Inhibition of Terminal Differentiation of Hamster Epidermal Cells in Culture by the Phorbol Ester 12-O-Tetradecanoylphorbol-13-Acetate

Enid E. Sisskin and J. Carl Barrett

Environmental Carcinogenesis Group, Laboratory of Pulmonary Function and Toxicology, National Institute of Environmental Health Sciences, Research Triangle Park, North Carolina 27709

ABSTRACT

The effect of 12-O-tetradecanoylphorbol-13-acetate (TPA) on the terminal differentiation of hamster epidermal cells in culture was studied. Epidermal cells were isolated from 1-day-old Syrian hamsters by separating the epidermis from the dermis by cold trypsin treatment. A large number of cells were isolated by this procedure without contamination with dermal fibroblasts. When grown in culture, the epidermal cells divided rapidly, stratified, and differentiated as measured by elaboration of abundant keratin-like amorphous material, red staining with rhodanile blue (which is characteristic of cornifying epithelium), and formation of cornified envelopes. These structures were measured by electron microscopy and quantitation of detergent-insoluble cell ghosts. TPA markedly inhibited this differentiation of the hamster epidermal cells in culture. When grown in the presence of TPA (5 to 1000 ng/ml) for three or more days, the epidermal cell monolayers failed to stain positively with rhodanile blue, and the cell stratification and production of keratin-like material was reduced. The differentiation of the epidermal cells was quantitated by measuring the percentage of cells with cornified envelopes; TPA reduced by up to 70% the number of these terminally differentiated cells. Phorbol didecanoate also inhibited the differentiation of hamster epidermal cells in culture, while phorbol was inactive. The effect of TPA was reversible. When TPA was removed from the media, the cells rapidly differentiated to the same extent as did untreated cells. TPA also stimulated DNA synthesis of the epidermal cells, especially after 10 days in culture when the vast number of cells in control cultures had ceased DNA synthesis. These results are discussed in view of the fact that TPA has not been demonstrated to promote epidermal carcinogenesis in Syrian hamsters.

INTRODUCTION

Tumor promoters, which act to complete the process of carcinogenesis initiated by a subthreshold dose of a carcinogen, may be important determinants in human neoplasms (27, 48). The most widely studied tumor promoter is TPA, a diterpene phorbol ester, which is a potent promoter of epidermal carcinogenesis in mice (4, 5). Studies with TPA and related phorbol derivatives have revealed pleiotropic effects of these compounds on mouse epidermis (1, 5, 7) and on numerous cell types in culture (7, 10-13, 46, 47, 51, 53, 54). It is difficult, however, to discern TPA-induced changes which are critical to tumor promotion from possible secondary effects of the chemical. A genetic approach to this problem might resolve this question. Certain strains of mice do not appear to be sensitive to carcinogen-promoter-induced epidermal carcinogenesis and might be useful for such studies (3, 4, 14, 21, 25). Furthermore, a species specificity for tumor promotion exists. Croton oil, of which TPA is the most active component, does not promote epidermal carcinogenesis in hamsters, guinea pigs, or rats under conditions for which it is a very active promoter in mice (9, 15, 34-36). It is unclear whether the lack of responsiveness of certain strains of animals to 2-stage carcinogenesis is due to insensitivity to the action of the carcinogen during the initiation phase, or to the action of the promoter during the promotion phase of carcinogenesis. The former explanation is very likely for some strains of mice (14), however, it is unlikely for hamsters which actively metabolize carci nogens, particularly polycyclic aromatic hydrocarbons, and are highly sensitive to carcinogen-induced tumorigenesis (9, 15, 17, 34-36). Using strain and species differences as experimental tools is an attractive approach, because they can be used to learn which critical effects of TPA may be lacking in the nonresponsive animal and also because they may yield information on the universality of tumor promotion.

For these reasons, we have begun a systematic study of the effects of TPA and other phorbol derivatives on hamster epidermis in vivo and hamster epidermal cells in vitro. Shubik et al. (9, 34, 36) have reported that croton oil does not promote epidermal carcinogenesis in hamsters. Goerttler et al. (15) have recently confirmed this observation with TPA, although promotion of melanoma formation was observed. We have preliminary data which also indicate that TPA does not promote hamster epidermal carcinogenesis, at least not during a 6-month treatment period (2).

