The Tumor-enhancing Principles of Croton tiglium L.

II. A Comparative Study

B. L. VAN DUUREN, L. LANGSETH, A. SIVAK, AND L. ORRIS

Institute of Environmental Medicine, New York University Medical Center, New York, New York

Summary

Distinct differences were observed in the biologic response elicited by the promoting agents croton resin and its active fractions on the one hand and croton oil on the other. With the active fractions of croton resin, in initiation-promotion experiments, there is a high incidence of malignancy and a low incidence of tumor regression. Applied alone, croton resin gives rise to very few tumors. In contrast, promotion with croton oil is reported to elicit a low incidence of malignancy, a markedly higher incidence of tumor regressions, and when applied alone is notably tumorigenic. These and other factors are discussed in the light of chemical composition, animal strain, degree of skin damage, and dose. It is shown that the croton oil used contains alkylating chemicals and it is concluded that the chemical composition of croton oil and of the active materials plays an important role in determining biologic activity.

Introduction

Many experiments have been carried out on the induction of skin tumors in mice by a single application of a subcarcinogenic dose of an aromatic hydrocarbon followed by repeated applications of croton oil (2, 21). Subsequent to the development of the 2-stage theory of carcinogenesis (2), a number of reports have appeared in which the terms initiation and promotion are questioned (16, 18, 20). It has been suggested, for example, that pure promoting agents do not exist since croton oil alone at times gave benign or even malignant tumors (18). Furthermore, the high rate of regression of tumors reported in some of these experiments has raised questions with respect to the role of croton oil as a tumor promoter. These and other factors, discussed below, have given rise to considerable discussion about the role of promoting agents and appear to have discouraged definitive experiments on their mode of action.

In a recent report from this laboratory, which constitutes Part I of this series (24), the isolation and biologic testing of highly active fractions from the seed of Croton tiglium L. was described. Some aspects of the chemistry of the active principles was discussed (24) and their similarities to the active materials prepared in another laboratory (9) were noted (24). These materials were obtained in our work by the solvent extraction of the seeds followed by a variety of fractionation procedures. Croton oil, which is a commercial product formerly used in veterinary medicine, is obtained by expression of the seeds and is a mixture of a wide variety of compounds.

In the course of a study of the biologic activity of the active principles (24, 25), a number of factors have come to light which merit attention and analysis. The biologic results with these fractionated materials, are, in some instances, in striking contrast to the findings of a number of workers who have used croton oil as a promoter. These differences, and some similarities, are summarized in Table 1.

This report describes the findings in several experiments with active cocarcinogens derived from croton seed extract and compares these results with those reported for croton oil by other investigators. In all, 17 literature references pertaining to biologic responses obtained with croton oil were used. These reports list extensive experiments that were useful in this comparative study and they cover a broad range of findings with croton oil from various sources. The possible underlying reasons for the wide differences in results are discussed.

Materials and Methods

PROMOTERS. The solvent extraction of croton seed and the preparation of croton resin and fractions A and C derived from it was described in detail in an earlier report (24).

HYDROPEROXIDE AND ALKYLATING AGENT ANALYSES OF CROTON OIL. Commercial quality croton oil (Magnus, Mabee and Reynolds, Inc., London, England) was used for these analyses. This sample of croton oil has been in the laboratory for a number of years. The quantitative hydroperoxide analysis (6) was carried out in duplicate. Hydroperoxides could not be detected in croton oil by this procedure. The quantitative analysis for alkylating agents in croton oil was carried out by the alkylation of 4-(p-nitrobenzyl)pyridine (10). The absorbance of the dye formed was measured at 565 mμ. Since the nature of the alkylating agent in croton oil is unknown, it was not possible to quantitate the agent accurately. However, in duplicate analyses, including appropriate blank determinations, 15.0 mg of croton oil gave an optical density of 0.05, ± 0.025 which is a low, but significant reading.

