems (17), the effects of cysteamine-HC1 on DMBA-induced toxicity in the rapidly proliferating epithelium of rat small intestine were examined (Chart 1). Administration of cysteamine-HC1 prior to and at 5 and 24 hr after the injection of DMBA did not prevent the DMBA-induced inhibition of thymidine incorporation into DNA. The extent of inhibition of thymidine uptake into DNA and the pattern of recovery of DNA synthesis were identical in both the DMBA and the DMBA-cysteamine-HC1 groups. In addition, histological examination showed that the DMBA-induced karyorrhexis and inhibition of mitosis were not affected by cysteamine-HC1 treatment. In contrast, the inhibition of mitosis induced by X-rays in rat small intestine is significantly decreased by cysteamine (7).

In spite of the inhibitory effect of cysteamine-HC1 on thymidine incorporation into DNA of rat small intestine at 6 hr (Chart 1), cysteamine-HC1 did protect rats against X-irradiation. In those rats receiving cysteamine-HC1 (150 mg/kg, i.p.) 2 min prior to X-irradiation (200 R), thymidine incorporation into DNA of small intestine at 6 hr was significantly ($p < 0.02$) higher than in those rats not pretreated with cysteamine-HC1. The results suggest than any protective effect afforded by cysteamine-HC1 against the DMBA-induced inhibition of DNA synthesis should have been detectable.

DISCUSSION

Wattenberg (20) has reported that butylated hydroxytoluene, a radical scavenger, prevents the in vivo effects of DMBA. In those studies, both the toxic (adrenal necrosis) and oncogenic (mammary tumors) effects of DMBA were reduced. DMBA-induced adrenal toxicity is caused by metabolites
Table 2
Effects of cysteamine-HCl on DMBA-induced adrenal necrosis and mammary tumors

<table>
<thead>
<tr>
<th>Treatmenta</th>
<th>No. of rats affected/no. of rats treated</th>
<th>4 mo.</th>
<th>11 mo.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Control emulsion + 0.9% NaCl solution</td>
<td>0/5</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>2. Control emulsion + cysteamine-HCl</td>
<td>0/4</td>
<td>0/10</td>
<td></td>
</tr>
<tr>
<td>3. DMBA-emulsion + 0.9% NaCl solution</td>
<td>18/18</td>
<td>12/18 (17)c</td>
<td>16/18 (38)</td>
</tr>
<tr>
<td>4. DMBA-emulsion + cysteamine-HCl</td>
<td>13/14</td>
<td>4/17 (7)</td>
<td>12/17 (19)</td>
</tr>
</tbody>
</table>

a Either 0.9% NaCl solution or cysteamine-HCl, 150 mg/kg, i.p., was given 20 min prior to and 5 and 24 hr after DMBA or control emulsion was administered i.v. The doses of DMBA used to induce adrenal necrosis and mammary tumors were 25 and 15 mg/kg, respectively.
b Analysis of the incidence of tumor-bearing rats indicates a significant difference ($p < 0.05$) between Treatments 3 and 4 at 4 months, but not at 11 months. Although the total number of tumors in Treatment 4 was less than that in Treatment 3 at both times, the differences are not statistically significant (Student’s $t$ test).
c Numbers in parentheses, total number of tumors.

Chart 1. Effect of cysteamine-HCl on DMBA-induced inhibition of thymidine-methyl-3H incorporation into DNA of rat small intestine.  ▶  Control animals receiving 0.9% NaCl solution and emulsion. The values of the control animals at each time interval were not significantly different and, therefore, were combined and are presented at 0 hr. ◄  Either 0.9% NaCl solution or cysteamine-HCl; ◄  DMBA and cysteamine-HCl. Either 0.9% NaCl solution or cysteamine-HCl (150 mg/kg) was given i.p. 20 min prior to and 5 and 24 hr after administration of either DMBA (25 mg/kg) or control emulsion i.v. The data are expressed as mean ± S.E.

formed by the microsomal mixed-function oxidases (21). Since butylated hydroxytoluene can influence microsomal enzyme activity (6), it may have altered the concentration of toxic metabolites.

In the in vitro studies described herein, in which DMBA-induced toxicity and transformation were studied under identical conditions, cysteamine-HCl effectively reduced the yield of transformed foci without affecting toxicity (plating efficiency). Since increased and decreased activity of microsomal mixed-function oxidases results in respective changes of DMBA-induced toxicity in vitro (11, 13), it appears that cysteamine-HCl does not affect this enzyme system and that the inhibition of DMBA-induced transformation, therefore, involves factors unrelated to microsomal metabolism. Since cysteamine is a radical scavenger, these studies suggest that the mechanism responsible for transformation involves DMBA-derived radicals. Our data obtained from in vivo studies, and those of Wattenberg (20) using butylated hydroxytoluene, are consistent with the proposal that DMBA-induced oncogenic changes may be mediated through radicals. The role of radicals in chemical carcinogenesis has been reviewed (18). Many carcinogens, such as polycyclic hydrocarbons (15, 16, 19, 22), derivatives of N-2-acetylaminofluorene (4), other aromatic amines and amides (2), O,O'-diacetyl-4-hydroxyaminoquinoline 1-oxide (1), and purine-N-oxides (5) readily form radicals, and it has been found that frequently one can correlate carcinogenicity with ease of radical formation (18).

These experiments are by no means conclusive. It is also conceivable that the target site responsible for transformation is inherently different from that for toxicity and can be modified by actions of cysteamine-HCl other than radical scavenging.

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