Effect of Tumor-promoting Phorbol Esters and of Acetic Acid on Mechanisms Controlling DNA Synthesis and Mitosis (Chalones) and on the Biosynthesis of Histidine-rich Protein in Mouse Epidermis

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SUMMARY

The effect of phorbol esters on the incorporation of thymidine-3H into DNA and on the mitotic activity in dorsal mouse epidermis as well as on the inhibition by epidermal G1 chalone of epidermal DNA synthesis was studied.

After application of 20 nmoles 12-O-tetradecanoylphorbol-13-acetate (TPA), a depression of DNA labeling for approximately 10 hr was observed, which was followed by an oscillating pattern of stimulated DNA synthesis exhibiting at least 3 peaks at 18, 30, and 42 hr (500, 1100, and 600% of the control). With a 10 times lower dose of TPA only a slight initial depression and only 1 peak at 18 hr (700%) occurred. No depression was found after application of 20 or 300 nmoles of the weak tumor promoter phorbol dibenzoate (PDB). With 20 nmoles PDB 1 peak at 18 hr (500%) and with 300 nmoles PDB 2 peaks at 18 and 42 hr (1150 and 400%) occurred. Acetic acid (400 μmoles) caused a very pronounced initial depression of DNA labeling and later a biphasic elevation with peaks at 18 to 24 hr (300%) and 42 hr (1000%).

After application of 2 nmoles TPA, an oscillating pattern of mitotic activity in epidermis was also observed, exhibiting at least 2 prominent peaks at 10 and 26 hr (600 and 2200% of the control). The early peak was obviously due to the stimulation of an epidermal cell population normally arrested in the G2 phase of the mitotic cycle.

After treatment of mouse skin with TPA, PDB, or acetic acid, the inhibitory effect of pigskin extracts, which have been found to contain an epidermis-specific inhibitor of DNA synthesis (G1 chalone), was transiently diminished or completely abolished. It could not be restored by increasing the dose of the inhibitor or by altering the time interval between the injection of the inhibitor and DNA labeling. Furthermore, the stimulatory effect of TPA on epidermal DNA synthesis could not be diminished significantly by repeated injections of skin extract prior to TPA application. In spite of this, G1 chalone could be demonstrated in TPA-treated epidermis.

TPA was more effective in disturbing the chalone mechanism than was PDB, whereas a nonpromoting phorbol ester such as 4-O-methylphorbol-12,13-didecanoate neither inhibited the chalone effect nor stimulated epidermal DNA synthesis. The effect of TPA on the chalone mechanism was dose dependent. The reappearance of the chalone effect at about 2 days after TPA treatment fell together with an enhanced precursor incorporation into histidine-rich protein.

INTRODUCTION

The tissues of multicellular organisms are subject to stringent control of growth and function. A permanent breakdown of this control ultimately leads to "dedifferentiation" and neoplastic growth, whereas a transient switching off results in reversible hyperplasia.

The nature of the control mechanisms is largely unknown; their elucidation, however, would greatly facilitate progress in experimental cancer research. Theoretically, the most satisfactory hypothesis is still the concept of Weiss and Kavanau (59), which states that mitotic and functional homeostasis in tissues is maintained by negative feedback between the differentiated and the proliferative cell populations. In the last 10 years, the idea that this feedback is brought about by tissue-specific endogenous inhibitors of cell division or chalones has gained increasing support (12). Especially in the case of epidermis and of the hemopoietic system, our knowledge of chalones has made considerable progress. Two local epidermis-specific inhibitors have been described which either prevent the flow of cells from the G1 into the S phase (G1 chalone) (12, 15, 36) or inhibit mitosis by setting up a block in the G2 phase (G2 chalone) (10, 28).

Since it is generally accepted that epidermal functionalization occurs in cells arrested in G1, the G1 chalone has become the favored candidate for an "epidermal chalone proper," whereas the role played by the G2 chalone in regulating tissue homeostasis is not as clear. The epidermal G1 chalone has been partially purified from pigskin (36) as well as from mouse epidermis (J. Schweizer and F. Marks, unpublished results). It is metabolically rather stable (15) and seems to be located predominantly in the keratinizing layers of epidermis (17), whereas the G2 chalone is more concentrated in the cells of the stratum basale (16). In pigskin extracts, the G1 chalone has been found to be highly aggregated with other proteins...
(36). At least in this form it is resistant to heat and proteolytic digestion (36). After cleavage of disulfide bonds, masking of free sulfhydryl groups, and disaggregation with sodium dodecyl sulfate, an apparent molecular weight of 10,000 daltons or less has been found by means of sodium dodecyl sulfate-polyacrylamide electrophoresis (F. Marks, unpublished results). When injected into mice, the G1 chalone inhibits thymidine incorporation into epidermal DNA after a lag phase of 2 to 5 hr; maximal inhibition is observed 12 to 15 hr after injection (36). As recently demonstrated by autoradiography, this inhibition is due to a true inhibition of DNA synthesis in the stratum basale (Ref. 40; K. Elgjo, personal communication). The inhibitory effect is obviously restricted to epidermis and closely related epithelia (36, 40). Since no evidence has been provided as yet that there is more than 1 inhibitor of DNA synthesis in pigskin extract, experiments with enriched skin fractions instead of the more purified chalone preparations are justifiable.