MICE. The mice used were females of an HA-ICR strain obtained from Millerton Research Farms, Millerton, New York. They were vaccinated against ectromelia and used for testing at age 8 weeks. Mice were housed on sterile wood chips in metal cages, 10 to a cage, and were fed Purina laboratory chow and water ad libitum. The animal rooms were maintained at 22°.
TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>Croton resin and fractions A and C, this study</th>
<th>Croton oil reported</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence of tumor regressions in initiation-promotion experiments</td>
<td>Low, 0-8%</td>
<td>Usually high, 14-65% (11, 12, 19, 20, 22)</td>
</tr>
<tr>
<td>Malignancy induction in initiation-promotion experiments</td>
<td>High, 40-60%</td>
<td>Low, 0-3% (1, 19, 20, 22)</td>
</tr>
<tr>
<td>Tumorigenicity of promoter alone Benign tumors</td>
<td>Very low, 0-5%</td>
<td>Usually high, up to 95% (12, 17-19, 20)</td>
</tr>
<tr>
<td>Av. No. of persisting papillomas/papilloma-bearing animal in initiation-promotion experiments</td>
<td>None</td>
<td>Some, 0-15% (5, 18)</td>
</tr>
<tr>
<td>Time to appearance of 1st tumors from beginning of promotion</td>
<td>Rapid; usually 31 ± 6 days</td>
<td>Rapid, 40-50 days (1, 5, 11, 18)</td>
</tr>
</tbody>
</table>

24.5°C. Water bottles and cages were machine-washed in water at 82°C with detergent at weekly and monthly intervals, respectively.

PROCEDURE. The backs of the mice were clipped with an Oster small animal clipper 2 days before the initial treatment and then as needed for the duration of the experiment. Different electric animal clipper blades were used for each experiment; they were cleaned frequently by soaking in benzene for 24 hr and were rinsed with acetone. The initiator which was DMBA in all experiments, was applied by micropipet in a single dose (150 or 300 μg) in 0.1 ml of acetone. This primary treatment was followed by 3 times weekly applications of promotors. The interval between initiation and promotion ranged from 12 to 380 days. Promotor solutions were applied onto the clipped dorsal skin with a No. 5 squirrel’s hair brush, delivering close to 100 mg of solution/application. Dosages for croton resin and fractions were based on results of short term toxicity tests (i.e., 3 times weekly paintings for at least 2 weeks). Materials were tested in solution in acetone, and mice were observed for gross skin damage. The most common gross skin reactions were crusting, hair loss, and superficial ulceration. Dosages were decreased until a level was reached where no macroscopic skin damage was apparent. In the case of croton resin a suitable dose was obtained at 0.1 ml of a 0.025% solution, i.e., 25 μg/application. Fractions A and C were used as 0.005% solutions, i.e., 5 μg/application in 0.1 ml of acetone, since A and C each constitute approximately 0.2% of croton resin (23, 25). The animals were observed regularly and the appearance of papillomas recorded. Tumors of a diameter greater than 1 mm were counted and the location charted at monthly intervals. The data dealing with regression and persistance of papillomas and progression to malignancy are based on these monthly chartings. Mice bearing more than 12 papillomas were recorded as having multiple papillomas and assigned a score of 12. The number of tumors/animal (tumor multiplicity) as reported here is the net effect of new tumors that appear and those that regress. Animals bearing tumors that appeared grossly to be carcinomas were sacrificed at approximately 2 months after the 1st appearance of the cancer. Representative samples of benign and malignant tumors were excised at death from all tumor-bearing animals and confirmed histologically.

Results and Discussion

The results obtained in 8 initiation-promotion experiments are given in Table 2 and control experiments in which the promoter alone was applied are listed in Table 3.

The columns listing total papillomas and total regressions in Table 2 give the data at 238-252 days from the beginning of the experiments. The data at these times were purposely used since after about 250 days it was at times difficult to keep accurate records of tumor multiplicity and regressions. The reason for this is that at about 250 days some papillomas coalesce and others become malignant. Experiments reported in the literature and used in this comparative study, were usually discontinued at 230-250 days.

Croton oil was not commercially available at the time that this work was carried out and could not, therefore, be used for direct comparison of tumorigenic activity with the active materials. The activity of these materials can, however, be compared with the findings of earlier workers who have used croton oil as a promoter under various experimental conditions.