An effect of tumor promoters which theoretically provides a basis for one aspect of tumor promotion is inhibition of cellular differentiation (7, 11, 29, 45). From ultrastructural studies of mouse epidermis exposed to TPA, Raick et al. (29, 30) have presented morphological evidence for an alteration of epidermal differentiation. Colburn (7, 8) has demonstrated that TPA and other hyperplas ionic agents reduce histidase activity of mouse skin. In the last few years, numerous studies have demonstrated that TPA can inhibit differentiation of many cell types in culture (6, 10, 19, 24, 26, 33, 49), including mouse epidermal cells (13, 51). However, stimulation of differentiation of human promyelocytic leukemia cells by TPA has also been reported (18).

In the present report, we present evidence for the inhibition of terminal differentiation of hamster epidermal cells in culture by TPA. The significance of these results to other effects of...
tumor promoters on hamster skin, including the inability of TPA to promote epidermal carcinogenesis in hamsters, is presented in the discussion.

**MATERIALS AND METHODS**

**Isolation of Epidermal Cells.** Epidermal cells were isolated by the method of Youspa and Harris (52). One-day-old hamsters, strains 15.16 or 87.20 (Telaco, Cambridge Mass.), were washed with Betadine surgical scrub and 70% ethanol and then anesthetized on cracked ice. The tail and limbs were removed, and the skin was slit up the back and peeled from each animal. Four skins were stretched, dermis side up, on a 150-mm Petri dish and covered with a piece of sterile filter paper. This was inverted and floated on 50 ml of 0.25% trypsin in PBS in a Petri dish at 4° for approximately 20 hr. The next day, the skins were removed from the filter and restretched, and the epidermis was physically separated from the dermis. The epidermis was minced with scissors and stirred at room temperature for 30 min in complete medium. After that time, the solution was filtered through Dacron gauze (157 mesh; Martin Supply Co., Inc., Baltimore, Md.) to obtain a single-cell suspension. The cells were counted using a Model ZBI Coulter Counter (Coulter Electronics, Hialeah, Fla.) and plated at 1.5 × 10⁵ cells/sq cm on Falcon tissue culture dishes. The cells were tested at various intervals from 1 day to 4 months in culture and were found to be free of *Mycoplasma*.

**Media.** Unless otherwise mentioned, the cells were grown in Eagle’s minimal essential medium without nonessential amino acids (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal calf serum (Reheis Chemical Co., Inc., Phoenix, Ariz.), hydrocortisone (0.1 µg/ml), and insulin (1 µg/ml; Sigma Chemical Co., St. Louis, Mo.). TPA, PDD, and phorbol (Chemical Carcinogenesis, Eden Prairie, Minn.) were dissolved in dimethyl sulfoxide (1 µg/ml) and diluted into medium. The concentration of dimethyl sulfoxide in the medium was less than 0.01%, a concentration shown not to affect cell growth. The concentration of dimethyl sulfoxide in the medium was less than 0.01%, a concentration shown not to affect cell growth. Media was changed in all cultures every 3 to 4 days.

**Light and Electron Microscopy.** Cells were fixed in situ with 3.5% glutaraldehyde buffered with 0.1 M sodium cacodylate (pH 7.4) for 2 to 3 hr at 4°. The cells were rinsed with 3-4 changes of 0.1 M sodium cacodylate at least overnight. The cells were then scraped and pelleted by centrifugation. Following postfixation with 1% OsO₄, the cells were dehydrated in a graded series of ethanol and 2 changes of propylene oxide and then embedded in Epon.

**Rhodanile Blue Staining.** Epidermal cells were stained for cornification by the method of Rheinwald and Green (31, 32). After the cells were fixed with 10% buffered formalin, a 1% solution of Rhodanile blue (Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added to the dish containing cells. After 30 min, the cells were rinsed under running water until the wash water no longer appeared red. Then the dishes were allowed to air dry and were examined under light microscopy.