The strong hyperplastic reaction seen after wounding or treatment of epidermis with certain agents such as the phorbol esters, which is frequently accompanied by a tumor-promoting effect (2, 25), may be taken as an indication of a pronounced effect (2, 25), may be taken as an indication of a pronounced relationship between the action of hyperplasiogenic agents and the chalone mechanism in mouse epidermis. Our results may lead to a new concept of the mechanism of tumor promotion.

MATERIALS AND METHODS

Chemicals. L-Histidine-3H hydrochloride (specific activity, ca. 3 Ci/mmmole) and thymidine-methyl-3H (specific activity, 6.7 Ci/mmmole) are products of New England Nuclear, Boston, Mass. Colchicine was purchased from Serva, Heidelberg, Germany. The phorbol esters TPA,3 PDB, and 4-O-methyl-phorbol-12,13-didecanoate were kindly supplied to us by Professor Dr. E. Hecker (Deutsches Krebsforschungszentrum, Institut für Biochemie, Heidelberg, Germany).

The preparation called “epidermal chalone” was a 72 to 81% ethanol precipitate of an aqueous extract from pigskin which has been shown to contain epidermal G1 and G2 chalone (28, 36). This material (Code CH 1333) was a generous gift of Dr. W. Hondius-Boldingh (N. V. Organon, Oss, The Netherlands).

Preparation of Extracts from Mouse Epidermis. The mice (strain NMRI) were killed by cervical dislocation. After back skin was dissected, the epidermis was scraped off by means of a scalpel and homogenized in 50 to 100 volumes of 0.1 M Tris-HCl buffer (pH 7.4). After centrifugation of the homogenate at 105,000 X g for 1 hr, the supernatant was ultrafiltered using an Amicon PM-10 filter with an exclusion limit of 10,000 daltons. The residue was washed on the filter with water until the filtrate was free of Tris-HCl and then lyophilized.

Animals and Treatment. Female NMRI mice 7 to 8 weeks old were used in all experiments. The animals were kept under an artificial day-night rhythm as described previously (36). The back skin of the mice was shaved 1 week prior to the experiment and only those animals that did not show a regrowth of hair were used.

The solution of the phorbol ester or of acetic acid (in 0.10 ml to 0.15 ml acetone) was routinely applied at 10 a.m. to the shaved dorsal skin by means of an Eppendorf micropipet. The control animals were treated only with the solvent acetone. The chalone preparation was dissolved in 0.3 ml 0.9% NaCl solution and injected i.p.

Pulse Labeling of Epidermal DNA. For pulse labeling of epidermal DNA, 1 µCi of thymidine-3H per g body weight (dissolved in 10 µl 0.9% NaCl solution) was injected i.p. Thirty minutes later the animals were killed by cervical dislocation. The back skin was immediately dissected and soaked in ice-cold 0.8 M perchloric acid for 2 to 5 min. The epidermis was then scraped off by means of a scalpel and homogenized in 2 ml of 0.4 M perchloric acid using a Potter homogenizer (B. Braun Apparatebau, Melsungen, West Germany). The homogenate was kept in an ice bath for 30 min and centrifuged. The sediment was then washed at 0—4°C with 2 ml 0.4 M perchloric acid (IX), 2 ml ethanol (2X), and 2 ml ether (2X). The dry residue was suspended in 1 ml of 0.3 M KOH, heated in a boiling water bath for 5 min, and then cooled in an ice bath. Then 60 µl of concentrated perchloric acid were added and the mixture was kept at 0—4°C for 1 hr. The precipitate obtained afterwards by centrifugation was washed at 0—4°C with 2 ml 0.4 M perchloric acid (2X) and 2 ml ethanol (2X), suspended in 1 ml 0.5 M perchloric acid, and heated in a boiling water bath for 10 min. After centrifugation 0.2-ml aliquots of the supernatant were used for duplicate determinations of radioactivity (26) and DNA content (11).

In control experiments it was checked that the acid-soluble radioactivity was completely washed out by this procedure.

Determination of Mitotic Activity. The mitotic activity in the interfollicular epidermis of mouse back skin was measured by means of the colchicine technique. For this purpose, 5 µg colchicine per g body weight were injected i.p. One or 4 hr later the animals were killed. The skin was dissected and fixed with 10% formalin. Five-µm sections were stained with hematoxylin-eosin, and the metaphase figures were counted under a microscope.

Pulse Labeling and Isolation of HRP. In order to measure the rate of biosynthesis of HRP, 15 µCi of histidine-3H hydrochloride (dissolved in 0.2 ml 0.9% NaCl solution) were injected i.p. The HRP fraction was isolated by a method based on that of Hoober and Bernstein (29). One hr after injection the animals were killed. The back skin was immediately dissected and soaked in 8 M urea. The epidermis was then scraped off as described above and suspended in 2 ml of 8 M urea in 0.2 M Tris-acetate (pH 8.5) and thoroughly homogenized using a Mini-Potter homogenizer. After standing for 1 hr, the homogenate was centrifuged at 40,000 X g and 15°C for 30 min. The supernatant was dialyzed at 2—4°C against 2 liters of 0.1 M ammonium hydroxide (IX) and 2 liters of 0.01 M
ammonium hydroxide (2X) for 36 hr and then lyophilized. The dry residue was suspended in 0.5 ml 0.1 M perchloric acid by shaking for 30 min at room temperature. After centrifugation, the supernatant was adjusted at pH 4.5 with 2 M sodium carbonate and kept in an ice bath for at least 10 min. The precipitate obtained by centrifugation at 0—4° was extracted with 0.5 ml of 0.02 M sodium carbonate for 4 to 5 hr (room temperature). After centrifugation, 0.2-ml aliquots of the supernatant were finally used for determination of radioactivity (26) and protein content (35).

