The Histogenesis of Benzpyrene-Induced Epidermal Tumors in the Mouse*

A. Glücksmann

(From the Strangeways Research Laboratory, Cambridge, England)

(Received for publication January 2, 1945)

The macroscopic and microscopic changes in mouse skin following the external application of tar or of carcinogenic hydrocarbons have been described and reviewed in some detail (8, 13, 66, 70). But though all accounts agree on the main facts, such as the cyclical changes in the hair coat, the induction of epidermal hyperplasia followed by the appearance of warts and later of malignant tumors, disagreement persists on the following points:

1. The specificity of the initial epidermal hyperplasia:

Wolbach (71, 72), Orr (52), and Cramer and Stowell (18) consider the initial epidermal hyperplasia as nonspecific and as similar to hyperplasia induced by noncarcinogenic irritants; Pullinger (57, 58) stresses the specificity of this reaction; Twort and Twort (68) hint at a possible distinction between the hyperplasia caused by oleic acid and that produced by noncarcinogenic skin irritants; and Paletta, Cowdry, and Lischer (56) report some differences between simple regenerative and precancerous hyperplasia.

2. The primary effects of the carcinogens:

Wolbach (71, 72), Orr (52), and Cramer and Stowell (18) consider the epidermal hyperplasia as regenerative and as subsequent to primary deleterious effects of the carcinogens; Pullinger (57, 58) finds little evidence of initial degenerative changes with fairly large doses of carcinogenic hydrocarbons applied in nonirritant solvents; Kuklianskis (36) reports the absence of cellular degeneration in mouse organs up to 38 hours after the injection of various carcinogens; and Cooper and Reller (15, 64) report a significant rise in mitotic activity within 48 hours of application of carcinogens without finding any prior tissue damage. On the other hand, undoubted growth-inhibiting effects of a specific nature were obtained by Haddow and others (31, 39) after the injection of carcinogens. Tests made on unicellular organisms (51, 67), tissue cultures (23, 24, 44), frog embryos (10), and other biological material (28, 29, 32, 53, 63), seem to indicate that primarily stimulating as well as deleterious effects occur depending on dose, concentration, and solvent used, and on the test object chosen.

3. The permanency of the initial changes and their dependence on a specific sensitivity of the epidermis:

While some mice react to a single application of carcinogenic hydrocarbons with the formation of warts and of malignant tumors (19, 37, 46), others require repeated paintings preferably at optimal intervals (17, 20) to produce the same result. In these animals warts and epidermal hyperplasia may regress in the absence of further applications. Nonspecific stimuli, varying from mechanical trauma (21, 22, 43, 45, 59, 65) to certain chemical irritants (3, 4), thermal agents (2, 42), or ionizing radiations (50) may maintain epidermal hyperplasia and warts initiated by the carcinogen, and even induce malignancy. Epidermal sensitivity to tar or carcinogenic hydrocarbons varies with species, strain, individual, and the region in the individual (7, 13, 27-29, 66, 68, 70). Since no parallel degree of variation in sensitivity is reported for subcutaneous tissue, the biological basis for the sensitivity variation should be correlated with the epidermal structure. Yamagiwa and his school (70) describe a difference in skin reaction to tar in rabbits and mice, and attribute the response in mice to the epidermis itself and in rabbits mainly to the hair follicles. Jonkhoff (35), on the other hand, demonstrates a definite correlation between periods of hair growth and periods of growth of papillomas in mice.

In the hope of obtaining some definite information on these points and on their significance in experimental carcinogenesis, a quantitative histological investigation of the effects of carcinogenic hydrocarbons and of relatively unspecified skin irritants in animals of a sensitive (mice) and of a resistant (rats) species was undertaken.

*Because of the difficulties of international communication the author has not read proof of this article.
MATERIAL AND METHODS

The mice used for these experiments were derived from an albino stock originally obtained from the National Institute, in Hampstead, and carried on in our laboratory by brother-sister matings. During a period of 5 years no spontaneous skin tumors have been observed in this strain. For the painting experiments litter mates of about 2 months, separated as to sex, were used. The rats were of mixed stock, and about 2 months old at the beginning of the experiment.

EXPERIMENTAL PROCEDURE

The solutions were applied to the interscapular region of the animals by means of a doubly bent pipette. In the mice the hairs were not clipped or shaved before or during the experiments, while in the rats the solutions were applied to the shaved skin. About 5 to 6 drops of the solutions were applied at every painting. The following experiments were made:

(a) On mice.---1. A single painting of a 1 per cent solution of benzpyrene (La Roche) in acetone was applied to 36 mice. The painted skin area was excised at intervals in 2 mice at a time and fixed for histological examination.

2. A 1 per cent solution of benzpyrene in acetone (as above) was applied at weekly intervals to a great number of mice and the treated area excised. The findings in 86 mice thus treated will be considered in this paper.

3. A group of 10 mice was painted once weekly with a 50 per cent solution of turpentine in acetone for 5 months, and individual animals of this group were killed at intervals of 7 days to 9 months.

4. A group of 10 mice was treated with a solution of pure acetone once weekly for 5 months, and all the animals were killed at the end of that period.

5. A group of 10 mice was depilated with a barium sulphide cream (Veet), and individual animals were killed at daily intervals.

