Induction of Skin Tumors in the Mouse with Minute Doses of 9,10-Dimethyl-1,2-benzanthracene Alone or with Croton Oil*

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Tumors have been induced frequently in the laboratory mouse following exposure to the carcinogenic hydrocarbons (6). In many of these studies, the chemicals were applied continuously and at a relatively high concentration. Cramer and Stowell (5) compared the tumorigenic effectiveness of 20-methylcholanthrene when applied continuously or discontinuously to the skin of mice and concluded that skin carcinomas could be produced by less carcinogen if the chemical was applied intermittently, e.g., once every 2–4 weeks. Recently, it was observed in this Laboratory that exposure of strain DBA mice once to 9,10-dimethyl-1,2-benzanthracene at a concentration too low to produce skin tumors conditioned the hosts to subsequent applications. Thus, one or more additional paintings, each equivalent to the first and applied at intervals as long as 3 months, resulted in the development of numerous skin tumors (7).

While Berenblum (1) first demonstrated the enhancing action of croton oil in skin tumorigenesis when combined with repeated applications of a carcinogen, Mottram (9) extended this observation by obtaining skin tumors with one subminimal application of a carcinogen followed by continued treatment with croton oil. In a similar experiment, Berenblum and Shubik (3) concluded that the one subminimal dose of carcinogen “initiated” tumorigenesis and the treatment with croton oil “promoted” development to the visible tumor stage. Although one subminimal dose of carcinogen is sufficient to initiate skin tumorigenesis in the mouse, the actual concentrations of carcinogen employed have been relatively large. In the present study, concentrations of 9,10-dimethyl-1,2-benzanthracene as low as 0.00006 percent have been tested for initiating action following one application and for cumulative effects by the use of repeated monthly applications. In addition, the tumorigenic response obtained when small amounts of carcinogen were applied to the skin monthly has been compared with the response observed to one such application and continued treatment with croton oil. A further phase of this investigation to be reported is concerned with the relative effectiveness of 9,10-dimethyl-1,2-benzanthracene when applied in one or in divided doses.

MATERIALS AND METHODS

The carcinogen 9,10-dimethyl-1,2-benzanthracene was dissolved in acetone, and a graded series of dilutions was prepared and checked at room temperature (26°C) using a Beckman Model DU Spectrophotometer at a wave length of 380 mμ. Each dilution was kept in a rubber-stoppered serum bottle and stored in the dark at 10°C. The various solutions were used on two separate occasions, after which a new series was prepared and checked as before. Immediately prior to skin painting, an aliquot from each solution was brought to room temperature (26°C), and the concentration of 9,10-dimethyl-1,2-benzanthracene was rechecked spectrophotometrically. No significant changes in concentration were observed in any of the samples tested.

Six-week-old Swiss Albino male mice were employed in this investigation. The back of each mouse was clipped free of hair prior to each skin painting. The carcinogenic solutions were withdrawn from the stoppered serum bottles with a 1-ml. tuberculin syringe and a 27-gauge hypodermic needle. The syringe was detached from the needle, and 0.2 ml. of solution was distributed as evenly as possible over the denuded area. All solutions were kept on ice throughout the period of painting.

The investigation was divided into two parts. In

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part I, the mice were separated into six groups of 30 mice each and exposed to a solution of 9,10-di-methyl-1,2-benzanthracene once, or once monthly for a total of ten applications (Table 1, Groups 1–6). Part II, which was independent of part I, consisted of six groups of 30 mice each (Groups 7–12). The mice in Groups 7–11 were painted once with 9,10-dimethyl-1,2-benzanthracene. Two weeks later they were painted once weekly for the duration of the experiment over the area exposed to the carcinogen with a 2.5 per cent solution of croton oil in acetone. The mice in Group 12 were exposed once weekly to croton oil, as in the previous groups, but received no preliminary treatment with 9,10-dimethyl-1,2-benzanthracene. This group was added to the investigation subsequent to Groups 7–11 in view of the observation that croton oil alone could produce skin tumors (1, 11–18). The period of observation extended from 9 to 989 months for Groups 1–11 and 7 months for Group 12, which still is under observation. The mice were inspected for visible tumors every 2 weeks, and the size and position of each growth were recorded. No tumor was included in the final data which did not persist for at least 1 month and which did not measure 1 mm. or more along the largest diameter. Tumors were fixed at autopsy, sectioned at 5 μ, and stained with hematoxylin and eosin prior to microscopic examination.