RESULTS

Stimulation of DNA Synthesis and Mitotic Activity by Phorbol Esters. Although the induction of epidermal DNA synthesis by tumor-promoting phorbol esters has been repeatedly studied (3, 24, 42, 43, 47, 49), the time course of the effect is still not known exactly. We have therefore reinvestigated the reaction by pulse labeling DNA of dorsal mouse epidermis with thymidine-3H in vivo. As shown in Chart 1, a dose of TPA as normally used for experimental tumor promotion (20 nmoles/animal) caused an initial depression of thymidine incorporation, followed by 3 peaks at 18, 30, and 42 hr after application. The fact that the 2nd peak is nearly twice as high as the 1st one might be interpreted by the assumption that after 18 hr DNA synthesis is still partially depressed due to the early toxic effect of TPA (as indicated by the initial depression of thymidine incorporation). Using one-tenth of this TPA dose (2 nmoles/animal), only a very slight initial depression and only 1 peak at 18 hr followed by a shoulder were observed (Chart 1). Under these conditions the stimulation of DNA synthesis began earlier than with the higher dose. The same experiment has been done with 2 different concentrations of the weak promoter (2) PDB (Chart 2). The temporal response seen after application of 20 nmoles PDB was quite similar to that after application of 2 nmoles TPA, but the effect did not last as long and was not as pronounced. With the very high dose of 300 nmoles PDB per animal, a strong stimulation was observed at 18 hr followed by a 2nd small peak at 42 hr. The initial depression observed after application of TPA could not be found with either 20 or 300 nmoles PDB.

The time course of the mitotic activity in the interfollicular epidermis of mouse back skin as observed after application of 2 nmoles of TPA is shown in Chart 3. The most striking feature of this curve is an initial mitotic peak (between 6 and 10 hr) which appears before the incorporation of thymidine into DNA is stimulated. Thereafter the mitotic activity declined to the control level between 14 and 16 hr, rising again to a shoulder at 20 hr which was followed by a sharp and prominent peak at 26 hr. After around 40 hr the stimulation ceased. With 20 nmoles TPA, peaks of mitotic activity were also seen to follow the peaks of DNA synthesis.

Effect of Epidermal G1 Chalone on Phorbol Ester- and Acetic Acid-Stimulated DNA Synthesis.4 For testing the activity of the epidermal G1 chalone, a 70 to 80% ethanol precipitate of aqueous pig skin extract (28) was injected i.p. Twelve hr later the epidermal DNA was pulse labeled as described above. It has been shown previously that the amount of extract injected (4 × the 50% inhibitory dose) causes, after an initial lag phase of 2 to 5 hr, a 60 to 80% inhibition of thymidine incorporation between 10 and 15 hr after injection (36). Up to 42 hr after application of 20 nmoles of TPA, the epidermis did not respond to the inhibitor (Charts 4 and 6). Only after 2 days did the inhibitory effect reappear rather suddenly. Even a 10-fold dose of the skin extract did not exert any effect during the period of no response (Table I).

One could argue that phorbol ester-treated epidermis does not respond to chalone because, being released from a “chalone block,” the cells have entered a chalone-insensitive

* Part of this was reported in a short communication (37).
Chart 2. The effect of a single local application of PDB on the labeling of DNA in mouse dorsal epidermis. For details see the legend to Chart 1. ---, 300 nmoles PDB; ----, 20 nmoles PDB.

Chart 4. The effect of chalone treatment on the labeling of DNA in mouse dorsal epidermis after a single local application of TPA. The animals were treated with 0.1 ml acetone or with 2 nmoles (right) or 20 nmoles (left) TPA in 0.1 ml acetone and killed at the times indicated. Twelve hr prior to sacrifice, 0.2 ml 0.9% NaCl solution or a 67 µg/g body weight dose of pigskin extract containing G alpha chalone (dose per animal dissolved in 0.3 ml 0.9% NaCl solution) were injected i.p., and 30 min prior to sacrifice 1 µCi thymidine-3H per g body weight was injected i.p. ---, labeling of epidermal DNA in mice given injections of 0.9% NaCl solution; ----, labeling of epidermal DNA in mice given injections of G alpha-chalone. The values are expressed as percentage of average specific activity in the acetone-treated mice (± S.D., n = 3 to 13).
phases of the cell cycle. In order to exclude this possibility 2 experiments have been performed. In the 1st experiment the animals were given injections of 2 mg pigskin extract each 12, 8, 4, and 0 hr prior to the application of 20 nmoles TPA, and pulse labeled 18 hr after that application. As shown in Table 1 this treatment caused a pronounced inhibition of DNA synthesis in the acetone-treated control mice, whereas in the TPA-treated epidermis no such inhibition could be observed. In the 2nd experiment up to 20 mg pigskin extract were injected 1 hr prior to treatment with TPA (2 nmoles) and the DNA labeling was measured 18 hr later. In this high dose the skin extract has been shown to cause a deep depression of DNA synthesis in normal epidermis between 5 and 30 hr after injection (36). By measurement of an unspecific inhibition by pigskin extract of epidermal adenyl cyclase (W. Grimm and F. Marks, unpublished data) it has been found, furthermore, that the extract arrives in epidermis by 20 min after i.p. injection. It could be expected, therefore, that the chalone was already in its target tissue when the phorbol ester was applied and that the time of pulse labeling was within the period of the maximal chalone effect. Nevertheless, in phorbol ester-treated epidermis no inhibition of DNA synthesis could be achieved (Table 1).