(b) On rats.---1. A group of 12 rats was shaved and treated once weekly with a 1 per cent solution of benzpyrene in acetone. Individual animals were killed during the next 3 weeks and the treated area was excised.

2. A group of 7 rats were shaved and individual animals were killed as controls for Experiment 1.

3. A group of 12 rats was painted once weekly with a 1 per cent solution of benzpyrene in acetone for 15 months and then killed.

In most of, though not all, the animals an additional piece of skin from the flank was fixed as control. It was found necessary to study in some detail the postnatal development of the skin in mice and rats, and for this purpose skin from various areas of 48 mice and 17 rats was fixed at daily, weekly, and monthly intervals. Fixation was always carried out 1 or 2 hours before noon.

HISTOLOGICAL TECHNIC

The excised skin, stretched on pieces of filter paper, was fixed in Zenker's solution or in Susa-mixture. While the pieces were being carried through the alcohols for paraffin embedding each was halved in a longitudinal direction. One half was subsequently sectioned longitudinally right through the painted area, while the other half was cut at right angles. Serial sections cut at 10μ were stained with hematoxylin-eosin, Heidenhain's Azan, carmalum-orange G-aniline blue, Weigert's elstica stain counter-stained with carmalum, by Feulgen's method counter-stained with a mixture of light green and naphthol green, or by Wilder's method.

RESULTS

I. HISTOLOGICAL ANALYSIS OF NORMAL MOUSE SKIN AND ITS POSTNATAL DEVELOPMENT

Before the experimental results are described it is necessary to consider the histological characteristics of normal mouse skin. In the adult mouse the epidermis of the interscapular region is very thin, and composed of 1 or 2 layers of cells covered by some layers of keratin. This thin layer of viable cells is described as undifferentiated (18, 57, 58) and formed by 1 cell type only (16); the appearance of many cell layers following the application of carcinogens is consequently regarded as a process of differentiation (18, 57). A consideration of the postnatal development of normal mouse skin, however, leads to a different interpretation of the normal histological structure of the adult skin.

At birth and during the first postnatal days the mouse epidermis shows distinct layer formation with a fully developed stratum basale, spinosum, granulosum, and corneum (Figs. 1a and b). At this time the hair follicles are in the process of producing the first hair coat (30). With the growth of the first pelage the number of epidermal layers is reduced by the shedding of the keratinized layers and the contraction into a single layer of the stratum basale, spinosum, and granulosum. This latter process is gradual and not quite uniform. Thus even in the adult epidermis some areas show 2 or more layers, of which the basal is composed of basal cells proper and of spinous cells, while the more superficial layer is formed by stratum granulosum. The basal cells proper are recognizable by their small amount of foamy cytoplasm and by their indistinct cell walls, while the cytoplasm of spinous cells is more condensed, more eosinophilic, and delimited by a distinct cell wall (Figs. 2a and b). These cellular characteristics are
equally obvious in the many-layered regenerating epidermis or in a depilated epidermis.

The presence of keratinized layers in the normal adult mouse skin, as well as the more obvious changes brought about by experimental conditions, indicates that in the mouse, as in most mammals, the process of keratinization proceeds by way of a stratum spinosum and granulosum. But instead of forming separate layers, the spinous cells and basal cells proper are contained in a single layer except in regions with reduced numbers of hairs (pads, ears, some areas in the abdominal skin, etc.) where the epidermis is thicker and its layering more obvious. The formation of a smooth hair coat in the interscapular region, which acts in a protective manner, is correlated with the reduction in number of epidermal cells and of epidermal layering.

The duration of the keratinization process in the adult mouse, i.e., the average life span of an epidermal cell from its mitosis to its casting off as keratinized debris, can be calculated roughly from the following data: (a) the mitotic index for the interscapular region is 0.2 per cent in our material; Champy and Vasiliiu (11) find 0.2-0.3 per cent, while Cooper and Franklin (14) calculate 0.11-0.14 per cent as an average over a day for the ear of the mouse. (b) The average duration of mitosis is given as approximately 1 hour by numerous authors (26, 38, 40, 41). Assuming that mitotic cells replace only keratinizing cells, and that the total number of cells for a given area of the adult epidermis remains constant, the life span of the average epidermal cell, i.e., the duration of keratinization, can be calculated as approximately 21 days. This period is of the same order as that of the hair cycle in the mouse (3 to 4 weeks).

For the quantitative histological analysis of the normal epidermis, as well as of epidermis subjected to experimental conditions, the most active areas are selected. These regions, characterized by the thickness of the basal layers and the number of mitotic cells, include the regenerating edges of healing ulcers. Such active areas are most likely to contribute directly to the further reaction of the skin, and in particular to the formation of warts and tumors. Necrotic and ulcerated areas have reached the limit of their developmental potentialities and can contribute only indirectly, if at all, to any further reaction.

Since the most active skin region is surrounded by a zone of gradually diminishing reaction that merges at the periphery into the normal untreated skin, the reaction of any treated and surviving skin area must range quantitatively between that of the most active area and that of the normal untreated skin.

Cell counts of selected areas were made in straight fields of epidermis measuring 0.45 mm. in length in longitudinal sections cut at 10 μ. Hair follicles and their mouths were excluded from the counts. With the exception of keratinized debris and of stratum granulosum cells with faded nuclei, all cells of the selected area were counted in 1 of the 4 following groups:
(a) Mitotic cells (M) of all stages from prophase to telophase.