**Cornified Envelope Formation.** The percentage of terminally differentiated epidermal cells with a cornified envelope were determined by the method of Sun and Green (41). Cells were removed from the dish with 0.05% trypsin and 0.1% EDTA and divided into 2 portions. One was counted directly in a hemocytometer, and the total cell number was determined. The other portion of cells was added to 10 mM Tris-HCl buffer (pH 7.4) containing 1% β-mercaptoethanol and 1% SDS. In this buffer, the cells were lysed and only cornified envelopes remained intact. The number of cornified envelopes was then counted using a hemocytometer, and the percentage of cornified envelopes per total number of cells was determined. The same procedure was used to measure the number of cornified cells which were in the media, i.e., not attached to the dish.

** Autoradiography.** Cells were grown on Labtek slides (Miles Laboratories, Naperville, Ill.). Prior to addition of fresh medium, [³H]thymidine (20.0 Ci/mmol; New England Nuclear, Boston, Mass.) was added to each culture to a final concentration of 1.0 µCi/ml. After 1 hr, the culture medium was removed, and the cells were washed twice with cold PBS containing 0.1% unlabelled thymidine. The cells were then fixed with 5% trichloroacetic acid, rinsed with water, and air dried. The slides were exposed and developed by a modification of the method of Stein and Yanishevsky (38). The slides were dipped into NTB-2 nuclear track emulsion (Eastman Kodak Co., Rochester, N. Y.) and allowed to drain for a moment, dried for 1 hr horizontally, and then placed in a slide box with desiccant for 1 week. The exposed autoradiographs were developed at 15–18° in Kodak D-19 developer for 2 min, rinsed in 0.1% acetic acid for 30 sec, soaked in Kodak rapid fixer for 5 min, rinsed in running water, and air dried. Nuclei overlaid with 20 or more grains were considered labeled. Background was less than 1 grain per nucleus.

**Incorporation of [³H]Thymidine.** Cells were grown in 60-mm tissue culture dishes and at different times were labeled with [³H]thymidine (1 µCi/ml) for 24 hr. After incubation, the radioactive medium was removed and the cells were washed twice with cold PBS containing 0.1% unlabeled thymidine. The cells were trypsinized, an aliquot was counted using a Coulter counter, and the rest were transferred to glass tubes. Trichloroacetic acid was added to precipitate the protein and nucleic acids, and the precipitate was collected and filtered through a 0.45-µm Millipore filter (Millipore Corp., Bedford, Mass.). The filters were counted in a Beckman scintillation counter and cpm/cell were determined.

**RESULTS**

**Isolation and Growth of Hamster Epidermal Cells.** Using the method of Youspa and Harris (52), which involves trypsin splitting (23, 44) of dermis from epidermis, a high yield (>10⁷ cells/skin) of epidermal cells from newborn hamsters was obtained. The isolated cells were usually only of epithelial origin, and no fibroblasts were observed. However, occasionally (approximately 1 preparation in every 10), fibroblast-like cells were observed. The growth of fibroblasts in these cultures was suppressed initially by the epithelial cells but, as the epithelial cells differentiated and sloughed off the dish, fibroblast growth became obvious. These cultures were not included in the experimental results presented in this report.

When the epidermal cells were plated on tissue culture dishes in complete media at 1.5 × 10⁵ cells/sq cm, they aggregated, attached, and began to divide, forming small islands (Fig. 1A). The plating efficiency, i.e., cells attached after 24 hr, was 10%. The cells which did not attach were large, squamous-appearing cells which were highly differentiated as shown by ultrastructural electron microscopic studies (Fig. 2A). The cells which attached to the plate began to divide rapidly.
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If the cells were allowed to attach for 24 hr and then labeled with [3H]thymidine for the next 24 hr, then greater than 90% of the cells incorporated the label as determined by autoradiography. This indicated that most of the cells were capable of DNA synthesis. After 3 days in culture, the cells had reached confluency (Fig. 1C), whereas only 10% of the dish had been covered with cells originally (Fig. 1A). DNA synthesis was continued for the next 4 to 5 days, but at a slower rate.

Differentiation [stratification and cell sloughing (Fig. 1, C and D)], was observed from Days 1 to 3 in culture but increased greatly after the cells reached confluency. During this time, the cells elaborated large amounts of keratin-like amorphous material which accumulated on the top layer of the cells, as observed also in mouse and human epidermal cultures (20, 31, 32, 50, 52). By 6 to 10 days in culture, the cells were considerably stratified and highly differentiated. The culture began to decrease in cell number gradually after this time, and by 3 to 4 weeks the cultures were completely degenerated. This progression is very similar to the growth and differentiation of mouse epidermal cells in culture as reported by Yuspa and Harris (50, 52).