RESULTS AND DISCUSSION

One exposure to 0.06 per cent 9,10-dimethyl-1,2-benzanthracene was sufficient to induce skin tumors in 69 per cent of the treated mice (Table 1, Group 1). Fifty per cent of the tumor-bearers already had one or more tumors by the 62nd day. When the concentration of carcinogen was decreased to 0.007 per cent (Group 2), fewer mice bore skin tumors, and the number of growths per tumor-bearer dropped from an average of 3.1 to 1.8. Tumors appeared sooner when the higher concentration of carcinogen was employed. Thus, it required an average of 150 days for the first tumors to appear in the five susceptible mice in Group 2, whereas eight of the mice in Group 1 already had tumors by the 51st day. In Group 3, the mice were exposed once monthly to the same concentration of 9,10-dimethyl-1,2-benzanthracene as was used in Group 2. Tumor incidence was more than doubled by the monthly treatments. Also, an increase in the number of tumors per mouse was observed in Group 3 as compared with Group 2. Eight of the mice in Group 3 had one or more tumors after an average observation period of 126 days, as compared with the 150 days for the five tumor-bearers in Group 2. The mice in Groups 4, 5, and 6 also received monthly applications of carcinogen but at a lower concentration than in Group 3. Tumor incidence and tumor multiplicity were both decreased in these three groups as compared with those in Group 3. The mice in Group 3 were exposed to approximately the same total dose of carcinogen as were those in Group 1 (Table 1). Although tumor incidences or tumor multiplicities were not appreciably different in Groups 1 and 3, the latent period was considerably less for the mice painted once as compared with those painted repeatedly. A similar comparison for Groups 2 and 4, both of which received a total dosage of 14 μg. of carcinogen, also revealed no appreciable change

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TABLE 1

| SKIN TUMORIGENESIS IN SWISS ALBINO MALE MICE EXPOSED TO 9,10-DIMETHYL-1,2-BENZANTHRACENE ALONE OR FOLLOWED BY 2.5 PER CENT CROTON OIL |
|---|---|---|---|---|---|---|---|
| **TREATMENT** | **9,10-Dimethyl-1,2-benzanthracene croton oil** | **Conc. (per cent)** | **Total dosage (μg.)** | **Application frequency** | **NO. EFFECTIVE** | **TUMORS** | **TUMOR MULTIPLICITY** | **LATENT PERIOD (days)** |
| **GROUPS** | No. applications | | | | **PER CENT** | **AV. NO.** | **AV. NO.** | |
| 1 | 1 | 0.06 | 120.00 | 1 | 20 | 15 | 1.0 |
| 2 | 1 | 0.007 | 14.00 | 2/week | 22 | 15 | 2 | 1.8 |
| 3 | 10; 1/month | 0.007 | 14.00 | 2/week | 22 | 15 | 2 | 1.8 |
| 4 | 10; 1/month | 0.0002 | 0.0 | 2/week | 22 | 15 | 2 | 1.8 |
| 5 | 10; 1/month | 0.00006 | 0.20 | 2/week | 22 | 15 | 2 | 1.8 |
| 6 | 10; 1/month | 0.00006 | 1.20 | 2/week | 22 | 15 | 2 | 1.8 |
| 7 | 1 | 0.06 | 120.00 | 1/week | 22 | 15 | 2 | 1.8 |
| 8 | 1 | 0.006 | 12.00 | 1/week | 22 | 15 | 2 | 1.8 |
| 9 | 1 | 0.0005 | 1.00 | 1/week | 22 | 15 | 2 | 1.8 |
| 10 | 1 | 0.0002 | 0.40 | 1/week | 22 | 15 | 2 | 1.8 |
| 11 | 1 | 0.00008 | 0.16 | 1/week | 22 | 15 | 2 | 1.8 |
| 12 | 0 | 0 | | 1/week | 22 | 15 | 2 | 1.8 |

* Groups 1–6 run concurrently; likewise Groups 7–11, which were run independently of the first six groups.