As highlighted in Table 1, the concentrated active materials represented by croton resin and fractions A and C elicit responses significantly different from those obtained by other workers who have used croton oil in their work. These differences are most likely related to 1 or more of the following factors: (a) animal strain, (b) dosage, (c) extent of skin damage, and (d) chemical composition of croton oil as compared to the active materials.

Since Swiss albino mice were used in our work, reference will be confined largely to earlier studies using Swiss albino mice, but keeping in mind that differences in response can be expected here also. The several aspects listed in Table 1 will be considered separately.

1. TUMOR REGRESSION. The 5 experiments in Table 2 for which regression data were carefully recorded showed a low incidence of regressions, 0-8% of total tumors. The active materials A and C from croton resin (Experiments 7, 8; Table 2) also gave few regressions; however, accurate numbers are not available for these 2 early experiments. In contrast to this finding, Saffiotti and Shubik (19) used Swiss mice and report regressions of 43%, of the total tumors; twice weekly applications of a 5% solution of croton oil in mineral oil were used. Similar results were obtained later when these workers fractionated croton oil and used a hexane-eluted chromatographic fraction of extract of croton seeds (20). The concentration of the solution was given in these studies but not the amounts delivered per application so that dosage could not be calculated. Frei and Ritchie (12) used 2 strains of Swiss mice and also obtained a high incidence of regressions. The
TABLE 2

<table>
<thead>
<tr>
<th>Experiment no.</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Initiating dose with DMBA</strong>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>150</td>
<td>150</td>
<td>300</td>
<td>300</td>
<td>300</td>
<td>150</td>
<td>300</td>
<td>300</td>
</tr>
<tr>
<td><strong>Promoting dose 3 times weekly, µg/100 mg of acetone</strong></td>
<td>22</td>
<td>14</td>
<td>12</td>
<td>14</td>
<td>46</td>
<td>380</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td><strong>Days between initiation and promotion</strong></td>
<td>30</td>
<td>35</td>
<td>26</td>
<td>31</td>
<td>68</td>
<td>35</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td><strong>Days to 1st tumor from beginning of promotion</strong></td>
<td>106 (240)</td>
<td>113 (250)</td>
<td>93 (220)</td>
<td>143 (250)</td>
<td>92 (250)</td>
<td>5 (250)</td>
<td>72 (250)</td>
<td>168 (250)</td>
</tr>
<tr>
<td><strong>Total No. papillomas (days)</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total regressions (at 250 days)</strong>&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.0</td>
<td>6.6</td>
<td>3.7</td>
<td>5.3</td>
<td>5.4</td>
<td>1.25</td>
<td>3.8</td>
<td>8.4</td>
</tr>
<tr>
<td><strong>Median survival time (days)</strong></td>
<td>&gt;338</td>
<td>276</td>
<td>254</td>
<td>275</td>
<td>349</td>
<td>458</td>
<td>365</td>
<td>225</td>
</tr>
<tr>
<td><strong>Duration of treatment (days)</strong></td>
<td>440</td>
<td>365</td>
<td>490</td>
<td>275</td>
<td>450</td>
<td>587</td>
<td>405</td>
<td>320</td>
</tr>
<tr>
<td><strong>Total No. mice with papillomas/total No. mice</strong></td>
<td>19/19</td>
<td>17/20</td>
<td>25/28</td>
<td>27/30</td>
<td>20/20</td>
<td>5/11</td>
<td>19/20</td>
<td>20/20</td>
</tr>
<tr>
<td><strong>Total papillomas; end of experiment</strong></td>
<td>164</td>
<td>126</td>
<td>117</td>
<td>154</td>
<td>128</td>
<td>5</td>
<td>84</td>
<td>174</td>
</tr>
<tr>
<td><strong>Total regressions; end of experiment</strong></td>
<td>10</td>
<td>2</td>
<td>2</td>
<td>13</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total animals with cancer</strong></td>
<td>10/19</td>
<td>9/20</td>
<td>8/28</td>
<td>6/30</td>
<td>11/20</td>
<td>1/11</td>
<td>12/20</td>
<td>9/20</td>
</tr>
</tbody>
</table>

<sup>a</sup> DMBA, 7,12-dimethylbenz(a)anthracene.

