Studies on the Promoting Phase in the Stages of Carcinogenesis in Mice, Rats, Rabbits, and Guinea Pigs

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In previous experiments by Berenblum and Shubik (4), tumors were induced in mice by a single application of a carcinogenic hydrocarbon, followed by repeated applications of non-carcinogenic croton oil. Friedewald and Rous (7) similarly showed that tumors may be induced in the rabbit by a suboptimal dose of carcinogen followed by wound healing and applications of non-specific agents, such as turpentine. Previously, Berenblum (1) had shown that turpentine, as well as croton oil, had an augmenting (cocarcinogenic) effect in carcinogenesis in mice; and several other examples of this activity have been reviewed in the literature by Berenblum in 1944 and Rusch (15). Arising from this work, a new concept of carcinogenesis has been proposed, involving an initiating stage, brought about by the hydrocarbon, with a small number of normal cells changing into latent tumor cells which are subsequently converted into morphological tumors by promoting action (e.g., croton oil, wound healing, etc.). The latent tumor cells remain irreversibly changed for 20 weeks (4) or even as long as 43 weeks (5).

In the mouse, only croton oil can be said to have considerable promoting action, whereas other factors previously investigated, such as turpentine (1) and wound healing (6, 13) have a minimal effect. In the rabbit, on the other hand, both wound healing and turpentine seem to have a marked effect.

Substances tested for possible promoting activity have usually been selected from those agents known to induce epidermal hyperplasia. Mice and rabbits have most frequently been employed for such tests. In the present study, a variety of substances, all inducing epidermal hyperplasia, was examined for possible promoting activity, and, in addition to mice and rabbits, rats and guinea pigs were also used.

METHODS

The mice used in this investigation were of the Swiss strain from the Medical Research Council, England, and were bred in this laboratory. Rabbits, rats, and guinea pigs were from the stock of the same laboratory. Throughout the investigation 9,10-dimethyl-1,2-benzanthracene as a 1.5 per cent solution in medicinal liquid paraffin (light mineral oil) was used as the initiating agent. Liquid paraffin was used as the solvent, except where solubilities made this impracticable. Test solutions were applied with a fine glass rod.

EXPERIMENTAL

MICE.—In this series, turpentine applications, already shown to have a mild cocarcinogenic effect in mice (1) and a noticeable action in rabbits (7), were reinvestigated as promoting agents after a single application of 1.5 per cent 9,10-dimethyl-1,2-benzanthracene. Next, a series of substances was examined in a similar way: (a) phenanthrene and fluorene, which are noncarcinogenic in mice (9, 10); (b) acridine, investigated by Kennaway (9) and Maisin et al. (11), and found to be noncarcinogenic but very irritating; (c) a series of vegetable oils, e.g., castor oil, its active principle ricinoleic acid, glyceryl monoricinoleate, and oleic acid, which had been shown (17) to augment carcinogenesis with inadequate dosage of 3,4-benzpyrene; (d) silver nitrate in aqueous solution; and (e) cauterization by electrocautery.

Each of these substances was first applied in serial dilutions to a small area of normal skin, in the interscapular region, which was clipped free of hair with scissors. After four applications, spaced over 2 weeks, the mice were killed, and pieces of tissue removed for histologic examination. The concentration yielding the maximum hyperplasia with the minimum of necrotic change was chosen for further testing. For the promotion tests, groups of ten mice were used, each receiving a single application of the carcinogen, and, after an interval...
of 3 weeks, twice-weekly applications of the test solution. The turpentine was used as a 20 per cent solution, phenanthrene as a 1 per cent, acridine as a 0.3 per cent, and fluorene as a 0.5 per cent solution, all in liquid paraffin. Silver nitrate was made up as a 10 per cent aqueous solution. The remaining test substances were used undiluted. Finally, a group of five mice was cauterized with a superficial linear streak 1 inch long in the center of the painted area at monthly intervals following a single application of the carcinogen. In this group no tumors have been recorded at 60 weeks following the original application.

The results of the tests for promoting activity, summarized in Table 1, were uniformly negative.

