Increasing Induction of Skin Tumors by Pretreatment with Croton Oil*

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SUMMARY

A number of investigators have concluded that, in contrast to the remarkable augmenting action of croton oil in experimental skin carcinogenesis when this agent is applied after the carcinogen, it has no influence when applied before exposure to the carcinogen. Three long-term studies were performed to test this view.

The findings clearly demonstrate that pretreatment with croton oil resulted in a small but significant increase in the incidence of skin tumors and of carcinomas only, as well as an increased proportion of malignant neoplasms. This increase, however, was of lesser magnitude than the striking enhancement of the incidence of skin tumors produced by post-treatment with croton oil. It was concluded that croton oil possesses both mild carcinogenic activity and strong augmenting properties.

The possibility that studies with croton oil may play a significant role in the understanding of experimental skin carcinogenesis has intrigued many investigators. In 1941 Berenblum reported that weekly applications, to the interscapular skin of mice, either of an acetone solution of 0.05 per cent benzpyrene and 0.5 per cent croton oil or of one containing the benzpyrene and 0.025 per cent croton resin, caused a decided augmentation of carcinogenesis in comparison with that observed in controls treated with 0.05 per cent benzpyrene solution alone (1). In the years that followed the oncologic characteristics of croton oil have been revealed through numerous and diverse experiments.

Its most striking property is the augmenting or promoting effect upon skin carcinogenesis, produced when this agent is applied to the skin of mice previously exposed to carcinogenic polycyclic hydrocarbons. Such a croton oil solution when given alone—in the same amount, concentration, and periodicity of application—produces papillomas in none or in only a low per cent of the mice exposed (1, 9, 18), and thus it must be considered a very weak carcinogen. Under special circumstances (unusual laboratory conditions or utilization of a particular strain of mouse) considerable carcinogenic activity has been reported (7, 15). With this background it is surprising that a number of investigators have concluded that croton oil has no effect upon skin carcinogenesis when applied prior to carcinogen treatment (2, 4, 16). There is only one investigator who presents an opposite view (12, 13).

About 10 years ago, as part of a broad, integrated program on factors and mechanism in carcinogenesis, we became interested in the significance of the action of croton oil. It was difficult to accept as valid the concept that croton oil (a carcinogen, albeit a weak one) does not contribute to the induction of tumors when utilized prior to a more potent carcinogen. Furthermore, the reported lack of influence of croton oil when applied prior to a carcinogenic polycyclic hydrocarbon has become one of the key arguments of some proponents (for example [3]) of a two-stage concept of skin carcinogenesis. Hypotheses embodying a two-stage mechanism have been advocated by a number of investigators with varying emphasis, degree of conviction, interpretation, and terminology (Rous and Kidd [17], MacKenzie and Rous [11], Berenblum [2], Mottram [12, 13], Tannenbaum [19], Kline and Rusch [10], Friedewald and Rous [8], and Berenblum and Shubik [5, 6]). It was hoped that a re-examination of the question with which this publication is concerned, as well as others related to croton oil, might add some clarification to our limited knowledge of stages and mechanisms in skin carcinogenesis.

MATERIALS AND METHODS

The experiments comprising this investigation were begun at different times: 1955, 1957, and 1959 for Experiments 1, 2, and 3, respectively. Essentially similar in design, each experiment or section (where both males and females were utilized) consisted of four groups. Groups of mice receiving croton oil alone were not included in these.

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skin tumors in the strains of mice employed. Moreover, our main purpose was to test the influence of pretreatment with croton oil. Since some readers may consider such data helpful, however, the results of our earlier studies on the influence of croton oil alone are presented (Table 1). These include groups that received doses comparable to those used in the present experiments, as well as much higher total doses (administered for longer periods and/or higher concentrations). These studies are not direct controls for the experiments described herein.