<sup>b</sup> From beginning of experiment (initiation).

TABLE 3

<table>
<thead>
<tr>
<th>Material</th>
<th>Fraction A</th>
<th>Fraction C</th>
<th>Croton resin</th>
<th>Croton resin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Dose, (µg/0.1 ml of acetone) 3 times weekly</strong></td>
<td>5</td>
<td>5</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td><strong>Duration of treatment (days)</strong></td>
<td>421</td>
<td>421</td>
<td>450</td>
<td>273</td>
</tr>
<tr>
<td><strong>Total No. mice with tumors/total No. mice</strong></td>
<td>1/20</td>
<td>3/20&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0/20</td>
<td>1/30&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Days to 1st tumor</strong></td>
<td>210</td>
<td>77</td>
<td>235</td>
<td>238</td>
</tr>
<tr>
<td><strong>Median survival time (days)</strong></td>
<td>&gt;421</td>
<td>380</td>
<td>&gt;273</td>
<td>386</td>
</tr>
</tbody>
</table>

<sup>a</sup> Only 1 tumor/animal.

striking differences in regressions between these reported findings and those of the present study are illustrated in Chart 1. These data show that the use of croton resin as a promoter results in a regression response that is consistently low, in contrast to the regression of up to 56% of the tumors formed in response to croton oil as a promoter (12). Klein (13), using a different strain of mouse (DBA), also reported a high incidence of tumor regressions which suggests that the differences in response between croton oil and croton resin are most likely due to animal strain alone.

The low incidence of tumor regressions obtained with croton resin as opposed to croton oil is of interest since the high regression rate with croton oil has been used as an illustration of the difference in behavior between a cocarcinogen and a total carcinogen (22). The low order of regressions with the active materials is comparable to the regressions obtained with repeated applications of dibenz(a,h)anthracene which is used routinely as a positive control in carcinogenicity assays in this laboratory. Thus, 0.1 ml of a 0.01% solution of this carcinogen in acetone applied 3 times weekly on 50 female Swiss Millerton mice gave, after 238 days, 25 animals with tumors. A total of 54 papillomas were counted in this group. Of these only 3 tumors regressed, giving a 94% cumulative yield of persisting tumors.

2. MALIGNANCY INDUCTION IN INITIATION-PROMOTION EXPERIMENTS. Shubik (22) reports a low malignancy induction using croton oil as a promoter subsequent to a single application of DMBA, whereas our findings with croton resin and fractions A and C reported earlier (24) show a substantial number of animals developing malignant tumors: 9/20, 12/20, 9/20, respectively. Frei and Ritchie (12) do not report carcinoma incidence; however, the termination dates for their experiments are prior to the time when a significant number of carcinomas are expected to appear.

3. TUMORIGENICITY OF PROMOTER ALONE. The low order of tumor induction with A, C, and croton resin alone is evident from the results given in Table 3. On the other hand Roe (17) and Boutwell et al. (5) obtained larger numbers of benign and some malignant tumors with croton oil alone. Boutwell et al. (5), using albino mice and 500 µg of croton oil alone, 2 times weekly, also

AUGUST 1966
observed carcinomas. Roe and Clack (18) used 2 strains of albino mice and also observed malignant tumors that arose after 1 year of painting with 250 μg of croton oil once weekly, after initiation. In addition, they observed large numbers of benign tumors in animals which had received the same amount of croton oil alone.

4. TUMOR MULTIPLICITY. In the present work the average number of tumors/tumor bearer ranged from 3.8 to 8.4 with 1 exception, Experiment 6; however, this group was different from the others since the animals were over 1 year old before the beginning of promoting treatment. There does not appear to be any striking difference in the tumor multiplicity, i.e., the average number of tumors/tumor bearer, between croton resin and its fractions and croton oil as reported by other workers (Table 1). The significance of any similarities in tumor multiplicity due to different promoters is difficult to assess because of the possible role of the initiating dose in determining the number of tumors/tumor bearer. For example, Ball and McCarter (1) and Klein (15) have reported a correlation between tumor multiplicity and dose of initiator. Animal strain and dose of promoter may be other factors influencing tumor multiplicity.