(b) Degenerating cells (Dg), i.e., cells in the process of nuclear pyknosis, karyorrhexis, and karyolysis.

(c) Resting cells (RC), i.e., basal cells proper that are capable of division and have not yet embarked on keratinization. They are recognized by their large and deeply staining nuclei; by their sparse, foamy, and basophilic cytoplasm; and by their ill-defined cell boundaries.

(d) Differentiating cells (Df), i.e., cells of the stratum spinosum and granulosum respectively. These have distinct cell walls, greater amounts of more condensed and eosinophilic cytoplasm, lighter staining nuclei, and, in the case of stratum granulosum cells, keratohyaline granules.

The results of the quantitative analysis of the normal postnatal development of the interscapular epidermis are given in Chart I. The average cell count for the unit length of skin is plotted against time. The extent of variation between different animals of the same age is indicated by the vertical lines (absolute maximal and minimal count). Throughout life mitotic and degenerative activity show some fluctuation (other than the diurnal rhythm), caused presumably by minor traumas or parasites. The fluctuations in number of resting and differentiating cells disappear virtually by the end of the first month, and a fairly constant level is maintained subsequently. The initial decrease in number of resting and differentiating cells is correlated with the development of the first hair coat (see above) and with the reduction of epidermal layering (30). During the first period of hair growth a differential spacing of hair follicles is noticed. At birth the number of follicles per unit length of skin is equal in longitudinal and transverse sections (on the average 18 per unit length of 1.2 mm). At the end of the first month the number of follicles for the same unit is reduced to 5 for longitudinal and to 10 for transverse sections. The hairs are thus not as densely spaced in the direction in which they overlap. This differential spacing of the hair follicles coincides with the distribution of the epidermal cells into fewer layers.

II. Control Observations on Mouse Skin Treated with (a) Acetone; (b) a Chemical Depilatory; (c) a Nonspecific Irritant

(a) Acetone painting.—Applied once weekly for a period of 112 days acetone failed to produce any notable macroscopic or microscopic changes in the painted area. The cell counts do not show any sig-
well within the limits of normal variation. The dermis is slightly infiltrated on the first day.

The early epidermal thickening, unaccompanied by increased epidermal mitotic activity, must be attributed to cellular migration from the hair follicles (18, 55). The hair bulbs show distinct mitotic activity during the first 3 days. But since mitotic activity in the hair bulbs varies with the phase in the hair cycle, and since it provides cells both for hair formation and for migration into the epidermis, it is impossible to correlate quantitatively the number of migrating cells with the increase in mitotic activity of the hair follicles.

(c) Repeated weekly paintings with a 50 per cent solution of turpentine in acetone.—These caused a slowly progressing epilation in the fourth week, followed by extensive ulceration in the sixth and seventh weeks. The ulcerations healed subsequently, and the hair coat was restored.

In histological specimens taken at weekly intervals the following changes are noticed: During the first 3 to 4 weeks a slight degree of epidermal thickening is associated with a fairly well marked inflammatory reaction in the dermis and with rarefaction of subepidermal collagenous fibers. By the 28th day the epidermis is pitted with numerous small ulcers, the dermis is highly infiltrated by round cells, the subepidermal collagenous fibers are rarefied, and the absence of elastic fibers indicates the formation of minute scars. Some of the hair follicles are epilated, though retaining their usual shape, and the number of mitotic cells indicates their regenerative activity (Fig. 3a). The epidermis at the edge of the ulcers is thickened (Fig. 3b). After 7 weeks most of the ulcers are healed and a new hair coat is formed. Epidermal thickening from adherent scurf persists for some time. The round cell infiltration of the dermis is replaced by fibroblasts, which form a new layer of subepidermal collagenous fibers that is complete by the tenth week.

The quantitative analysis of the epidermal reaction is given in Chart III. During the first 3 weeks a rise in the resting (RC) and degenerate (Dg) cell count is not correlated with an increased epidermal mitotic (M) activity or with an increase in the number of differentiating (Df) cells. Cellular migration from the hair follicles must be responsible for the replacement of degenerate cells and the epidermal thickening of this initial period. In the subsequent period of raised mitotic activity in the epidermis more resting cells are converted into differentiating cells, though the absolute count of resting cells remains constant. During this phase, which coincides with the onset of epithelial changes, the supply of epidermal cells is provided not only by the hair follicles but also by the epidermis itself. The differentiating cells disappear from the count after 7 weeks by becoming keratinized. During the period of ulceration even the most actively regenerating skin areas show many degenerating cells, which disappear with the healing of the ulcers. After 10 weeks the cell counts return to normal proportions.
Figs. 3A-6
The epidermal thickening is thus caused in the initial period by cellular migration and subsequently also by increased epidermal mitotic activity.

III. Observations on Benzpyrene-Treated Animals

(a) The reaction of mouse skin to a single application.—After the rapid evaporation of the acetone the benzpyrene powder remains visible as a yellow stain on the hairs for about 24 hours. In some mice epilatory changes may begin on the third day and a new hair coat be formed by the end of the third week, while in others epilation may start in the second or third week and a new hair coat be formed in the fifth to seventh week. No ulceration, wart, or tumor formation has been observed in this series.