The experimental procedures were divided into three periods or stages, separated by a 2-week interval during which the mice received no treatment. When the schedule called for applications of agents during a particular period, these were carried out at the same time for the four groups of an experiment. During the basic second or middle period the mice of all groups received identical treatment with the carcinogen—a single drop (1/50 ml.) of 0.2 per cent benzpyrene, dissolved in redistilled acetone, to the skin of the interscapular area. In all three experiments this basic, second-period treatment was given twice weekly for 10 times (4 1/2 weeks from first to last).

In other respects the treatment for the four groups of a particular experiment, and between experiments, differed: either (a) no treatment, (b) application of acetone (as solvent control), or (c) an acetone solution of croton oil to the interscapular skin during the first period (which preceded benzpyrene treatment) and third period (which followed benzpyrene treatment). In short, the design of treatment was such as to achieve: Group 1, carcinogen control; Group 2, pretreatment with croton oil; Group 3, post-treatment with croton oil; and Group 4, both pre- and post-treatment with croton oil. The concentration of croton oil solutions and the number of applications are given in Table 2. All applications to an individual mouse were to the same interscapular area, 1 drop (1/50 ml.) delivered from a dropping pipette. This covered a circular skin area approximately 2 cm. in diameter. In a particular study the same dropping pipette was used for all the mice receiving 0.2 per cent benzpyrene during period 2 and a second pipette for the mice receiving the croton oil solution during the first and third periods. The agents utilized were: reagent-grade acetone, redistilled; croton oil, N.F.VII; benz(a)pyrene, recrystallized, m.p. 177°-179° C.

The mice employed were from inbred strains maintained in our laboratory by brother-to-sister mating. Those of Experiments 1 and 3 were male and female F1 hybrids of C57BL females and C3H males (C57BL×C3H) F1, but designated as C×H for simplification; and of Experiment 2, DBA females. The mice were weaned at about 30 days of age, at which time they were divided into groups by litter-mate distribution, insofar as possible, and identified by ear punch. From weaning and for the duration of the study they were allowed Purina Laboratory Chow Checkers and distilled water ad libitum. The animals were housed in sets of five in metal cages with solid bottoms; bedding consisted of a shallow layer of sterilized wood shavings and peat moss. The mice were kept in a temperature-controlled laboratory at 80° F.

From previous experience we were aware that the application of various agents caused differing conditions of the skin in respect to epilation and hair growth, smoothness or scaliness, pigmentation, thickening, and other changes in the epidermis and dermis. Thus, acetone has little effect, whereas croton oil solutions or 0.2 per cent benzpyrene has definite, though differing, influences including rate of epilation and hair regrowth. To insure, insofar as possible, the uniformity and equality of the applications of 0.2 per cent benzpyrene, the interscapular skin area of all mice was made free of hair by means of an Andis electric clipper 2 days before this treatment began. The clipper was carefully washed after use on each group.

During the course of the studies the mice were weighed and inspected at 2-week intervals. Average body weights between groups within a given experiment did not vary significantly at any of the determinations. Superficial tumors were observed and described periodically, and a record was made of their size and nature. Papillomas had a typical gross appearance and generally grew progressively. Their conversion to carcinomas was recognized by induration at the periphery, extension downward, keratinization, and tendency to ulcerate centrally. Mice that died during the experiment and those sacrificed at its

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<th>TREATMENT*</th>
<th>Papilloma-bearing mice</th>
<th>DURATION OF EXPERIMENT (WEEKS)</th>
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* Treatment consisted of twice-weekly applications of 1/50 ml. of croton oil (CO) solutions in acetone, delivered from a dropping pipette to the interscapular skin. Concentration of croton oil and total number of applications are indicated.
† Average time, from the beginning of the experiment, at which papillomas appeared.
‡ C×H is abbreviation for (C57BL×C3H) F1.
§ Croton oil applications were given only during the first 20 weeks of the experiment.
‡ Croton oil applications were given throughout the entire duration of the experiment.

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362

TABLE 1
SKEIN TUMOR RESPONSE TO CROTON OIL ALONE

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termination were examined for skin tumors, other neoplasms, and general pathology. Specimens were taken from all tumors, and often from other tissues, for histologic study. The tissues were fixed in 10 per cent formalin, processed and sectioned, and stained with hematoxylin and eosin.

