The Inhibition of Croton Oil-promoted Mouse Skin Tumorigenesis by Steroid Hormones

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SUMMARY

Tumors in female Swiss Millerton mouse skin were initiated with 25 µg 7,12-dimethylbenz(a)anthracene and, 2 weeks later, were promoted 3 times/week with 0.2 ml of 0.5% croton oil. Four steroid hormones in 0.2 ml of acetone were applied percutaneously at 6 and 30 µg 5 times/week. A fifth steroid was applied at the 30-µg dose. Tumor inhibition by the hormones was dose dependent in the order dexamethasone > Schering No. 11572 > prednisolone > hydrocortisone > cortisone. There was a general correlation between the antiinflammatory and antitumor activities of the steroids.

INTRODUCTION

There appears to be an association between epidermal hyperplasia and mouse skin tumor promotion (18). Although all promoters appear to be irritants which induce epidermal hyperplasia (3, 4, 12, 18, 26), not all irritants or hyperplastic agents are promoters (3, 18, 26). Berenblum (3) has suggested that a specific form of irritation or hyperplasia forms one of the component phases involved in the carcinogenic process. Boutwell (5) and Hennings and Boutwell (18) indicated 2 functions for promoters, a conversion to dormant tumor cells followed by mitotic stimulation.

Recent studies (19, 20, 31) in our laboratory have shown that small amounts (1 µg or less) of phorbol ester produced a marked inflammatory response in mouse ears, which is in agreement with the observations of Hecker (16) and Liberman et al. (21). The phorbol ester also produced an increase in tosyl arginine methyl esterase activity. Tumor promotion was inhibited by protease inhibitors, which suppressed both the inflammatory response and the esterase activity induced by croton oil or phorbol ester.

These observations led us to examine the promotion-inhibitory activities of certain steroids which are antiinflammatory (27-29), and antimitotic (7, 8, 15, 25) for the skin.

Thus far only cortisone and hydrocortisone have been tested, and they were found to inhibit tumor promotion by croton oil (5, 13, 30). Cortisone also inhibits mouse skin tumors by the complete carcinogens, benzpyrene (6), methylcholangthrene (1) and 9,10-dimethyl-1,2-benzanthracene (11, 13). Nakai (22) found that cortisone and 5 other steroid hormones inhibited methylcholangthrene-induced s.c. sarcomas in mice. His data indicated a general correspondence of tumor inhibition in mice with the antiinflammatory activities of the compounds in rats.

This paper reports the inhibitory effects of 5 steroid hormones on croton oil-promoted tumors and compares these effects with their antiinflammatory and antimitotic activities in mice.

MATERIALS AND METHODS

Materials

DMBA2 was obtained from Eastman Kodak (Rochester, N. Y.) and recrystallized from acetone-water. Croton oil was obtained from T. Schuchardt and Co. (Consolidated Midland Corp., Katonah, N. Y.). The 5 steroid hormones were a gift from Dr. B. Katchen, Schering Corp. These included a new synthetic steroid designated Schering No. 11572.3

Methods

Initiation and Promotion. Female Swiss Millerton mice (Millerton Farms, N. Y.), 6 to 8 weeks old, were given 25 µg of DMBA in 0.2 ml of acetone applied to the shaven backs. Two weeks later skin tumorigenesis was promoted in the mice with 0.2 ml of 0.5% croton oil in acetone applied 3 times a week. The steroids were applied to the backs percutaneously, at 2 concentrations, 5 times a week starting at the time croton oil was applied. The amounts used were 6 and 30 µg only. Solvent alone was applied to the controls. Twenty-four mice were used in each experiment.

Inflammation Assay. Six mice (6 to 8 weeks old) were used for each assay. The left ears were painted with solvent alone (95% acetone-water), and the right ears were painted with 1% croton oil in solvent or with 1% croton oil containing the steroids. The solutions were applied with a cotton-tipped applicator by swabbing twice both sides of the ear. About 0.10 ml is the estimated dose. After 4 hr the ears were cut off at the

1 This investigation was supported by project grants from the NIH (USPHS Research Grants ES-00505 and CA-08621) and a grant from the Allied Chemical Corporation and is part of a Center Program by the National Institute of Environmental Health Sciences Grant ES-00260. A preliminary report of this work has appeared (2).