In histological specimens taken 0.5, 2, 4, 6, 8, and 10 hours after application no obvious changes are noticed. Some of the animals examined after 15 and 18 hours show vascular dilatation and a slight degree of round cell infiltration in the deeper dermal layers, and minute preulcerative areas in the epidermis. In these loci the basal cell layer has disappeared and the dermis is covered by keratin only, but there is not yet any round cell infiltration and the adjacent epidermis is thickened. These minute preulcerative lesions seem to heal very quickly, since there is no trace of them in specimens taken after 24 hours or later.

The epidermis thickens considerably on the second day, and this thickening persists in varying degree for 8 to 9 weeks. It is caused at first by an increase in the number of cells in the stratum basale and spinosum, followed on the fourth day by a thickening of the stratum granulosum, which is converted into stratum corneum on the seventh day. Particularly during the first week some of the cells in the stratum spinosum are greatly enlarged.

The epidermal changes are closely related to changes in the hair follicles and sebaceous glands: thickening and keratinization occur in the mouths of the follicles on the second day, extend down to the orifices of the sebaceous glands on the third, and involve the hair bulbs on the fourth day. The thickening of the epithelial hair sheaths results from multiplication of the cells by mitosis, from an increase in cell size, and also from a shortening of the follicles. The increased keratinization of the epithelial sheaths, combined with the shortening of the follicles, forces out the hair (Figs. 4a and b). This process may be complete as early as the third or fourth day, but there is great regional variation as regards epilation, so that some hair follicles may be closed by keratin plugs while some are not yet affected and others have started to regenerate. Usually the shortened and epilated hair follicles remain closed by keratin plugs up to the ninth or 11th day, when they become elongated and show active signs of regeneration leading to the production of new hairs by about the 14th day (Fig. 5). Sebaceous gland cells undergo a squamous metaplasia from the third day onwards when they resemble spinous cells, which subsequently keratinize. This change is probably consequent on the epilatory change rather than a direct effect of the carcinogen. With the regeneration of hairs new sebaceous glands are formed by basal cells at the orifices of the glands, usually by the 14th day.

In the same area the conversion of stratum spinosum into stratum granulosum and of the latter into stratum corneum as a rule occurs simultaneously in the epidermis, hair follicles, and the metaplastic sebaceous glands. As with the epidermal changes, occasional areas of epilatory or regenerative activity in the hair follicles may be visible for about 8 or 9 weeks. Dermal changes, consisting of vascular dilatation, round cell infiltration, and slight rarefaction of subepidermal collagenous fibers are seen for roughly the same period.

The results of a quantitative analysis of the epidermal reaction are given in Charts IV and V. During
the first 24 hours (Chart IV) the number of differentiating (Df) and resting (RC) cells is not significantly altered. An almost immediate rise in the number of degenerate (Dg) cells is followed within 6 hours by a progressive increase in mitotic activity (M). Calculations based on a duration of mitosis of 1 hour and of degeneration of 7 hours (38) show that mitotic activity in the epidermis accounts satisfactorily for the increased number of resting cells on the second day, and for the subsequent increase in the number of differentiating cells (from the third day onwards). A peak in the resting cell count is reached on the fourth day and is followed by a peak value for the differentiating cell count on the seventh day (Chart V). At this time the stratum granulosum is converted into stratum corneum, which for some time may remain adherent to the skin as scurf but is excluded from the cell counts. Resting and differentiating cell counts remain at a fairly high level for another 5 weeks, when they return slowly to normal proportions. The mitotic activity reaches its normal value after about 3 weeks, while the count of degenerate cells regains the upper limits of normal variation at about the seventh day, but remains at the relatively high level throughout the further period of observation. The return to normal proportions of the cell counts is subject to individual variation, and while in some mice it occurs as early as 6 weeks after painting, others may show altered counts after 9 weeks.

To summarize, the epidermal thickening that ensues upon benzpyrene painting results from an early rise in mitotic activity, followed subsequently by a distinct rise first in the resting and later in the differentiating cell count. There is no evidence that cellular migration from the hair follicles contributes to the increase in epidermal thickness. There is also an almost immediate, though not very definite, increase in the number of degenerate cells. Mitotic activity is not inhibited at any stage following painting.

(b) The reaction of mouse skin to repeated applications at weekly intervals.—The macroscopic changes consist of a cycle of epilation and regeneration of the hair coat followed by, or later coinciding with, the formation of warts and of tumors. Jonkhoff's (35) data on tarred mice suggest a hair cycle in the treated animals of about 22 days, and his findings are confirmed in our own material. In a series of 10 litter mates painted at weekly intervals from the fourth postnatal day onwards, the treated area in all the animals was completely epilated after 21, 44, and 65 days; that is, at intervals of 21 to 23 days. Warts first appeared after 48 days, and were present in most mice after 60 days. The first tumor, histologically confirmed, was excised on the 70th day. Ulcerations in the treated area were seen in the later periods of the experiments; they healed rather quickly in spite of continued painting except for those in or around fairly large tumors.