<table>
<thead>
<tr>
<th>Substance Tested for Tumor Promotion in Mice</th>
<th>Skin Promotion Test</th>
<th>Skin Histology</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 per cent Turpentine in liquid paraffin</td>
<td>No tumors recorded</td>
<td>Minimal histological change</td>
</tr>
<tr>
<td>0.3 per cent Acridine in liquid paraffin</td>
<td>Marked epidermal hyperplasia</td>
<td></td>
</tr>
<tr>
<td>0.5 per cent Fluorene in liquid paraffin</td>
<td>Slight epidermal hyperplasia</td>
<td></td>
</tr>
<tr>
<td>1 per cent Phenanthrene in liquid paraffin</td>
<td>Marked epidermal hyperplasia</td>
<td></td>
</tr>
<tr>
<td>Castor oil</td>
<td>Good hyperplasia, most marked in the skin</td>
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<tr>
<td>Oleic acid</td>
<td></td>
<td></td>
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<tr>
<td>Silver nitrate (10 per cent aqueous)</td>
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*Groups of three mice; 41 weekly applications.
† Groups of ten mice; given semiweekly applications for 20 weeks, 3 weeks after a single application of 0.10-dimethyl-1,2-benzanthracene in liquid paraffin.

Rabbits.—The experiments of Friedewald and Rous (7), showing that wound healing may act as a promoting agent in this species, were repeated but modified by using only a single application of carcinogen as initiator. Turpentine has been reinvestigated under these conditions, and the effects of croton oil studied in the rabbit. Similarly, chloroform and inorganic arsenic were reinvestigated under these conditions.

Berenblum (2) showed that 9,10-dimethyl-1,2-benzanthracene is a potent carcinogen for rabbit skin, and a single application of this hydrocarbon was therefore used as standard initiator for this part of the investigation also.

The effects of wound healing and turpentine.—Four areas of skin on the back of one rabbit, clipped free of hair with electric clippers, were used for the determination of the optimal dosage of turpentine; four strengths, undiluted, and 50, 20, and 10 per cent turpentine in liquid paraffin, were applied to these areas twice weekly. The undiluted turpentine induced ulceration within 1 week, and it was discontinued. The other three solutions induced macroscopic reddening and thickening of the skin. After 3 weeks the 50 per cent solution also caused some ulceration, and it, too, was discontinued; a skin biopsy was made for histological examination. The remaining concentrations were applied for up to 10 weeks, and the animal then killed. Histologically, extensive epidermal damage was noted in the skin treated with 50 per cent solution, and only minimal hyperplasia; with the 20 per cent solution there was considerably less damage, but, again, only minimal hyperplasia. The 10 per cent solution induced almost no change. In no case was an appearance produced resembling that described by Rous (14), where turpentine caused the epidermis to become from five to ten cells thick.

In spite of the unsatisfactory nature of the first test, but because of earlier favorable reports, the experiment was continued, using the 20 per cent solution, applied to three areas of skin on the backs of four rabbits, which had been treated 1 month previously with a single application of the carcinogen. The fourth area on each rabbit was maintained as a control for one application of carcinogen. These applications were continued twice weekly for 20 weeks; one rabbit died in the sixth week of the experiment and yielded no results. Concurrently, a hole 1/4 inch in diameter was punched through the ears of each rabbit previously treated only once with carcinogen, using a sharp cork borer. Among the three surviving rabbits, one developed definite papillomas on both ears at the seventh week around the site of the punched hole, and another developed a papilloma on one ear at the eighth week following the trauma. All of these tumors continued to grow until the twentieth week when the animals were killed, but none became malignant. On the third rabbit tumors did not develop. There was no evidence of any tumor formation in any of the areas treated with turpentine.

The effects of croton oil on rabbit skin.—Again preliminary skin tests were carried out on one rabbit, with a 5 per cent solution of croton oil applied to two areas of skin and a 10 per cent solution to two others, at twice-weekly intervals for 2 weeks. Both solutions were made up in liquid paraffin. The skin showed some macroscopic reddening and thickening following the 5 per cent solution, and ulceration following the 10 per cent. Histologically it was noted that both solutions induced some epidermal necrosis, far more pronounced with the stronger solution; and in both cases there was...
patchy epidermal hyperplasia. Four areas of skin on three rabbits were then treated with a single application of the carcinogen, and, after an interval of 1 month, applications twice weekly of croton oil (5 per cent) were begun. No tumors were recorded after 20 weeks.