In addition, no inhibitory effect was observed if the time interval between injection of skin extract and pulse labeling of DNA was decreased from 12 to 6 hr and less, thus excluding the possibility that TPA shortens the lag phase of the G₁ inhibition (15, 36). In acetone-treated control animals, the DNA synthesis could be depressed to about 30% of the normal value on injection of skin extract. Two days after treatment with TPA, the stimulated DNA labeling could be reduced from 380% to about 170% on injection of the G₁ chalone. This degree of inhibition could not be increased by raising the dose of the skin extract, which indicates that the effect was not caused by an insufficient dose of the inhibitor but was, rather, because only a certain proportion of the proliferating cells was able to respond to the chalone at this time (Table 1). Carrying out the same experiment with boiled extract, in which the G₂ chalone but not the G₁ chalone is destroyed, (10, 28, 36), provided essentially the same results.

These results favor the idea that the abolition of the chalone effect was due to a blocking by the phorbol ester of the chalone receptor mechanism instead of an inactivation of the chalone itself. This was confirmed by the following experiment. Mice were treated with 20 nmoles TPA or acetone in the usual manner. A "chalone extract" was prepared from the treated epidermis and injected into untreated animals. As shown in Table 2, the DNA synthesis was inhibited equally well by epidermal extracts from acetone-treated and TPA-treated mice (since the chalone-dependent inhibition of DNA synthesis always fluctuates between 50 and 80% the differences cannot be considered to be significant). This result indicates that TPA-treated epidermis still contains G₁ chalone, probably in concentrations comparable to those of normal tissue.

The abolition of the G₁ inhibition depended on the dose of TPA. It was also observed after treatment with 2 nmoles (Chart 4). However, under these conditions the period of no response was considerably shorter. Finally, after application of 1 nmole TPA, the stimulated epidermal DNA synthesis could still be inhibited (from 470% to 250% of the acetone control at 18 hr).

PDB was considerably less active in inhibiting the chalone effect. A complete abolition for a rather short period of time could be achieved only with 300 nmoles PDB (Charts 5 and 7). With 20 nmoles the effect of exogenous chalone did not disappear but was only diminished (Charts 5 and 7). Again, as after treatment with TPA, the G₁ inhibition could not be restored by increasing the dose of skin extract or by chalone injection prior to PDB application (Table 1). Finally, a nonpromoting phorbol ester such as 4-O-methyl-phorbol-didecanoate did not exert any effect on the G₁ inhibition and did not stimulate epidermal DNA synthesis. These results are summarized in Charts 6 and 7, showing the time course of the chalone effect after application of the 2 phorbol esters. During the period of no response following TPA application, a stimulation of thymidine incorporation was repeatedly seen after injection of G₁ chalone (see also Table 1). However, in

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**Table 1**

<table>
<thead>
<tr>
<th>Application of Pigskin extract (mg/animal)</th>
<th>DNA specific radioactivity Mean ± S.D.</th>
<th>n</th>
</tr>
</thead>
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<tr>
<td>TPA (2 nmoles) a</td>
<td>0</td>
<td>689 ± 147</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>809 ± 186</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>996 ± 125</td>
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<tr>
<td>TPA (20 nmoles) a</td>
<td>0</td>
<td>510 ± 66</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>515 ± 135</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>628 ± 178</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>657 ± 131</td>
</tr>
<tr>
<td>TPA (20 nmoles) b</td>
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<td>382 ± 62</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>378 ± 104</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>162 ± 59</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>157 ± 32</td>
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<tr>
<td>TPA (20 nmoles) c</td>
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<td>362 ± 71</td>
</tr>
<tr>
<td>Acetone</td>
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</tr>
<tr>
<td>TPA (2 nmoles) d</td>
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<td>689 ± 147</td>
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<tr>
<td></td>
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<td>846 ± 153</td>
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<tr>
<td>PDB (20 nmoles)</td>
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</tr>
<tr>
<td></td>
<td>2</td>
<td>360 ± 112</td>
</tr>
<tr>
<td></td>
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<tr>
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<tr>
<td>PDB (20 nmoles)</td>
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<td></td>
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<tr>
<td>PDB (300 nmoles)</td>
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</tr>
<tr>
<td></td>
<td>20</td>
<td>1114 ± 74</td>
</tr>
</tbody>
</table>

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a Pulse labeling 18 hr after application of phorbol ester or acetone.
b Pulse labeling 48 hr after application of TPA or acetone.
c Two mg each injected 12, 8, 4, and 0 hr prior to TPA application.
d Injected 1 hr prior to application of phorbol ester or acetone.
view of the large standard deviations some doubts may be raised as to the significance of this finding.