Dermal changes, in the form of an inflammatory reaction, start in the deeper dermal layers, spread towards the subepidermal zone, and may involve the epidermis. Renewed paintings tend to exacerbate the inflammatory reaction until it reaches an almost constant level after about 5 weeks. The collagenous fibers, particularly of the subepidermal zone, are dissolved and replaced first by fibroblasts and from the fifth week onwards by new, thin-fibered collagenous tissue. At about this time the elastic fibers of the dermis begin to show some irregularities in arrangement and distribution. In some places there are only few and granular fibers, while in others there are more fibers which, however, tend to clump together. These changes are probably secondary to (a) the effect of the inflammatory reaction on the collagenous fibers, the arrangement of which determines to some extent the arrangement of the elastic fibers, and (b) the cycle of epilatory and regenerative changes in the hair follicles around which the elastic fiber
malignant tumors. Epidermal hyperplasia always pre-
and rather diffuse dermal reaction.

The epidermal reactions to the first painting have been described above. The subsequent changes consist in increased epidermal hyperplasia and hyper-
keratosis, in similar changes in the epidermal appendages, and in the formation of papillomas and malignant tumors. Epidermal hyperplasia always pre-
cedes tumor formation, which occurs only in a hyper-
plastic epidermal region, or in hyperplastic hair fol-
lies. Papillomas may become malignant growths, they may remain—at least for some considerable time—
papillomas without malignant change, or they may regress. Malignant growths may also arise directly
in hyperplastic regions without the previous forma-
tion of a wart.

The epidermal thickening that follows the first application of benzpyrene is maintained at about the same level by the subsequent paintings for the first 6 weeks. The epidermal hyperplasia is generalized over the whole treated area, but is greater in some regions than in others. The increase in cell size most noticeable during the first week tends to diminish during subsequent weeks, though many large and bi-
nucleate cells are always encountered. Among the dividing cells a number of abnormal mitotic figures is seen. Some variation in thickness of the most active epidermal region is caused by the periodical conversion of stratum granulosum into stratum corneum, which occurs on about the 14th, 28th, and 42nd day.

The cyclical changes in the hair coat consist of a hypertrophy of follicles followed by hyperkeratosis and loss of hair, and later by the regeneration of follicles and sebaceous glands and the formation of hairs. At least 2 complete cycles are usually observed during the first 6 weeks. During this initial period the number of hair follicles per unit length of skin and the process of hair formation are not substantially altered.

Warts usually begin to form about the seventh week, in definitely thickened epidermal areas and in hypertrophic hair follicles (Figs. 6, 7, 8). The further increase in thickness of epidermal areas leads to the formation of partly keratinized excrescences as well as to downward projections. Similarly the hyper-
trrophic hair follicles enlarge still further, their number tends to increase, and only abortive hair formation is found. The central parts of the follicles keratinize in the same manner as the epidermis and they are thus transformed into epidermal interpapillary processes, which remain recognizable by the occasional presence of hairs or their rudiments, by the attachment of parts of normal or metaplastic sebaceous glands, and by the persistence of a central structure derived from the original lumen or mouth of the hair follicle and now filled with keratin (Figs. 7, 8, 9). This central structure is not found in downward projections of hyper-
plastic epidermal regions. Very frequently the neigh-
borung areas of hypertrophic epidermis and hyper-
trrophic follicles become confluent and form the center of a wart (Figs. 8, 9, 10). These formations are never sharply defined at their periphery, but taper out into less hypertrophic regions.

The form of the ensuing papilloma is determined by the localization of its most actively growing part. A relatively greater growth rate in this periphery, as compared with the center, produces an inversion of the central parts and the formation of a cyst-like lumen filled with keratin (Figs. 11, 13), while a rela-
tively greater growth rate at the center produces a broad-based wart with a center projecting outwards (Figs. 9, 10). Small keratinized cysts result from single enlarged and keratinizing hair follicles (Figs. 11, 12).

There is no evidence of anaplastic changes in the cell layers of the papillomas, which differ only quanti-
tatively from those of the hyperplastic epidermis. The direction of growth towards the stratum corneum, i.e., in a vertical direction from the basal layer, remains unchanged. Lateral expansion of papillomas is a result of increased growth of adjacent hyperplastic regions and not of lateral migration of "papilloma" cells and their multiplication. Apart from a dissolu-
tion of the basement membrane at the tip of the epidermal projections, and the diffuse inflammatory reaction, the dermal tissue does not seem to be in-
volved in this purely epithelial hypertrophy.

Malignant foci appear as early as 10 weeks after the first painting in some mice. They arise in a hyper-
plastic area of the epidermis (Fig. 12), or more frequently in the growing parts of papillomas (Fig. 13). In either case the malignant change takes place in the basal layer, i.e., among the resting cells, while at first the more differentiated layers remain unaltered. The induction of malignancy appears to be a gradual process, since it is impossible to find a clear demarca-
tion line between malignant cells and their non-
malignant neighbors. Even fairly small foci are not sharply outlined. The malignant change may take place in the walls of keratinizing cysts and here, as in papillomas, the older parts go on keratinizing without reflecting any sign of malignant change. In branching cysts or papillomas malignancy may be induced at first in one branch only.