All three studies proceeded without untoward events, and no differential mortality was introduced by deaths from spontaneous mammary carcinomas or lymphomas in the DBA mice of Experiment 2.

RESULTS

The findings in all three studies (five sections or subgroups) are relatively consistent (Table 2). With the incidence of mice bearing either papilloma or carcinoma or of those bearing only carcinoma used as a criterion, it is apparent that pretreatment with croton oil increased skin tumor formation in comparison with controls exposed to benzpyrene only (Group 2 versus Group 1). This higher tumor incidence is also confirmed by comparing Groups 4 and 3. The mice of these latter two groups received the same benzpyrene treatment in the second period and croton oil applications in the third period, thus differing only whether or not they were pretreated with croton oil.

In brief, periodic and prolonged pretreatment of the skin with croton oil solutions produced a small but consistent increase in the incidence of mice with skin tumors. The data on the average time of appearance of tumors are of little value, being neither large nor consistent in direction. Differences in the multiplicity of neoplasms (skin tumors per mouse) would also be helpful in interpretation,

TABLE 2

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<td>CO</td>
<td>156</td>
<td>98</td>
<td>63 44</td>
<td>80</td>
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* BP = benzpyrene, 0.2 per cent solution in acetone, applied twice weekly for 10 times. CO = croton oil, in acetone solution; numbers of twice-weekly applications are indicated. Interval between periods, during which no treatment was given, was 2 weeks.

† Number of mice in each group at the time the first skin tumor was detected in the particular experiment.

‡ Either papillomas or carcinomas.

§ Average time, from the beginning of the experiment, at which the neoplasms appeared.

¶ Average time, from the beginning of the experiment, at which carcinomas were recognized clinically.

†† Numbers in parentheses represent single values.

** Each group in this section represents the combined corresponding groups of Experiments 1, 2, and 3.
but these were not present—the mice almost invariably developing only one tumor.

Post-treatment of the skin with croton oil (i.e., following the basic application of 0.2 per cent benzpyrene) resulted in a striking enhancement of skin carcinogenesis. This is now an oft-repeated and well established observation, but of special and additional interest are the facts: (a) most of the papillomas became carcinomas; and (b) the augmenting influence is equally valid whether mice with skin tumors (papillomas or carcinomas) or with carcinomas only are considered (compare Group 2 with Group 1, and Group 4 with Group 3, in each experiment in Table 2).

The neoplasms were definite and readily recognized grossly. They appeared only in the treated area. In Experiments 1 and 3, with C X H mice, the papillomas grew progressively, and none regressed. Only an occasional tumor was a carcinoma at first observation. The conversion of papillomas into carcinomas proceeded in no unusual manner. In all these respects there were no noteworthy differences between the groups.

Experiment 2 differed from the others in a number of ways. Each of the four groups of DBA female mice developed spontaneous mammary carcinomas and lymphomas, but these occurred at a late age and apparently did not interfere with the development of skin tumors. Each of the four groups of this experiment had from fourteen to seventeen mice with spontaneous mammary carcinoma and six to nine mice with malignant lymphoma. A remarkable occurrence in this investigation was the nature of the skin lesions. Most of them were carcinomas at onset, presenting themselves not as typical papillomas but as indurated, malignant lesions—papillomatous, nodular, or ulcerated. We were surprised by the predominance of these lesions.

The time interval between the beginning of an experiment and the final application of agents was 47, 37, and 27 weeks for Experiments 1, 2, and 3, respectively. Corresponding figures for the entire duration of the experiments are 87, 82, and 77 weeks, respectively. The fact that only about 40 per cent of all skin tumors and 4 per cent of all carcinomas were observed before treatment was terminated illustrates that the majority of neoplasms appeared between the termination of all treatment and the end of the experiment.