Finally, a similar experiment was carried out with acetic acid (400 μmoles/animal; 15% solution in acetone) instead of phorbol esters. Acetic acid induces cell proliferation in epidermis but is generally considered to possess no tumor-promoting activity (53). As shown in Chart 8, acetic acid caused an extended and very deep initial depression (approximately 10% of the control) followed by a broad elevation of DNA labeling with a pronounced peak 42 hr after treatment. Again, the inhibitory effect of the G1 chalone was suspended for the whole period of stimulation (Chart 8).

Effect of TPA on the Synthesis of HRP. From the theoretical point of view, the chalone effect has to be cell-line specific. This means that the whole mechanism is an expression of cell differentiation or even modulation and must be established during the maturation of skin cells to committed and differentiated tissue cells (7). This view is consistent with the observation that the epidermal G1 inhibitor is localized predominantly in the keratinizing epidermal cells (17). Therefore, the reappearance of the G1 inhibition 2 days after application of TPA might coincide with a wave of keratinization, which would be expected to follow cell proliferation when the stimulatory effect of the phorbol ester falls off. To test this possibility, the incorporation of labeled histidine into the so-called HRP was measured. This protein has been shown to be involved in the biosynthesis of keratohyalin, which is thought to be a precursor of keratin (5).

Table 2
Effect of in vivo acetone or TPA treatment on the ability of epidermis fractions to inhibit incorporation of thymidine-3H into epidermal DNA

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>DNA specific radioactivity % of NaCl solution (control)</th>
</tr>
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<tbody>
<tr>
<td>Acetone-treated mouse epidermis</td>
<td>20.3 ± 6.2</td>
</tr>
<tr>
<td>Epidermis 6 hr after TPA</td>
<td></td>
</tr>
<tr>
<td>application</td>
<td>24.1 ± 7.6</td>
</tr>
<tr>
<td>Epidermis 18 hr after TPA</td>
<td>27.8 ± 8.3</td>
</tr>
<tr>
<td>application</td>
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<tr>
<td>Epidermis 24 hr after TPA</td>
<td>37.0 ± 12.0</td>
</tr>
<tr>
<td>application</td>
<td>6</td>
</tr>
<tr>
<td>Epidermis 30 hr after TPA</td>
<td>31.6 ± 7.6</td>
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<tr>
<td>application</td>
<td>5</td>
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<tr>
<td>Epidermis 48 hr after TPA</td>
<td>24.6 ± 13.2</td>
</tr>
<tr>
<td>application</td>
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</tr>
<tr>
<td>Epidermis 72 hr after TPA</td>
<td>24.9 ± 9.6</td>
</tr>
<tr>
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</table>

Chart 6. The effect of pigskin extract (G1 chalone) on the labeling of DNA in mouse dorsal epidermis after a single local application of TPA. The animals were treated with 0.1 ml acetone or a solution of 2 nmoles (-----) or 20 nmoles (- - - - - - ) TPA in 0.1 ml acetone and killed at the times indicated. For the injection of NaCl solution (controls), pigskin extract and labeled thymidine see the legend to Chart 4. The values are expressed as percentage of average inhibition of DNA labeling by pigskin extract in TPA treated animals. (± S.D., n = 3 to 13). Shaded area, average S.D. of the “inhibition” of DNA labeling in TPA-treated mice that were given injections of 0.9% NaCl solution only.

Chart 5. The effect of chalone treatment on the labeling of DNA in mouse dorsal epidermis after a single local application of 20 nmoles (right) or 300 nmoles (left) PDB. For details see the legend to Chart 4 (n = 3 to 6).
Chart 7. The effect of pigskin extract (G₁ chalone) on the labeling of DNA in mouse dorsal epidermis after a single local application of PDB. ●, 300 nmoles PDB; ○, 20 nmoles PDB. For details see the legends to Charts 5 and 6 (n = 3 to 6).

At least the accumulation of keratohyalin seems to run parallel to keratinization. The biosynthesis of HRP has therefore been suggested to be a biochemical parameter for epidermal functionalization.

Indeed, after treatment with 2 nmoles of TPA a broad peak of histidine incorporation into HRP was observed 18 hr after the peak of DNA synthesis (Chart 9). With the higher dose (20 nmoles) a more pronounced peak appeared after 2 days which was obviously related to the high maximum of DNA synthesis at 30 hr (Chart 10). Thus, with both TPA concentrations a constant time interval was observed between the peaks of thymidine and histidine incorporation. In both cases the maximum of HRP synthesis was seen approximately at the time when the chalone effect was reappearing. This result may be taken at least as an indication that the assumption described above is correct.

The kinetics of stimulation of HRP synthesis differs considerably from the kinetics of stimulation of synthesis of total epidermal protein, which shows a peak prior to the peak of DNA synthesis (3, 25).