The morphological characteristics of the malignant change consist of (a) cellular changes such as in-
crease in volume of cytoplasm, nucleus, and nucleolus, (b) qualitatively and quantitatively abnormal differ-
etiation, (c) invasive properties. The character of the malignant formations varies with the develop-
Fig. 7.—Mouse skin painted with benzpyrene at weekly intervals and fixed on 112th day of experiment. Hyperplastic epidermis forms a number of downward projections (interpapillary bodies). Hair follicles also hyperplastic and some show abnormal keratinization. (Other parts of same section shown in Figs. 11 and 12.) Mag. X 46.

Fig. 8.—Mouse skin painted with benzpyrene at weekly intervals and fixed on 70th day of experiment. The center of a wart is formed by a number of adjacent hyperplastic hair follicles and interpapillary bodies. Mag. X 27.

Fig. 9.—Mouse skin painted with benzpyrene at weekly intervals and fixed on 100th day of experiment. A wart formed by neighboring interpapillary bodies and hyperplastic hair follicles. Later recognized by attachment of sebaceous glands (Seb) and hair rudiments (hr) and by keratin configuration at their mouths. Here, as in Figs. 6, 7, and 8, downward projections of epidermis appear as separate units and show same direction of growth and keratinization as normal epidermal structures. Mag. X 46.

Fig. 10.—Mouse skin painted with benzpyrene at weekly intervals and fixed on 84th day of experiment. A wart showing secondary fusion of hyperplastic hair follicles and of interpapillary bodies. Mag. X 35.

Fig. 11.—Same section as shown in Fig. 7. Note downward growth of papillomatous formations next to branching keratinized cyst. Hair follicles at periphery of cyst are hyperplastic and show abortive attempts at hair formation. Mag. X 26.5.

Fig. 12.—Same section as shown in Figs. 7 and 11. On left, a small invasive, anaplastic focus arises in the hyperplastic epidermis. On right, abnormal hair follicles and interpapillary bodies. Figs. 7, 11, and 12 illustrate the difference in degree of generalized reaction of mouse skin to benzpyrene painting. Mag. X 51.5.

Fig. 13.—Same section as in Fig. 10. Anaplastic invasive extensions growing from papillomatous region into dermis and into wall of keratinized cyst. The originally separate downward projections of epidermis fuse secondarily. Mag. X 26.

Fig. 14.—Mouse skin painted with benzpyrene at weekly intervals and fixed on 93rd day of experiment. On left, a keratinizing squamous cell carcinoma that invades panniculus carnosus. On right, a wart, joined by hyperplastic epidermal region to the carcinoma. Note localized inflammatory reaction induced by carcinoma. Mag. X 14.5.
Glücksmann—Histogenesis of Benzpyrene Mouse Tumors

395

mental stage and environment of the focus. Young foci are recognized by notable mitotic activity, by a proportionately large number of resting cells, and by a correspondingly small number of differentiating cells, which may proceed to normal keratinization in regular layers. More frequently the layer formation is disturbed and instead of normal keratinization cells undergo a parakeratotic change, i.e., they form dense, almost hyaline material without having passed through the stage of forming keratohyaline granules, and they retain a pyknotic nucleus. Old foci consist of parakeratotic cells or keratinized debris, and are usually infiltrated by round cells.

The invasive properties of young malignant foci are due to their high mitotic rate, combined with histolytic powers and their ability to evoke a localized inflammatory reaction. The growing malignant focus shares with any growing epithelial formation the ability to dissolve the basement membrane at the growing tip. It remains rather doubtful how much the malignant focus owes its invasive action to the presence of intracellular histolytic agents, and how much to the effect of the inflammatory reaction. But whether the mechanism of tissue dissolution is direct or indirect, the invasive power is undoubtedly a new and even the final sign of the malignant change, and the invasion of dermal muscles is usually taken as the criterion of epidermal carcinoma in the mouse.

Malignant loci may appear simultaneously or at short intervals in the treated area at varying distances from each other; they frequently become confluent and form a single large tumor. Warts may persist unchanged for some time close to malignant foci (Fig. 14). The developmental stage of the foci, the different types and degrees of anaplasia, the nutritional conditions, and the stroma reaction contribute to the great variety in the appearance of tumors even in the same animal. For our quantitative analysis, which cannot demonstrate the anaplastic change or the acquisition of invasive properties, the youngest and most active tumor parts are chosen.

Cell counts made up to and including the stage of the formation of small papillomas refer to the same unit of skin as used in the other experiments. For the carcinomas the cell counts cannot be related to such a unit, and the results for this series are therefore expressed as percentage cell counts. In Chart VI the total cell count per epidermal unit before carcinoma formation is also given. The counts refer to epidermal hyperplasia up to day 49, to papillomatous regions from day 49 to day 70, inclusive, and to carcinomatous foci afterwards.

The initial quantitative changes are described above (first week in Chart V), and they are maintained with fluctuations around the same level for the first 6 weeks. The 3 troughs in the total cell count (Chart VI) are a result mainly of the conversion of stratum granulosum into stratum corneum. The period of papilloma formation is characterized by an absolute and relative increase in the number of resting cells, while the number of differentiating cells is reduced only relatively. Mitotic and degenerate cell percentages are not greatly altered during this period.