Although the three studies differ in a number of experimental characteristics, they are fundamentally similar. For this reason, and in order to achieve a concise, over-all presentation, the data related to the three experiments are combined and shown in the lower section of Table 2. These data reveal that pretreatment with croton oil resulted in a small but significant increase in the incidence of mice bearing skin tumors, irrespective of type (13 versus 6 per cent, and 63 versus 50 per cent), or carcinomas only (8 versus 2 per cent, and 51 versus 31 per cent). Further more, pretreatment increased the proportion of malignant tumors (12/21 versus 3/9, and 80/98 versus 49/79). These effects, however, were of lesser magnitude than the striking influences produced by post-treatment with croton oil: for mice with skin tumors, 50 versus 6 per cent, and 63 versus 13 per cent; the corresponding values for carcinomas only were 31 versus 2 per cent, and 51 versus 8 per cent.

DISCUSSION

The experiments clearly demonstrate that prior exposure to a limited but adequate course of croton oil treatments causes a small but significant increase in skin tumors as compared with the incidence in mice given only the benzpyrene applications, which summate to a threshold response. A similar course of croton oil treatments given after the applications of benzpyrene results in a far greater increase in skin tumors. The large augmentation of skin carcinogenesis produced by the sequence carcinogen–croton oil has been demonstrated often and is commonly designated as promotion. Obviously the croton oil effect depends on whether it is given before or after the carcinogenic hydrocarbons; only for simplification may one designate the former as carcinogenicity and the latter as augmentation (promotion).

Previous studies.—In the first experiment concerned with this problem Berenblum (2) concluded that pretreatment with croton resin failed to influence significantly the development of skin tumors; however, this was modified to “was not influenced” in the opening sentence of a follow-up publication (4).

Later, Mottram (12, 13) employed a three-stage experiment in which the left flanks of the mice were utilized as controls for the experimental procedures applied to the right flanks. To the right flank an acetone solution of croton oil was applied for 5 times. Two days later both flanks were given a single application of benzpyrene; subsequently this was followed by thrice-weekly applications of the croton oil solution to both flanks. These treatments resulted in a greater number of papillomas and malignant tumors of the skin of the pretreated right flanks than of the non-pretreated left flanks. Mottram explained that the use of the left flank to control the right was a wartime measure to save food and that preliminary experiments had shown that cross-contamination did not occur. Although there are reasons for concern about the small number of mice, we ascribe validity to Mottram’s over-all conclusion that pretreatment with croton oil increased skin tumor formation.

Berenblum and Shubik (4) extended Mottram’s experiments, utilizing his technic of applying only a single dose of carcinogen to the mice but employing separate groups of animals. For pretreatment they gave five paintings of 5 per cent croton oil in liquid paraffin. They concluded, from two separate experiments, that there was no significant difference in skin tumor yield between the mice pretreated with croton oil and those without such pretreatment. In the light of our findings a more prolonged pretreatment with croton oil (for example, 20 weeks rather than 2) would probably have produced a small effect.

The experiment of Berenblum and Haran (3) was not designed to include a control group with mice treated with carcinogen alone. Thus the specific influence of pretreatment with croton oil was not determined. Many more tumors arose in the mice chronically exposed to croton oil following carcinogen in comparison with those similarly exposed preceding carcinogen. It is of interest to speculate what the tumor response might have been if the croton oil carcinogen group had been carried on for a period.
greater than 15 weeks following the major carcinogenic exposure (single carcinogen application).

A study by Roe (16) utilized three groups, treated as follows: Group 1—single application of carcinogen, as control; Group 2—similar application of carcinogen and, 3 weeks later, a course of treatment with croton oil solution; Group 3—treatments with croton oil solution followed, after a 3-week interval, by a single application of carcinogen. The unusual and disturbing appearance of tumors outside the treated area and the fact that Groups 1 and 2 were exposed to the carcinogen at a different age from Group 3 combine to make it difficult to interpret the data. In our opinion the results suggest that pretreatment resulted in a higher tumor yield, but the author states, “One may conclude quite confidently that as far as the treated area is concerned, croton oil promoted tumor formation when given after a single application of 9,10-dimethyl-1,2-benzanthracene but not when given before it.”