DISCUSSION

The time course of epidermal DNA synthesis seen after stimulation with the strong hyperplasiogenic and tumor-promoting phorbol ester TPA indicates that the stimulated cells pass through several mitotic cycles in a more or less synchronous manner. Although studies on phorbol ester-stimulated DNA synthesis have been undertaken in several laboratories (3, 24, 42, 43, 47, 49) this pattern of synchronization has not been described as yet, perhaps because in those experiments the intervals between the thymidine pulses were too long. The cycle time of 10 to 12 hr and the 8-hr interval between S phase and mitosis are consistent with data recently obtained by Frankfurt and Raitcheva (18) by means of the labeled mitosis technique after application of the promoting agent TPA 60.

Surprisingly, with the low dose of TPA a peak of mitotic activity was seen prior to the 1st peak of DNA synthesis, indicating an initial stimulation of a cell population normally blocked in G₂. A similar observation has been reported after treatment of mouse skin with vitamin A acid (64). These findings agree with the concept that in normal stratum germinativum there exist 2 discrete epidermal cell populations, in which the cells are blocked at different positions in the mitotic cycle: a "G₁ population" and a "G₂ population" (20). Recently, it has been suggested that the G₂ population consists partially of pluri-potential epidermal stem cells (46).

The pattern of mitotic (and DNA-synthetic) activity found after application of a low dose of TPA may then be interpreted as a superposition of the effects on both of these populations. Initially, the G₂-population is stimulated to undergo a 1st wave of division between 6 and 10 hr and, after having passed 1 cycle, perhaps a 2nd wave at 18 to 22 hr. The latter would be expected to be superposed partially by the pronounced peak of mitotic activity seen after 26 hr, which is obviously caused by the stimulation of the G₁ population. After application of a higher dose of TPA (20 nmoles) only the "G₁ peak" at 24 to 26 hr was observed, whereas the "G₂ peak" was probably abolished by a pronounced depression of mitotic activity between 0 and 12 hr (47), which corresponds to the initial depression of thymidine incorporation. It is possible that the use of excessive TPA doses may be the reason that the G₂ peak has escaped detection until now.

An initial inhibition of mitoses (47) and of DNA synthesis (3, 24, 42, 43, 47, 49), as seen with the higher dose of TPA, was not observed after treatment with PDB, which is a considerably weaker promoter. Since, however, tumor promo-
tion can be achieved with the lower dose of TPA as well as with 20 nmoles PDB (2), this initial depression does not appear to have anything to do with the cocarcinogenic activity but may rather reflect some transient toxic effect on the cells. Furthermore, the stimulation of DNA synthesis by PDB or by the low dose of TPA is of short duration and does not exhibit a pronounced pattern of synchronization as seen after application of the higher dose of TPA. Whether this effect is due to a special biological property of TPA or is caused by a prolongation of its biological lifetime or of its presence as an active agent in skin must remain open at present. The observed synchrony of cell division might be taken as evidence that the epidermal cells are released from a physiological block rather than that they are stimulated directly. The concept of 2 epidermal cell populations controlled either in G1 or in G2 is furthermore consistent with the demonstration of 2 tissue-specific local inhibitors (chalones) in epidermal extracts acting either on DNA synthesis (G1 chalone) (15, 36) or on mitosis (G2 chalone) (10, 28). We therefore believe that our data lend strong support to the assumption that the induction of hyperplasia caused by phorbol esters or acetic acid is due to a transient inactivation at least of the mechanism of G1 inhibition. The same may be true for the G2 inhibition, as indicated by the appearance of the G2 peak of mitotic activity.

At the moment 2 tentative explanations may be envisaged: (a) the hyperplasticogenic effect of phorbol esters and acetic acid is directly due to an interference with the chalone mechanism. In this case our results favor the idea that the hypothetical chalone-receptor is inactivated, rather than the G1 chalone itself. (b) phorbol ester-treated epidermis does not respond to the G1 chalone because its cells have been converted to a physiological state which by itself is insensitive to chalone inhibition. Such conversion may have taken place in the course of some kind of “dedifferentiation,” morphological evidence for which was obtained in recent studies (47).

Experiments with chalones of the hemopoietic system (12) have supported the theoretical postulate that only the committed but not the noncommitted (pluripotential) stem cells (34) respond to these tissue- and cell-line-specific inhibitors. On the basis of studies on cell kinetics as well as on histological data, Potten (46) recently suggested the existence also in epidermis of a 2-compartment proliferative cell population similar to that of the hemopoietic system. The possibility must therefore be considered that under certain circumstances (for example, after treatment with phorbol esters) the chalone-sensitive committed stem cells of the stratum basale are converted into chalone-insensitive noncommitted stem cells. The latter are thought to be characterized by their potency to differentiate into the cells of the interfollicular epidermis, the hair follicles, or the sebaceous glands. This assumption is supported by several lines of evidence. First, as in the hemopoietic system, each of the 3 epidermal cell types seems to be controlled by its own chalone system (7—9). As we shall report in another paper (S. Bertsch and F. Marks. Lack of an Effect of Tumor Promoting Phorbol Esters and of Epidermal G1-Chalone on DNA-Synthesis in the Epidermis of Newborn Mice, in preparation), epidermis of newborn mice does not respond at all to the epidermal G1 chalone (and cannot be stimulated by phorbol esters). Since
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Chart 10. The effect of a single local application of 20 nmoles TPA on the labeling of HRP in mouse dorsal epidermis. For details see the legend to Chart 9.