A further absolute and relative increase in number of the now anaplastic resting cells marks the period of malignant change. There is a further relative decrease in the number of differentiating cells, while the mitotic and degenerate cell percentages persist on roughly the same high level. In the absence of a progressive change in percentage of mitotic and degenerate cells, papillomas and carcinomas are formed by the absolutely and relatively increased number of resting cells; this fact indicates that the “resting” phase is prolonged; i.e., fewer resting cells become differentiating cells at any given time and the onset of differentiation is delayed (see below). The disturbance of the differentiation processes is shown qualitatively in the abortive hair formation; the scattering of sebaceous gland cells in epidermal regions; the increased number of epidermal interpapillary bodies (twice the number of normal hair follicles in the same unit of skin, Fig. 8); their increase in diameter (amounting to 2 or 3 times that of the normal hair follicle); and in the irregular keratinization of foci (parakeratosis and absence of proper stratification).
The reaction of mouse epidermis to repeated benzpyrene paintings may be summed up as an early and maintained rise in mitotic activity, followed by an absolute and relative increase in the number of resting cells, which, combined with a progressive relative decrease in the number of differentiating cells, leads to the formation of papillomas and later of carcinomas. The malignant change of resting cells occurs in hyperplastic epidermal regions, in hyperplastic hair follicles, or in papillomas. The differentiation process, previously disturbed only quantitatively, now undergoes qualitative changes. The early rise in number of degenerate cells is maintained, but does not interfere with the growth of the active areas.

(c) The reaction of rat skin to single and repeated applications.—The findings to be described under this heading are mainly negative, since the epidermis fails to react to the application of benzpyrene. There is no epilation, epidermal hyperplasia, or tumor formation. The only noticeable effect consists in a slight dermal infiltration immediately after application of the solution, indicating that the absence of an epidermal reaction is not referable to a failure of the solution to penetrate the thicker keratin layers of the rat’s skin. Equally thick layers of keratin in treated or very young mouse skin fail to interfere with the penetration and action of the benzpyrene solution.

**DISCUSSION**

*The specificity of the initial epidermal hyperplasia.—*

For a convenient comparison of the quantitative changes elicited in the same unit of mouse skin by the various treatments, the average cell counts with their standard deviations are given in Table I. The table, in conjunction with the charts, shows that the same degree of epidermal thickening (see Groups 3, 4, and 5b in Table I) can be brought about in 3 ways: (a) by cellular migration from the hair follicles without any rise in epidermal mitotic activity (Group 3), a process that also deals adequately with the repair of minor trauma and burns (1); (b) by cellular migration supplemented later by increased cellular proliferation in the epidermis (Group 4, Table I). This process is also observed in the repair of larger wounds and burns; (c) by a definite and immediate increase in epidermal mitotic activity while the hair follicles enlarge and keratinize (Group 5).

The epidermal hyperplasia following benzpyrene painting is brought about differently from the usual type of regenerative hyperplasia. Benzpyrene hyperplasia is a result of increased mitotic activity and an absolute and relative increase in the number of resting cells. In regenerative hyperplasia the epidermal thickening is correlated in extent and duration with the extent of tissue loss. In benzpyrene hyperplasia the epidermal thickening following a single application in a nonirritant solvent is not related to any noteworthy degenerative effect and lasts for considerable periods. Finally, benzpyrene hyperplasia is specific, in that it is absent in animals of a nonsensitive species (rats), and is not elicited in sensitive animals by painting with noncarcinogenic hydrocarbons (57, 58).

**Table I: Showing Average Cell Counts with Standard Deviations for Treated and Untreated Mouse Epidermis**

<table>
<thead>
<tr>
<th>Group</th>
<th>Differentiating cells</th>
<th>Mitotic cells</th>
<th>Resting cells</th>
<th>Degenerate cells</th>
<th>Total cell count</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Adult controls</td>
<td>92 ± 7.1</td>
<td>1.3 ± 0.22</td>
<td>16 ± 1.3</td>
<td>5 ± 1.3</td>
<td>114 ± 7.9</td>
</tr>
<tr>
<td>2. Acetone-painted mice</td>
<td>82 ± 5.9</td>
<td>1 ± 0.3</td>
<td>15 ± 1.4</td>
<td>4 ± 0.5</td>
<td>102 ± 5.9</td>
</tr>
<tr>
<td>3. Chemically epilated mice</td>
<td>149 ± 14.2</td>
<td>1 ± 0.4</td>
<td>23 ± 3.7</td>
<td>6 ± 1.3</td>
<td>180 ± 18</td>
</tr>
<tr>
<td>4. Turpentine-painted mice</td>
<td>134 ± 15</td>
<td>2 ± 0.35</td>
<td>36 ± 2.6</td>
<td>13 ± 3.8</td>
<td>185 ± 20</td>
</tr>
<tr>
<td>5. Benzpyrene-painted mice (single application)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a) 0.5 to 24 hours after painting</td>
<td>99 ± 4.2</td>
<td>3 ± 0.44</td>
<td>25 ± 1.5</td>
<td>10 ± 0.9</td>
<td>136 ± 5.5</td>
</tr>
<tr>
<td>(b) 2 to 7 days after painting</td>
<td>137 ± 11.5</td>
<td>5 ± 0.62</td>
<td>49 ± 2.7</td>
<td>12 ± 1.3</td>
<td>203 ± 13.5</td>
</tr>
<tr>
<td>(c) 11 to 63 days after painting</td>
<td>133 ± 11.1</td>
<td>2.4 ± 0.93</td>
<td>32 ± 3.6</td>
<td>6.6 ± 0.5</td>
<td>174 ± 14.2</td>
</tr>
</tbody>
</table>

*Primary effects of benzpyrene on mouse skin.—*

The local application of benzpyrene in a nonirritant solution causes no significant cellular degeneration, nor has it the specific growth inhibitory effect that follows injection of carcinogenic hydrocarbons (31, 39). The toxic effect of hydrocarbons on mouse skin varies with the compound, its concentration, dose, and solvent, and also with the region (15, 17-20, 64) to which it is applied. But the absence of definite toxic effects in our own experiments confirms previous reports (15, 57, 58, 64), and shows that if such deleterious effects occur under other experimental conditions they are probably without significance for the subsequent tumor formation.