2 The abbreviation used is: DMBA, 7,12-dimethylbenz(a)anthracene.

3 The structure of this compound was obtained after this paper was prepared. Its name is 16 a-methyldichlorosone-17,21-dibutyrate (structure on request).
Tumor Inhibition by Steroids

Preliminary experiments had shown that the maximum response was reached before 4 hr and continued for several hours thereafter. A similar result was obtained by Janoff et al. (19). This differs from rat ears (29), where the maximum response to croton oil is reached in 6 to 8 hr and declines thereafter. After being weighed, the ears were dried for 2 hr at 60° and then for 2 hr at 110°. The ears were then weighed again. This procedure is a combination of those of Tonelli et al. (29) and Janoff et al. (19).

The inflammation index $R$ is calculated as:

$$R = \frac{\text{Wet weight} - \text{dry weight}}{\text{dry weight}}$$

Chart 1. Effect of steroid hormones on tumor incidence of croton oil-promoted tumorigenesis in mice, expressed as percentage of mice with tumors. Abscissa, days after start of croton oil painting. Tumorigenesis was initiated in groups of 24 mice with 25 µg of DMBA in 0.2 ml of acetone. Two weeks later, the mice were treated thrice weekly with 0.5% croton oil in 0.2 ml of acetone. The hormone treatment was begun at the same time and consisted of 5 weekly applications of the indicated doses in 0.2 ml of acetone. A, 6 µg per daily dose (0.015 µmole of cortisone acetate, 0.015 µmole of hydrocortisone acetate, 0.017 µmole prednisolone, and 0.015 µmole of dexamethasone); B, 30 µg per daily dose. The numbers under $A$ and $B$ and in parentheses in $B$ are those of living mice at the last given point.

Chart 2. Same legend and symbols as Chart 1, expressed as number of papillomas per mouse.

Table 1

<table>
<thead>
<tr>
<th>Steroid</th>
<th>6 µg/application</th>
<th>Mice with tumors (%)</th>
<th>30 µg/application</th>
<th>Mice with tumors (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4</td>
<td>80</td>
<td>8.4</td>
<td>80</td>
</tr>
<tr>
<td>Cortisone</td>
<td>8.4</td>
<td>75</td>
<td>2.8</td>
<td>64</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>6.4</td>
<td>80</td>
<td>3.0</td>
<td>46</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5.2</td>
<td>60</td>
<td>2.0</td>
<td>36</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.4</td>
<td>18</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Schering No. 11572</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Effect of steroids on tumor incidence at 60 days after promotion with croton oil

Tumorigenesis in groups of 24 mice was initiated with 25 µg of DMBA in 0.2 ml of acetone. Thrice weekly treatments with 0.5% croton oil in 0.2 ml of acetone were begun 2 weeks later. The hormones were applied 5 times a week in 0.2 ml of acetone, starting at the same time as the croton oil.

MARCH 1972 451

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Histological Examination of Skin for Mitoses and Inflammatory Cells. Six- to 8-week-old female Swiss Millerton mice were treated at 10 a.m. with 0.2 ml of 0.5% croton oil in acetone on their clipped backs. Controls received acetone alone. Twenty-four hr later, 0.1 mg of colchicine in 0.2 ml of water was injected i.p., and the animals were sacrificed 4 hr later. The skins were fixed in formalin, sectioned at 5 µm, and stained with hematoxylin-eosin. The hormones were applied to the backs about 15 min after croton oil at 30 and 150 µg in 0.2 ml of acetone. Mitotic counts were done on the epidermis.

The inflammatory cell counts were performed by Dr. Harry Demopoulos, Department of Pathology, New York University Medical Center, New York, N. Y. The slides were examined blind and at random. Areas of dermis were chosen so that s.c. fat, epidermis, and skin appendages did not encroach on the field. Two counts were done on each slide at X 1200 under oil immersion.