this epidermis is not yet fully developed one might expect that the proliferative population is built up exclusively of noncommitted stem cells which will be replaced by committed stem cells during maturation of the animal. Additional support is derived from observations on wound healing. It is a well-known fact that, after wounding, cells of the hair follicles as well as of the sebaceous glands can be brought up to repair damage of the interfollicular epidermis (7, 45) and perhaps vice versa (6, 39). For example, after surgical removal of human skin the whole epithelium is regenerated by residual hair roots. For this purpose the cells must certainly dedifferentiate transiently to become pluripotential epidermal stem cells. Such a conversion of the type of differentiation (metaplasia), in which a differentiated cell population “reverses its commitment and assumes a type of differentiation distinct from the original one” (62), has been extensively studied in the case of lens regeneration from iris epithelium (14, 62, 63). During a process of this kind a cell line-specific autoregulation, for example by chalones, must be transiently switched off. A special kind of healing is the regeneration of epidermis observed after “stripping” the horny layer. Experiments performed in our laboratory indicate in fact that this process, also, cannot be prevented by the G1 chalone, indicating again the transient appearance of chalone-insensitive pluripotential stem cells (S. Bertsch and F. Marks, manuscript in preparation). These results are fully consistent with evidence presented recently by Potten (46) that the “central basal cells” of the epidermal proliferative units (which he believes to be noncommitted stem cells) are possibly responsible for wound repair. Finally, our assumption is strongly supported by morphological studies (47) which show that, after treatment of epidermis with TPA, basal and even superficial cells are “rejuvenated,” acquiring morphological characteristics resembling those of embryonic epidermis. This again agrees with our finding that TPA-treated epidermis resembles neonatal epidermis in that it shows no response to epidermal G1 chalone (S. Bertsch and F. Marks. Lack of an Effect of Tumor Promoting Phorbol Esters and of Epidermal G1-Chalone on DNA-Synthesis in the Epidermis of Newborn Mice, in preparation).

According to Bullough’s (7) chalone concept, it may be postulated, therefore, that the G1 chalone acts by arresting committed stem cells at a point of the mitotic cycle where they get the opportunity to take the road towards functionalization. After wounding or treatment with hyperplasiogenic agents such as the phorbol esters, all cells that are still in a state of reversible functionalization (on this side of the point of no return of the “suicide maturation” pathway (34) of keratinization) will fall back transiently into a state of noncommitment (pluripotentiality). Proliferative cells, which are in this state, do not respond to the control signals of the chalone system. As soon as the stimulus decreases the cells will redifferentiate, reestablishing their chalone receptors and forming again the different cell types present in the epidermis. That the latter is indeed the case is shown by morphological and biochemical evidence. Epidermal hyperplasia (after wounding, after phorbol ester treatment etc.) is always followed by a wave of keratinization and hair growth, the latter even in the telogen phase of the hair growth cycle (1).

The interrelationships between hyperplasiogenic and tumor-
promoting activities are still not fully understood. All we can say at present is that hyperplasia always accompanies promotion (19, 41, 51, 54). However, there are several agents, for example acetic acid (53), that although causing hyperplasia do not seem to exhibit promoting activity. The argument arising from these findings, i.e., that hyperplasia cannot be a sufficient condition for promotion, is certainly not conclusive. The 2-stage experiment of chemical carcinogenesis involves a sequence of complex processes and requires repeated applications of the promoter over a long period of time. Only very little is known about the molecular events occurring in epidermis during the later phases of this treatment. However, these events might be of crucial importance in determining whether or not an agent exhibits tumor-promoting activity. When the application of TPA is continued over several weeks the initial inhibition of thymidine-incorporation disappears and the subsequent stimulation of DNA labeling is decreased considerably (L. Krieg and F. Marks, unpublished work). It has furthermore been shown that the interval between individual applications is of crucial importance for the success of the promotion experiment (57, 58). For example, PDB is a very weak promoter if applied in the same manner as TPA (twice weekly), but it turned out to be rather active when applied more frequently (2).

Another source of error is the possibility that certain hyperplasicogenic agents may preferentially kill tumor cells due to cytotoxic side effects. This is probably the reason for the relatively low promoting activity of cantharidin (25). The same may be true for acetic acid; this agent causes a very pronounced inhibition of thymidine incorporation within the 1st 12 hr after application, which may be taken as an expression of its cytotoxic effect. The depression of DNA synthesis lasted longer than that observed with TPA. The tumor-promoting capacity of a compound which is at the same time cytotoxic and hyperplasicogenic may well depend on the relationship between both these effects and on the ability of skin to gain resistance against cytotoxicity during repeated applications.

Finally, experiments with hairless mice (21) as well as histopathological studies (K. Goerttler, unpublished results) strongly indicate that the great majority of papillomas and carcinomas generated during the classical 2-stage experiment of chemical carcinogenesis are derived from hair root cells instead of cells of the interfollicular epidermis, whereas the effect of a single application of a promoter is generally measured in the latter part of the tissue. In view of the intricate mechanisms involved in the hair growth cycle, for example, the situation (i.e., the relationship between the hyperplasicogenic and the cytotoxic effect of a promoter as well as the time course of growth stimulation) may be expected to be indeed much more complex in hair follicles.