Our finding of a small, consistent, and significant increase in skin carcinogenesis through croton oil applications preceding exposure to carcinogen is in agreement with that of Mottram (12, 13) but not with those of others (2, 4, 16). It is probable that demonstration of this relatively small effect was due to good experimental conditions, adequate numbers of animals, croton oil treatments consistent with its low carcinogenicity, and the long duration of the experiments.

Other considerations.—The multi-application method of administering carcinogen to the skin produced papillomas that were classical in appearance; none regressed, and, after a few weeks or months of progressive growth, they were converted into carcinomas. This description is also valid for the lesions in mice pretreated and/or post-treated with croton oil. (The unusual observations regarding the lesions in Experiment 2 have already been described in “Results.”) In contrast to our general experience, many investigators have reported studies in which a single application of carcinogen, followed by a course of croton oil applications, resulted in the appearance of large numbers of papillomas on a total or per mouse basis. A high proportion of these regressed, however, and only a relatively small percentage became carcinomas. Is the nature of this type of skin papilloma really the same as that induced by the conventional multi-application of carcinogen, followed or not by croton oil applications? This question is of engaging interest not only for academic reasons but also because the former results have been considered in the development of hypotheses regarding carcinogenesis.

An experimental plan may be such as to give one great confidence that it is well designed, critical, and controlled; yet the events that actually occur may bring up many new and difficult questions regarding its adequacy. In particular, the problem arises whether or not one can deliver equivalent tissue dosages of carcinogen to a tissue—in one instance relatively normal skin (untreated or to which only acetone has been applied), in the other instance skin that has been previously exposed to repeated applications of an acetone solution of croton oil. The latter treatment produces many modifications of the epidermis and dermis, a few being epidermal hyperplasia, changes in the sebaceous glands and hair follicles, and alterations in pigmentation. These modifications and others may influence the absorption, distribution, and metabolism of the carcinogen.

These comments are not theoretical or academic. As an example, applications of croton oil in the first period resulted in two to three cycles of epilation and hair regrowth during the 10–20 weeks of this phase of the experiments. At the end of this period clipping of the interscapular hair of the mice revealed, in the two groups exposed to croton oil (2 and 4), a circular, pigmented skin area 1–2 cm. in diameter. The corresponding skin area of the two groups not treated with croton oil (1 and 3) did not exhibit this finding. The disparity in pigmentation (manifestation of an early stage in the hair growth cycle) and other characteristics emphasize the possibility that the two conditions of the skin may respond differentially to carcinogen. Supporting this view are the intriguing “Results” and “Discussion” concerned with the influence of the application of croton oil to the skin of mice 18–48 hours prior to the injection of urethan (14).

Application of carcinogen to the four groups of a study, in the second period, again produced a differential response. The mice of the groups previously treated with croton oil appeared to be more resistant to the toxic and inflammatory action of benzpyrene than the mice of the groups not previously treated with croton oil. The skin of the former exhibited less hyperemia and scaliness and the epilation-hair-regrowth cycle occurred at a slower rate; by the tenth and final benzpyrene application all obvious gross differences had disappeared.

What influence did these gross changes, produced by pretreatment with croton oil (and their microscopic and functional counterparts), have on tissue dosage of carcinogen and on carcinogenic potentiality? Were they without effect, acceleratory or inhibitory? We do not know nor do we believe that fluorescence studies or others utilizing labeled carcinogen would necessarily clarify this situation. Nevertheless, this concern for possible modes of action in no way negates the practical effect of pretreatment with croton oil demonstrated in these studies—increase in skin tumor formation.

Croton oil is a mixture of many substances. From a review of the numerous studies in the literature and the results of the present investigations it can be accepted that croton oil possesses slight carcinogenic action as well as striking augmenting properties. Whether these actions reside in one or more compounds and whether the carcinogenic and augmenting characteristics are functions of the same or different compounds has not been determined. It is prudent to await further work and clarification.

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