RESULTS

Tumor Inhibition by Steroid Hormones. The results in Charts 1 and 2 clearly demonstrate the tumor-inhibitory activities of the steroids. The last point in each chart is the maximum value observed. Beyond this time, the papillomas were coalescing into large masses, and some regressions occurred. The inhibitory potency is dose dependent and is in the order dexamethasone > Schering No. 11572 > prednisolone > hydrocortisone > cortisone. This is more clearly seen from the data at 60 days shown in Table 1.

Antiinflammatory Effect of Steroids. The antiinflammatory effect of the 5 steroids as measured by ear weights is shown in Table 2. The order of activity is Schering No. 11572 > dexamethasone = prednisolone > hydrocortisone = cortisone = control. Cortisone did not exhibit an antiinflammatory effect at the 2 concentrations given in the table. This is in

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>R ± a.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% croton oil</td>
<td>2.23 ± 0.13</td>
</tr>
<tr>
<td>1% croton oil + 0.15% cortisone</td>
<td>2.26 ± 0.12</td>
</tr>
<tr>
<td>1% croton oil + 0.75% cortisone</td>
<td>2.25 ± 0.00</td>
</tr>
<tr>
<td>1% croton oil + 0.15% hydrocortisone</td>
<td>2.19 ± 0.06</td>
</tr>
<tr>
<td>1% croton oil + 0.075% prednisolone</td>
<td>2.11 ± 0.13</td>
</tr>
<tr>
<td>1% croton oil + 0.15% prednisolone</td>
<td>1.65 ± 0.13</td>
</tr>
<tr>
<td>1% croton oil + 0.075% dexamethasone</td>
<td>1.93 ± 0.14</td>
</tr>
<tr>
<td>1% croton oil + 0.15% dexamethasone</td>
<td>1.64 ± 0.14</td>
</tr>
<tr>
<td>1% croton oil + 0.15% Schering No. 11572</td>
<td>1.15 ± 0.14</td>
</tr>
</tbody>
</table>

Six mice in each experiment.

b Solvent applied to left ear, croton oil and steroids to right ear.

c R, index of increased weight in treated ear; a.d., average deviation.

Table 3

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Rat ears*</th>
<th>Thymus*</th>
<th>Merck Indexb</th>
<th>Rat skin*c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisone</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.7</td>
<td>1.6</td>
<td>1.5</td>
<td>4</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>5.8</td>
<td>6.7</td>
<td>5.5</td>
<td>12</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>65</td>
<td>131</td>
<td>30</td>
<td>800</td>
</tr>
</tbody>
</table>

Data of Tonelli et al. (29)

b Data from Merck Index (28); organ and species not given.

c Data compiled by Nakai (22).

Table 4

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of cells/field</th>
<th>No. of acute inflammatory cells</th>
<th>No. of chronic inflammatory cells</th>
<th>Chronic minus control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Croton oil</td>
<td>158</td>
<td>48</td>
<td>110</td>
<td>90</td>
</tr>
<tr>
<td>Croton oil + cortisone</td>
<td>112</td>
<td>45</td>
<td>67</td>
<td>47</td>
</tr>
<tr>
<td>Croton oil + hydrocortisone</td>
<td>93</td>
<td>38</td>
<td>55</td>
<td>35</td>
</tr>
<tr>
<td>Croton oil + prednisolone</td>
<td>64</td>
<td>26</td>
<td>38</td>
<td>18</td>
</tr>
<tr>
<td>Croton oil + dexamethasone</td>
<td>92</td>
<td>55</td>
<td>37</td>
<td>17</td>
</tr>
<tr>
<td>Croton oil + Schering No. 11572</td>
<td>40</td>
<td>4</td>
<td>36</td>
<td>16</td>
</tr>
</tbody>
</table>

Numbers are averages for 2 counts which were not significantly different.

b Control skin contains round cells which are mostly normal tissue histiocytes and not chronic inflammatory cells.
contrast to its effect on rat ears (29), where it does have this effect. Dexamethasone and prednisolone have equal activities at 0.015%, and dexamethasone is slightly more active than prednisolone at 0.075%. These results are also in contrast to their effect in rat ears (29), where dexamethasone is 10 times more potent than prednisolone. The relative order of antiinflammatory activity is, however, similar to that in rat ears, rat thymus, and rat skin (Table 3).