The epithelial nature of the cells was confirmed by electron microscopy (Fig. 2). The cells stratified in multilayers (Fig. 2B) and were joined by desmosome-like structures (Fig. 2, C and D) and interdigititation (Fig. 2D). The cells also produced abundant keratin as shown by SDS-polyacrylamide gel electrophoresis and immunological staining and by radioimmunoassay4 (55).

Effect of TPA on Cell Differentiation. When grown in the presence of TPA (0.1 µg/ml), the differentiation of the hamster epidermal cells was inhibited. In the presence of TPA, the cells appeared smaller, failed to stratify after they became confluent, and elaborated much less amorphous material (Fig. 3). Although the cells produce abundant keratin, we have chosen not to use keratin production as a quantitative measure of differentiation. This is because even basal cells and epithelial cell lines in culture produce and elaborate keratin in considerable amounts (39, 40, 42, 43). Instead, we determined the degree of cell differentiation by 2 techniques which measure cornification. The first involved a histological stain, Rhodanile blue, which detects the process of cornification (22, 31). During vertical stratification, hamster epidermal cells elaborated large amounts of an amorphous red-staining material upon Rhodanile blue staining, indicating cornification. A significant reduction of the red-staining material was observed in the TPA-treated cultures (Fig. 3). The effects of TPA on the differentiation of the hamster epidermal cells were observed over a dose range of 0.05 to 1.0 µg/ml. The inhibition of differentiation increased from 0.05 to 3.0 µg/ml and then was constant at higher doses; no cytotoxicity was observed at up to 1.0 µg/ml.

A second, more quantitative, measurement of cornification was also performed. Terminally differentiating keratinocytes form a structure called the cornified envelope beneath the plasma membrane (41), which is composed of a protein with highly stable ɛ-Glu-glutamyllysine cross-links. The cornified envelopes are resistant to detergents and reducing agents due to the isopeptide bonds. Treatment of cells with SDS and β-mercaptoethanol causes cellular lysis, but cornified envelopes remain intact (41). Treatment of a known number of cells with these agents enables the number of cornified envelopes to be counted, and the percentage of cells with cornified envelopes can be determined. In control cultures, the percentage of cells with cornified envelopes varied in an apparent cyclic manner from 4 to 15% (Chart 1). This pattern of production was reproducible; however, the timing of the peaks varied from experiment to experiment for unknown reasons. When the cells were grown in the presence of TPA (0.1 µg/ml), the percentage of cells with cornified envelopes was reduced (Chart 1). The inhibition of cornified envelope production by TPA was most marked (70% inhibition) after 6 to 7 days in culture (Chart 1). Chart 1 represents the results of one experiment; however, a total of 5 experiments of this type were conducted with the same result. TPA inhibited the percentage of cells with cornified envelopes by 20 to 70%, depending on the day of culture. The results of all the experiments could not be combined due to the variation in the timing of the cornified envelope production in the control cultures.

In some experiments, the cells which were sloughed into the media were also collected, counted, and analyzed for cornified envelope formation. At early time points (Days 1 to 3 in culture), analysis of the total cells, attached and sloughed, indicated that TPA increased the loss of cornified cells as well as reduced the absolute percentage of these cells. At later time, TPA inhibited the percentage of total cells with cornified envelopes. The number of sloughed cells in the TPA cultures was too small to determine if increased sloughing of cornified cells occurred.

By 2 to 4 weeks in culture, untreated cells had differentiated extensively and few small, undifferentiated, basal cells were present. By 3 to 4 weeks in culture, the cells had completely sloughed from the dish and only a few large squamous-appearing cells remained. The process also occurred in the TPA-treated cultures, but the rate of terminal differentiation of the culture was decreased and culture life was extended. In the presence of TPA (0.1 µg/ml), the number of small undifferentiated cells at 2 weeks was increased, and a few areas of such

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cells were seen even at 4 weeks in culture. The cells could be maintained in the presence of TPA for up to 5 weeks.