5. TIME or TUMOR APPEARANCE. One of the striking findings in our experiments concerns the number of days from the beginning of promotion until the appearance of 1st tumors. In 7 out of 8 experiments this occurred at 31 ± 6 days, with croton resin, A and C at comparable dosages.

Klein found the latent period to be determined by the initiating dose when croton oil treatment was held constant (15). However, Klein used initiating doses that were carcinogenic for his strain of mice. When a subcarcinogenic initiating dose is used, latent period is independent of the initiating dose. This is well substantiated by the work of Berenblum and Shubik (3) and Ball and McCarter (1); the same conclusion was also drawn from the present work, in which 150 μg of DMBA was used for some experiments and 300 μg in others.

In the earlier studies, dosages of croton oil ranging from 250 μg (18) to 5000 μg/application (12) were used, as compared to croton resin dosages of 5–25 μg used in the present work. The dosages of croton oil used in other studies and those of the active materials used in the present study are in the same range as far as active principles from croton resin are concerned since croton oil, according to various workers (8), contains between 1.0 and 10% croton resin.

It is not possible to ascribe the observed differences in biologic response between croton oil and the active materials to a single factor.

Skin damage as a factor in tumorigenesis should be considered, although its role is difficult to assess. Many investigators have reported macroscopic skin damage with croton oil including epilation, inflammatory swellings, and gross hyperplasia (5, 12) in mice following chronic croton oil administration. With the sample of croton oil available to us, it was found that a 5% solution of croton oil in acetone applied 3 times weekly for 2 weeks (100 mg solution/application) gave rise to severe skin damage similar to that described by these earlier workers (5, 12).

The microscopic changes brought about in mouse skin by single and repeated applications of croton resin and of fraction C were also determined. Animals were sacrificed at various time intervals between 4 hr and 7 days after single applications of 25 μg and 5 μg of croton resin and fraction C, respectively. The skins were examined histologically for: epidermal hyperplasia, changes in keratin, hair follicle changes, sebaceous gland changes, and dermal changes.

The earliest histologic changes observed after a single treatment with promoter were inflammatory reactions in the dermis.
These were seen in more than 90% of the animals as early as 4 hr after treatment, reached a peak in severity at 24 hr and generally subsided by about 72 hr. Diffuse epidermal hyperplasia, however, was not observed until 24 hr. With chronic exposure to croton resin or fraction C (up to 270 days) the only histologic abnormality observed was diffuse epidermal hyperplasia. The details of these findings will be published elsewhere.

From the results reported by other workers, it is clear that croton oil and obtained similar gross skin damage in all 4 strains, while the similarity in tumor multiplicity and time to appearance of 1st tumors among several animal strains (Table 1) suggest that these factors are largely independent of strain.

One of the factors most frequently overlooked in earlier studies is that of chemical composition. Croton oil is a complex mixture of lipids, fatty acids (saturated and unsaturated), and other materials. It is reasonable to assume that the chemical composition of the oil will vary from 1 batch to another, from source to source and will also change on aging. Croton oil also contains an interesting compound, crotonoside, which is isoguanine riboside (7). The biologic properties of this compound remain to be explored. The presence of weakly carcinogenic materials in croton oil remains to be determined. Experiments carried out in this laboratory and described above have established the presence of alkylating agents in croton oil. The most likely type of material that might be a natural product alkylating agent is an epoxide. The several reports of carcinogenic activity of croton oil may well be due to the presence of an epoxide or other type of carcinogen in croton oil. Until malignant tumors are induced with known active materials, results obtained with the mixture of chemicals, known as croton oil, must be viewed with reserve.

References


14. ——. Influence of Continued and Intermittent Painting with Croton Oil on Skin Tumorigenesis in Mice. Ibid., 14: 83–89, 1953.

15. ——. Induction of Skin Tumors in the Mouse with Minute Doses of 9,10-Dimethyl-1,2-benzanthracene Alone or with Croton Oil. Cancer Res., 16: 123–27, 1956.


The Tumor-enhancing Principles of *Croton tiglium L.*: II. A Comparative Study

B. L. Van Duuren, L. Langseth, A. Sivak, et al.


Updated version: Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/26/8_Part_1/1729