Nevertheless, there is one strong evidence that induced cell proliferation alone is a sufficient condition for promotion. This is provided by the well-documented fact that normal wound healing has a pronounced tumor-promoting potency (23, 25). Considering the observations discussed above, especially those supporting the idea that wound healing is due to a transient dedifferentiation of committed epithelial cells (14, 63), we would like to put forward the following theory of tumor promotion.

It is generally accepted that neoplastic growth is due to a change in the state of differentiation rather than to a disturbance of tissue growth, which may be a secondary effect (see, for example, Ref. 44). This means that a dormant tumor cell generated in the classical 2-stage approach of chemical skin carcinogenesis has gained a new state of commitment (a new epidermal cell type has been created). Under normal conditions, at least in the adult animal, the committed state of epidermal differentiation is apparently extremely stable. It can be switched into another direction including that of the malignant state only, if the cell is allowed to dedifferentiate transiently to a pluripotential stem cell.5 Therefore, each manipulation that is able to bring about a reversal of epidermal functionalization beyond the committed state should be potentially tumor promoting, unless the epidermis develops some resistance or tumor cells are killed before they can be promoted. Since such manipulation is always accompanied by cell division, our conception agrees with the finding that mitosis is necessary for the expression of the malignant state (41).

At present our data are not sufficient to decide whether the inactivation of the chalone mechanism itself is the triggering event for the dedifferentiation or whether it is a secondary effect in the course of formation of pluripotential stem cells. Further investigations on the relationships between the chalone mechanism and functionalization may shed more light on the whole problem which is certainly one of the central questions of experimental cancer research.

There is a growing body of evidence that the control mechanisms for growth and function are localized in the outer cell membrane and that cancer is perhaps a “membrane disease” (52). On the basis of certain observations made during studies on isolation and purification of the epidermal G1 chalone, we have recently proposed that this factor may also act at the membrane level (36). If we assume that hyperplasicogenic agents interfere directly with this mechanism this would be fully consistent with experiments indicating that phorbol esters primarily attack the cell membrane (32, 33, 50, 55, 56, 60). Recently, it has been found in this laboratory that the β-receptor-adenyl cyclase complex, which may be considered to be a marker enzyme of the epidermal cell membrane, was completely inactivated or “uncoupled” shortly after treatment with phorbol esters in vivo (22, 37). Furthermore, it has been found that in salivary glands the mitogenic effect of isoproterenol (which obviously acts on the membrane) is counteracted by a chalone-like mitotic suppressor (4). A similar factor which is an antagonist of the mitogen phytohemagglutinin has been isolated from lymphocytes (30). Evidence has been provided that this “lymphocyte chalone” is bound at the outside of the plasma membrane, perhaps interacting with lectin receptors (J. C. Houck. The Lymphocyte Chalone. Lecture Presented at the International Symposium on Chalone Control Mechanisms, Lane End,

5 After wounding (13, 38) or treatment with TPA (47, 48, 57) epidermal cells with a new phenotype have been reported to appear which cannot be found in normal skin. This may be taken as additional support for the concept of a TPA-induced dedifferentiation to noncommitted cells followed by a redifferentiation to normal and perhaps abnormal epidermal cell types.
England, October 12 to 13, 1973). Moreover, the proliferation of thymic lymphoblasts has been found to be stimulated by the phorbol ester TPA (61). Finally, the inhibitory effect of a chalone-like factor found in diploid human fibroblasts (30, 31) seems to be suspended by a fibroblast growth factor isolated from serum (J. C. Houck. A Fibroblast Growth Factor. Lecture Presented at the International Symposium on Chalone Control Mechanisms, Lane End, England, October 12 to 13, 1973), and it is generally accepted that the stimulation of fibroblasts by serum factors is due to an effect on the cell membrane.

The systems salivary gland plus isoproterenol, lymphocytes plus phytohemagglutinin, and fibroblasts plus serum factors, among others, are classical examples of the so-called “pleiotypic response” (27). This term is meant to represent the sequence of molecular events that occurs when a more or less “quiescent” cell population is triggered to undergo cell division. This process has been shown to be very similar in all stimulated systems and appears to be independent of the means of triggering the cells. Experiments with tissue cultures lend support to the idea that the trigger mechanism of the pleiotypic response is localized in the outer cell membrane (27). The biochemical reactions seen after treatment of epidermis with phorbol esters, such as synthesis of DNA, RNA, and protein; stimulation of phospholipid metabolism; alteration of membrane permeability coupled with an increased influx of water and ions; phosphorylation of nuclear proteins; and alterations of the level of cyclic nucleotides, are strikingly similar in their degree and sequence to those biochemical reactions observed in “pleiotypic systems.” The question of whether the phorbol ester-stimulated epidermis can be considered to be a new pleiotypic system and whether such systems generally can be triggered by interference with the normal mechanism of G1 inhibition in a manner similar to that described for the epidermis will be the subject of further investigations in this laboratory. Typical pleiotypic stimulators such as estrogens, androgens, or prolactin exhibit a pronounced tumor-promoting activity in their target tissues.

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Effect of Tumor-promoting Phorbol Esters and of Acetic Acid on Mechanisms Controlling DNA Synthesis and Mitosis (Chalones) and on the Biosynthesis of Histidine-rich Protein in Mouse Epidermis

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