Reversibility of TPA-induced Inhibition of Differentiation. The pronounced inhibition of differentiation by TPA observed at 4 to 8 days in culture was rapidly reversible. Fig. 4A is a control culture at 4 days and shows stratification and cornification (amorphous material). The same culture grown in the presence of TPA for 4 days (Fig. 4B) was much less stratified and differentiated. If the cells were maintained in TPA media for 2 more days, they remained undifferentiated (Fig. 4D). However, if the TPA was removed from the media, the cells rapidly stratified and differentiated, and within 2 days they were indistinguishable from cultures which had not been treated with TPA (Fig. 4C). Thus, TPA appeared only to suppress differentiation rather than irreversibly alter the process. This reversibility was observed even after 2 weeks of TPA treatment.

Effect of Phorbol and PDD on Cellular Differentiation. Two other compounds were tested in this system. Phorbol (0.1 μg/ml), of which TPA is a diester derivative, is not a tumor promoter and had no effect on the differentiation or growth of hamster epidermal cells in culture (Fig. 5A). PDD, which, like TPA, is another diester of phorbol and an active tumor promoter, significantly inhibited the differentiation of hamster epidermal cells (Fig. 5B). In fact, the inhibition by PDD (0.1 μg/ml) was equal to TPA (0.1 μg/ml) for the first 7 to 10 days of the cultures and was greater than TPA after longer time periods as measured by the persistence of small, undifferentiated cells in the culture. This is due to the shorter half-life of TPA compared to PDD in the presence of these cells.5

Effect of TPA on Division of Hamster Epidermal Cells in Culture. In addition to its effects on terminal differentiation, TPA (0.1 μg/ml) had a pronounced effect on DNA synthesis. TPA effects on DNA synthesis were measured by counting the number of cells labeled during a 1-hr pulse of [3H]thymidine, followed by autoradiography and by a 24 hr incorporation of [3H]thymidine into acid-insoluble material. TPA inhibited DNA synthesis slightly during the first 2 days in culture (Chart 2). This result is consistent with TPA inhibition of DNA synthesis early after treatment in other cell systems (7, 11, 13, 51, 53). After 2 to 3 days in culture, TPA-treated cells incorporated [3H]thymidine at a high rate while incorporation by control cells decreased dramatically (Charts 2 and 3). By 10 days, the difference was most pronounced (Chart 3). At this time, very few cells were synthesizing DNA in the untreated cultures (<0.02%), while more than 2% of the cells still were synthesizing DNA in the presence of TPA.

DISCUSSION

In this report, we describe a cell culture system for the study of hamster epidermal cells and the effects of tumor promoters on these cells. The value of an in vitro model system depends on how closely it mimics the in vivo model on which it is based (50). Like intact epidermis, the cultured epidermal cells divide and undergo terminal differentiation. This latter process can be followed morphologically by staining with Rhodanine blue and quantitatively by determining the percentage of cells in the culture forming a cornified envelope. On the basis of these criteria, the hamster epidermal system should prove to be a useful tool to study epidermal growth, differentiation, and subsequently neoplastic transformation.

The growth and differentiation of hamster epidermal cells are influenced by the tumor promoter TPA. TPA-treated cells maintain a high rate of DNA synthesis as measured by autoradiography, and their differentiation, as measured morphologically, by Rhodanine blue staining, and by formation of cornified envelopes, is inhibited. These 2 opposing effects of TPA on epidermal cells may be related, since Hennings et al. (16) have shown that terminal differentiation is associated with rapid loss of thymidine incorporation in mouse epidermal cells. The inhibition of differentiation is also observed when the cells are grown in the presence of PDD, another potent tumor promoter, but not when they are grown in the presence of phorbol, which

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We feel that the most probable explanation for the different effects in vivo and in vitro are a combination of hypotheses d and e. The hypothesis that inhibition of differentiation is important in tumor promotion is attractive on theoretical grounds as discussed above. Through the study of the effects of TPA on hamster skin in vivo and hamster epidermal cells in vitro, we hope to elucidate the correct mechanism.

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is inactive as a tumor promoter. The inhibition of differentiation is also reversible when treatment of the cells has ceased. The effects of phorbol esters on differentiation correlate with the known effects of these compounds in promoting carcinogenesis, suggesting that inhibition of differentiation may be important in tumor promotion.