The antiinflammatory effects on dermal inflammatory cells are shown in Table 4. Control skin contains round cells which are mostly normal histiocytes and not inflammatory cells. Croton oil produces a marked increase (about 8-fold) in the number of cells, most of which are chronic inflammatory cells. All the steroids inhibited this response to croton oil. The number of acute inflammatory cells was not greatly affected, with the exception of Schering No. 11572. The major inhibition was in the number of chronic inflammatory cells and is in the order Schering No. 11572 = dexamethasone = prednisolone > hydrocortisone > cortisone.

**Antimitotic Effects.** The number of mitotic cells in normal epidermis is about 4/1000. Croton oil induced about a 10-fold increase in 24 hr (Chart 3). Cortisone at low and high concentrations acted as an additional stimulus, producing a further 2-fold increase in mitoses. Hydrocortisone exerted a slight stimulation at low concentrations and a slight inhibitory effect at high concentrations. Prednisolone had a variable effect at low concentrations but an inhibitory effect at high concentrations. Dexamethasone and the Schering compound were inhibitory at both concentrations.

**DISCUSSION**

The inhibition of croton oil-promoted tumorigenesis in mouse skin by small amounts of locally applied natural and synthetic corticosteroid hormones is clearly established by the results in this paper. The most potent antitumor steroid, dexamethasone, at 30 μg per application delayed the onset of tumors by 90 days and produced a marked inhibition beyond that time.

Our results are in agreement with the observations of Ghadially and Green (13), Boutwell (5), and Trainin (30) that cortisone and hydrocortisone inhibit croton oil-induced papillomas.

Our data indicate a general correlation between the antiinflammatory and antineoplastic activities of the steroids in mice. This is best seen from the histological data in Table 4. With the exception that the activity of prednisolone and dexamethasone are equal, the correlation is very good. Nakai (22) found a similar inhibition of methylcholanthrene-induced s.c. sarcomas in mice by steroids when given in doses that were equivalent to their antiinflammatory potencies in the rat, i.e., dexamethasone >> triamcinolone > methyl prednisolone > prednisolone > hydrocortisone > cortisone (Table 3). These data suggest a correlation between antineoplastic and antiinflammatory activities, but these 2 effects were not measured in the same species, and methylcholanthrene was used as a complete carcinogen. The results in Table 2 and the data in Table 3 show that the rat and mouse respond differently to the antiinflammatory action of the steroids.

The variable responses of the mice to the effects of the steroids on croton oil-induced mitoses merely suggest a contributory role of antimitoses to their antitumor activities. The observation that cortisone acts as a further stimulus to mitoses would indicate that this hormone acts by other means. This effect has been observed by others (24).

The hierarchial activities of the steroid hormones should be useful in understanding the mechanism of promotion, provided that good correlations can be obtained between their antitumor and other biological effects in the specific animal and tissue involved. Experiments in progress indicate such a correlation with inhibition of hair growth after plucking. This may be significant if the claim of Giovanella et al. (14) that neoplastic cells arise from the piliary apparatus is correct.

Promotion has been operationally separated into 2 parts, a conversion step and a propagation step (5, 18). The hormones may act on 1 step or both. Cortisone, for example, which does not inhibit croton oil-induced mitoses, may inhibit the conversion step only. This possibility is under investigation.

The similar behavior of the steroid hormones and protease inhibitors (31) that suppress tumorigenesis suggest that they may act by related mechanisms. Both groups of compounds are antiinflammatory (29, 31), inhibit phytohemagglutinin-induced mitosis and macromolecular
Sidney Belman and Walter Troll

synthesis in lymphocytes (10, 17, 23), and prevent the increase in croton oil-induced mouse skin protease (unpublished observations). The relationship between these 2 groups may be the stabilization of lysosomal membranes by steroids which would prevent the release of croton oil-induced protease (9, 32).

ACKNOWLEDGMENTS

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REFERENCES

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