The effects of arsenic and chloroform.—Arsenic trioxide and arsenic pentoxide were each made up as a 5 per cent preparation in a Lanette wax SX ointment base. The chloroform was applied undiluted. Four areas of skin of two rabbits were clipped free of hair, and to three of these was applied a single application of the carcinogen. After an interval of 1 month, the three areas were treated with one or another of the test compounds. The areas not treated with carcinogen were used as controls for the effects of the arsenic alone. The experiment was concluded at 20 weeks from the commencement of the second series of applications. No tumors were recorded.

Guinea Pigs.—

The effects of croton oil.—Studies on tumor formation with the guinea pig have been few, although Haagensen and Krebhiel (8) and Shimkin and Mider (16) have shown that the induction of sarcomas, using 3,4-benzpyrene and 20-methylcholanthrene, is possible within limits comparable to those occurring among other laboratory animals. Recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene is an effective carcinogen for guinea-pig skin, although the average latent period is as long as 51 weeks.

Preliminary skin tests on normal guinea pigs were carried out with 5 and 10 per cent solutions of croton oil in liquid paraffin. The 5 per cent solution induced considerable hyperplasia with minimal damage. Solutions were applied to both flanks of the animals, which were clipped free of hair with electric clippers. The effects of a limited number of applications of the carcinogen, followed by repeated applications of croton oil, were then investigated. Four groups of five guinea pigs were used for this:

Group 1.—both flanks were given three applications of carcinogen at twice-weekly intervals: after a 4-day interval only the left flanks received, twice weekly, 5 per cent croton oil applications.

Group 2.—the left flanks only were given three applications of the carcinogen: after a 4-day interval both flanks received twice-weekly applications of croton oil.

Group 3.—both flanks were given three applications of carcinogen: after a 4-day interval both received croton oil applications.

Group 4.—both flanks received but a single application of carcinogen, followed by the croton oil.

The experiment was continued for 38 weeks from the commencement of the croton oil applications, and no tumors were recorded. However, by the end of the experiment the animals were all in bad condition, having scratched the treated areas considerably. The procedure was then repeated on another group of 30 guinea pigs, divided into three groups of ten; only a single area of skin, in the interscapular region, was used for the applications in each animal. One group received ten applications of carcinogen, another five, at twice-weekly intervals, as it was considered that the three applications used originally might have been insufficient in this species. The third group received croton oil only. The experiment was continued for 36 weeks, but again no tumors were induced, and the skins of the animals were found to be in bad condition: a sensitivity to croton oil apparently developed after a few applications in some guinea pigs, which necessitated periodic cessation of treatment to prevent frank ulceration.

Rats.—

The effects of croton oil.—Studies on tumor formation of the skin of rats are more numerous than those with guinea pigs, and recently Berenblum (5) has shown that 9,10-dimethyl-1,2-benzanthracene

<table>
<thead>
<tr>
<th>Species</th>
<th>Agent tested</th>
<th>Skin histology</th>
<th>Promotion test</th>
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</thead>
<tbody>
<tr>
<td>Rabbits</td>
<td>Wound healing</td>
<td>Slight epidermal hyperplasia</td>
<td>No tumors induced</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>20 per cent turpentine in liquid paraffin</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td></td>
<td>5 per cent croton oil in liquid paraffin</td>
<td>=</td>
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</tr>
<tr>
<td></td>
<td>Chloroform</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td></td>
<td>5 per cent arsenic trioxide in Lanette wax SX</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td></td>
<td>5 per cent arsenic pentoxide in Lanette wax SX</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Rats</td>
<td>5 per cent croton oil in liquid paraffin</td>
<td>Some atrophic changes in dermis</td>
<td>=</td>
</tr>
<tr>
<td>Guinea</td>
<td>5 per cent croton oil in liquid paraffin</td>
<td>Marked epidermal hyperplasia</td>
<td>=</td>
</tr>
</tbody>
</table>