There are 2 mechanisms by which cellular differentiation might play a role in neoplastic transformation. First, aberrant differentiation has been suggested as a causative mechanism for cellular neoplasia (28). Carcinogenesis involves a loss of growth control and, since normal growth control is a balance between renewing cells and differentiating cells, a shift in this balance will change the growth pattern of the population. Thus, carcinogenesis may involve a failure to maintain normal differentiation patterns. Of course, aberrant differentiation may be a secondary result of the carcinogenic change. Second, differentiation is an antagonist to neoplastic progression. A cell which undergoes terminal differentiation, resulting in a loss of the ability to divide, is usually eliminated from the population and thus cannot progress to malignancy. In addition, a nondifferentiating, rapidly dividing cell population may be more susceptible to carcinogenic insults. All of these mechanisms may be important in tumor promotion.

The significance of the inhibition of differentiation of hamster epidermal cells by TPA must be considered with the observation that TPA does not promote epidermal carcinogenesis of hamster skin under conditions where it is very effective on mouse skin. There are several possible explanations for these seemingly disparate observations. (a) Inhibition of differentiation plays no role in tumor promotion. We do not favor this hypothesis because evidence from many studies indicates that tumor promoters modulate differentiation (7, 11, 15) and inhibition of terminal differentiation of a preneoplastic cell, which would permit progression of that cell to malignancy and provide a theoretical basis for tumor promotion. (b) TPA is metabolized to inactive products in vitro but not in vivo. Preliminary results on the metabolism of TPA on hamster skin indicate that this is not the case. (c) Metabolism occurs more rapidly in vitro than in vivo. (c) Inhibition of differentiation of hamster epidermal cells occurs in vitro but not in vivo. Our studies (2, 37) of hamster skin exposed to TPA indicate a pronounced TPA effect on hamster epidermis including hyperplasia and morphological changes analogous to those observed by Raick et al. (29, 30) in mouse skin exposed to TPA (2). These changes are consistent with a TPA-induced alteration of differentiation occurring in both mouse and hamster epidermis in vivo. (d) Tumor promotion requires an inhibition of cellular differentiation, but other effects are also required and these effects are not induced in hamster epidermis. (e) The transitory effects of TPA on hamster epidermal cells in vivo do not persist for a sufficient time to allow tumor promotion. This hypothesis suggests that TPA induces all the changes necessary for tumor promotion in vivo but that the effects are reversed more rapidly in hamsters than in mice. This is a possible explanation and is currently under investigation (2). Our recent results indicate that a single exposure of hamster skin to TPA induces a hyperplastic response in the epidermis but that multiple exposures to TPA are not hyperplasigenic to hamster epidermis (37). This adaptation to the effects of TPA by hamster epidermis may be very important to understanding the lack of tumor promotion by this tissue (2).
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Fig. 1. Appearance of epidermal cells at various times after plating. A. cells 24 hr after plating; B. cells 48 hr after plating; C. cells 3 days after plating; D. cells 5 days after plating. Phase contrast microscopy. × 25.
Fig. 2. Electron micrographs of epidermal cells. A, group of cells which have sloughed from the culture. × 11,000. B, cross-section of cells (5 days in culture) showing stratification. × 6,450. C, cells scraped from the dish after 5 days in culture. × 12,540. D, 2 cells attached to each other by desmosome-like complexes and interdigitation. × 6,840.

Fig. 3. Six-day-old cultures grown under various conditions, fixed, and stained with Rhodanile blue. A, untreated cells. × 25. B, TPA (0.1 μg/ml)-treated cells. × 25. C, details as for A. × 50. D, details as for B. × 50.
Fig. 4. Reversibility of TPA inhibition of terminal differentiation of epidermal cells. A, a 4-day-old untreated culture; B, a TPA (0.1 \( \mu \)g/ml)-treated culture, also at 4 days; C, a culture grown for 4 days in the presence of TPA (0.1 \( \mu \)g/ml) and then for 2 days in control medium; D, a culture grown continuously for 6 days in the presence of TPA (0.1 \( \mu \)g/ml). \( \times 25 \).
Fig. 5. Effect of phorbol and PDD on the morphological differentiation of epidermal cells in culture. A, a culture grown in the presence of phorbol (0.1 μg/ml) for 5 days; B, a culture grown in the presence of PDD (0.1 μg/ml) for 5 days. Phase contrast microscopy. × 25.
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