1 Mice alive at time of appearance of first tumor in the group.
2 Average number of tumors per tumor-bearer.
3 Time when 50 per cent of the mice bore one or more tumors.
in tumor incidence, while the number of tumors per tumor-bearing dropped from an average of 1.8 in Group 2 to 1.0 in Group 4. Although latent periods cannot be compared for these two groups, a tumor incidence of 50 per cent not having been attained, tumors first appeared in the four susceptible mice in Group 4 after an average observation period of 192 days as compared with 150 days for the tumor-bearers in Group 2. Thus, tumors appeared sooner when the carcinogen was administered in one as compared with divided doses, as had been noted previously (Groups 1 and 3). The three tumors in Groups 5 and 6 were observed after an average observation period of 178 days and 184 days, respectively. It is evident that a cumulative process was concerned in skin tumorigenesis for Groups 3 to 6 inclusive and that the process was predominantly progressive rather than regressive, despite the monthly intervals between treatments. The demonstration that minute quantities of a carcinogen may be effective in skin tumorigenesis and that their effect is cumulative is especially important in that it emphasizes the potential dangers inherent in trace "contaminants" or impurities in some of our industrial products and in our immediate environment.

It is apparent from these data that as little as 0.12 μg. of 9,10-dimethyl-1,2-benzanthracene applied monthly over a large area of skin was sufficient to induce visible skin tumors in Swiss Albino male mice. An experiment with another group of Swiss males, not included in this study, was started when it was observed that tumors could be induced at the 0.00006 per cent concentration (Group 6). The new group has already been exposed to six monthly applications of 0.00001 per cent 9,10-dimethyl-1,2-benzanthracene (0.02 μg./application) without the development of a visible tumor. Poel and Kammer (10) applied 3,4-benzpyrene to the skin of different strains of mice and observed that C57BL/6 males developed skin tumors with as little as 18 μg. when the carcinogen was applied 3 times weekly, 0.2 μg./application. On the other hand, strain DBA/2JAX males were much more resistant to 3,4-benzpyrene when similarly exposed (10). While one application of 0.007 per cent 9,10-dimethyl-1,2-benzanthracene was sufficient to induce skin tumors in Swiss Albino mice in the present study (Group 2), no tumors were obtained following one application of 0.15 per cent of the same carcinogen to the backs of DBA/2JAX male mice. From these observations, it would appear that, while the C57BL/6 strain and perhaps others might prove as responsive, or more so, to the small quantities of carcinogen employed in the present investigation, other strains of mice probably would display a greater resistance under the same conditions of exposure.

Skin tumors were induced in all the groups which received one application of 9,10-dimethyl-1,2-benzanthracene followed by repeated applications of croton oil (Groups 7–11, inclusive). Among the mice treated with croton oil and no carcinogen (Group 12), one out of an effective total of 28 bore one papilloma each. In a recent study on the influence of croton oil in tumorigenesis of the forestomach, Berenblum and Haran (3) referred to the experiments, now in press, of Rusch et al. (11), in which skin tumors also were obtained in the mouse with croton oil in the absence of carcinogen. Other investigators (1, 12, 13) similarly have observed such tumors following repeated exposures to croton oil alone. In view of these observations and the results obtained in Groups 11 and 12 in the present study, it is not possible to ascribe an initiating influence to the 0.16-μg. dose of 9,10-dimethyl-1,2-benzanthracene (Group 11). It is possible that such an influence would have been demonstrated had more mice been employed, though this remains to be proved. The effectiveness of a minute amount of 9,10-dimethyl-1,2-benzanthracene in initiating skin tumorigenesis is more evident where the mice were exposed to a total dosage of 0.4 μg. (Group 10). This still represents a sizable amount of carcinogen on the molecular level. Thus, assuming an even distribution of the 0.4 μg. over the 120 sq. mm. of skin exposed, the approximate amount reaching each cell is of the order of 10¹³ molecules of 9,10-dimethyl-1,2-benzanthracene.