* On normal skin after 47 weekly applications of the agents.
† On skin painted with a single application of 9,10-dimethyl-1,2-benzanthracene (or more; see text), and then treated with the test agent twice weekly.
‡ Performed by punching a hole, 1 inch in diameter, through the ear with a sharp cork borer.
is an effective carcinogen for the skin of this species, although the average latent period is 30 weeks. Preliminary skin tests with croton oil induced macroscopic thickening and reddening; and an eczematoid lesion of the skin occurred with the 5 and 10 per cent solutions, more marked with the stronger. After four applications of croton oil given over 2 weeks some atrophic changes of the dermis were noted histologically, with minimal damage to the epidermis. In spite of this finding, twenty rats were each given three applications of the carcinogen at twice-weekly intervals, followed after 4 days by twice-weekly applications of 5 per cent croton oil. Another group of ten rats was given the croton oil only. This experiment was complicated by the fact that eczematoid skin lesions arose in all of the animals treated with croton oil. No tumors were recorded at 30 weeks from the commencement of the croton oil treatment.

DISCUSSION

Under the conditions of these experiments, croton oil possesses a definite specificity for the mouse, for which it is the most potent promoting agent yet investigated. The other substances tested for promoting activity in the mouse all proved negative following a single application of the carcinogen, although some of them had been shown earlier to have a co carcinogenic effect, in combination with repeated applications of carcinogenic hydrocarbons. It is possible that turpentine, for example, is a weak promoting agent, whose activity can only be revealed after more potent initiation.

In the rabbit, wound healing has proved to be by far the most effective method of promoting tumors, although it is likely that the turpentine sample used in this experiment differed in properties from that used by Rous and his collaborators. Inevitably, the continued use of such complex and uncontrolled organic mixtures will make the comparison of results haphazard. However, samples of croton oil, which are quite effective in the mouse, prove completely inactive in the rabbit. Chloroform, too, seems only a very weak promoting agent, requiring more than a single application of carcinogen preceding it, if its action is to be revealed (7).

The rat and the guinea pig are known to differ in their response to the carcinogenic hydrocarbons. In the previous analysis of the mechanism of carcinogenesis in mice (4) it was seen that the latent period was a function of the promoting action, whereas the total number of tumors induced was an expression of the initiating action only. Therefore, it might be concluded that, with an effective carcinogen, the latent period in relatively unresponsive species might be altered by effective promotion. Croton oil, however, appears to have no effect in either the rat or the guinea pig, although neither of the experiments could be described as final, in view of the poor health of the animals.

The results suggest that epidermal hyperplasia and tumor-promoting activity are not related in any simple fashion. Several of the substances investigated in the mouse, other than croton oil, induced marked hyperplasia, and yet were ineffective in promoting tumors following a single application of the carcinogen. It must be mentioned, however, that none of these substances seemed to induce as great a hyperplasia, and with such speed, as that encountered with croton oil, although this aspect requires further and more detailed histologic investigation. Nevertheless, this investigation raises the possibility of a specific metabolic role for croton oil in the mouse, rather than any simple explanation, as hitherto envisaged but not discussed. The only confusing factor is the undoubted effectiveness of wound healing as a promoting agent in the rabbit under these same conditions.

SUMMARY

1. Turpentine, acridine, fluorene, phenanthrene, castor oil, ricinoleic acid, glyceryl monoricinoleate, oleic acid, and silver nitrate have been tested for tumor-promoting activity following a single application of 9,10-dimethyl-1,2-benzanthracene in the mouse. They were ineffective as promoters of tumors.

2. The effects of wound healing, turpentine and croton oil applications, following a single application of 9,10-dimethyl-1,2-benzanthracene, have been tested for tumor-promoting activity on the rabbit. Wound healing alone has been found to be an effective stimulus.

3. The effects of repeated applications of croton oil, following a minimal number of applications of 9,10-dimethyl-1,2-benzanthracene have been investigated in the guinea pig. No tumors were induced.

4. The effects of croton oil, following three applications of 9,10-dimethyl-1,2-benzanthracene in the rat, have been investigated. No tumors were induced.

5. The specific effect of croton oil on the mouse and of wound healing on the rabbit, as promoters of tumor formation are discussed.

ACKNOWLEDGEMENTS

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5. ———. The persistence of Latent Tumour Cells Induced in the Mouse's Skin by a Single Application of 9,10-dimethyl-1,2-benzanthracene. Ibid., 1949 (in press).
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