Tumor incidence among the mice treated with croton oil decreased from a high of 83 per cent, when 120 μg. of 9,10-dimethyl-1,2-benzanthracene was applied initially, to 17 per cent when the total dosage was lowered to 0.16 μg. The number of tumors/tumor-bearing decreased in similar fashion, while at the same time the latent period increased. In the experiments of Berenblum and Shubik (4) with one application of a carcinogen and repeated applications of the promoter, croton oil, it was observed that when the concentration of carcinogen was altered and the mice subsequently received the same treatment with croton oil, tumor incidence varied, but the latent period remained approximately the same. From this and earlier observations (3), it was concluded that the promoter croton oil affected only the latent period, whereas the one application of carcinogen affected only tumor incidence. This was not observed in the present investigation, since the latent period varied despite the constancy of treatment with croton oil. It may

1 Unpublished data.
be concluded from this that the concentration of initiator had a decided influence on latent period, the latter increasing with a decrease in the concentration of carcinogen applied (Table 1, Groups 7, 8, 9). In support of this conclusion, Shubik and Saffiotti (15) recently reported that the latent period of skin tumorigenesis could be shortened in mice exposed repeatedly to 0.01 per cent 9,10-dimethyl-1,2-benzanthracene by first painting the animals once with a 1.0 per cent solution of the same carcinogen. The amounts of 9,10-dimethyl-1,2-benzanthracene employed as initiator in the experiments of Berenblum and Shubik (4) were considerably greater than those used in the present study. This might account for the differences in latent period observed. It is evident that one application of a carcinogen not only may initiate the process of skin tumorigenesis but, further, may develop such alterations as are produced in the direction of the visible tumor stage, the extent depending on the amount of carcinogen applied. Thus, visible skin tumors may be induced in the mouse following but one application of a carcinogen ([8] Groups 1, 2, Table 1). It is reasonable to assume that, when a carcinogen is applied once, increased developing action will result when more rather than less of the agent is employed. This probably explains the increases in latent period in Groups 7-11, Table 1, as the concentration of 9,10-dimethyl-1,2-benzanthracene was decreased progressively.

In a previous experiment it was observed with strain DBA mice that repeated monthly exposures to 9,10-dimethyl-1,2-benzanthracene were more effective in skin tumorigenesis than one such exposure followed by monthly exposures to croton oil (7). In the present investigation, monthly applications of 9,10-dimethyl-1,2-benzanthracene at lower concentrations than in the preceding experiment (7) proved less effective than one application of this carcinogen followed by weekly applications of croton oil (Table 1). Thus, the incidence of skin tumors and tumor multiplicity were consistently higher and the latent periods lower among the mice exposed to carcino- gen and croton oil (Table 1), as compared with those exposed to 9,10-dimethyl-1,2-benzanthracene alone (Groups 3-6), despite the fact that the mice in the latter groups received a greater total dosage of carcino- gen.

All the skin tumors among the mice in Groups 1-6 as well as most of the tumors in Groups 7-12 were benign papillomas. As Shubik (14) observed previously, many of these papillomas regressed, others remained stationary or nearly so, while still others enlarged progressively. With the data available in the present study, it is not possible to state the frequency with which these tumors regressed or the average time of persistence. Squamous-cell carcinomas were observed in three out of 23 animals in Group 7 and in one out of 22 in Group 8. In addition, one subcutaneous fibrosarcoma was obtained from a treated mouse in Group 8.

**SUMMARY**

Solutions of 0.06-0.00006 per cent 9,10-dimethyl-1,2-benzanthracene were applied to the skin of 11 groups of Swiss Albino mice once, once monthly, or once followed by repeated application of croton oil. An additional group received croton oil alone. Skin tumors arose among all the groups. Tumor incidence and tumor multiplicity decreased and latent period increased as less carcinogen was applied. The influence of a single dose of carcinogen on latent period is discussed.

No appreciable differences in tumor incidence or multiplicity were encountered whether the same or approximately the same total dose of carcinogen was applied once or in divided amounts. However, the latent period was shorter following the single application.

Fewer tumors were elicited with repeated monthly applications of 9,10-dimethyl-1,2-benzanthracene than with one such application followed by weekly treatment with croton oil.

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