The stratification of the thickened mouse epidermis is an outcome of an increase in the number of cells but is not necessarily equivalent to increased differentiation, that is, to a relative increase in the number of differentiating cells. The epidermal hyperplasia following benzpyrene painting is due to (a) increased mitotic activity of resting epidermal cells (Table II), and (b) a delay in the onset of differentiation; i.e., a relative prolongation of the resting phase (Charts V and VI, Tables I and II). These effects are followed during the final period of tumor
formation by interference with the differentiation processes.

The regular cyclical conversion of stratum granulosum into stratum corneum in benzyrene hyperplasia, together with the regular period of the hair cycle (about 21 days) indicates that the duration of keratinization is not greatly altered during the early periods up to the appearance of warts. The relative increase in the number of resting cells and the relative decrease in the number of differentiating cells during this period indicate a prolongation of the resting phase by about 2 days (average duration of keratinization = 21 days, average percentage of resting cells = 14 per cent, average duration of resting phase = 3 days: with an average of 24 per cent of resting cells in benzpyrene hyperplasia the duration of the resting phase is increased to about 5 days).

Prolongation of the resting phase, which makes more cells available for division at a given time, does not account entirely for the increased number of mitotic cells in the painted skin, as the percentage of resting cells in division (Table II) is significantly increased during the period of epidermal hyperplasia, and particularly during the first day. Thus the epidermal thickening is due to both prolongation of the resting phase and more rapid proliferation of the cells. During wart and tumor formation the mitotic index in resting cells does not differ significantly from that of the controls. This finding agrees well with direct observations on the duration of the intermitotic period and of mitosis. The duration of the intermitotic period is of the same order normally (except quantitatively) and both the epidermal hyperplasia and the warts are of a reversible nature. Most of these cellular changes are presumably lethal (5, 9), and the significance of the nonlethal cytological abnormalities remains obscure.

The delay in onset and the subsequent change in character of the differentiation processes may be secondary to the growth-stimulating effects of the carcinogens or may be interpreted as a separate direct effect on the factors determining the differentiation of mouse skin or on the response to them of the epidermal cells. The existence of such determining or "organizing" factors in mouse skin, and their variation with region, have been established in skin grafting experiments between mice of genetically pure lines (60-62). The location of epidermal cells in a skin region has been found to determine the differentiation of the cells or their descendants.

The evidence for such a direct, separate effect of carcinogens on differentiation in mouse epidermis is not conclusive, although there is a striking correlation between regional variation in sensitivity and regional variation in epidermal structure. The sensitivity to carcinogens decreases in a caudal and abdominal direction towards the pads of the feet (68, 70), and it is interesting that the thickness of epidermal layering and size of hairs increases, while the density of the hair coat decreases, in the same direction. A similar correlation may hold true for different strains of mice (7, 33, 34) and for different species, where size of hairs increases with decreasing sensi-

<table>
<thead>
<tr>
<th>Group</th>
<th>Differentiating</th>
<th>Degenerate</th>
<th>Resting</th>
<th>Mitotic</th>
<th>Resting cells in mitosis, (per cent)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Adult controls</td>
<td>92 ± 7.1</td>
<td>5 ± 1.3</td>
<td>16 ± 1.3</td>
<td>1.3 ± 0.2</td>
<td>8 (6-10)*</td>
</tr>
<tr>
<td>2. 0-24 Hours after single benzyrene painting</td>
<td>99 ± 4.2</td>
<td>10 ± 0.9</td>
<td>25 ± 1.5</td>
<td>3 ± 0.4</td>
<td>12 (7-15)</td>
</tr>
<tr>
<td>3. 2-7 Days after single benzyrene painting</td>
<td>137 ± 11.5</td>
<td>12 ± 1.3</td>
<td>49 ± 2.7</td>
<td>5 ± 0.6</td>
<td>10 (8-12)</td>
</tr>
<tr>
<td>4. 8-49 Days after repeated benzyrene painting</td>
<td>142 ± 7.6</td>
<td>21 ± 1.4</td>
<td>53 ± 2.3</td>
<td>6 ± 0.6</td>
<td>11 (10-13)</td>
</tr>
<tr>
<td>5. Papillomatous regions, 63-70 days after repeated paintings</td>
<td>148 ± 15.1</td>
<td>28 ± 4.2</td>
<td>143 ± 6.2</td>
<td>9 ± 2.0</td>
<td>6 (5-8)</td>
</tr>
<tr>
<td>6. Carcinomatous regions, 74-119 days after repeated paintings</td>
<td>83 ± 8.7</td>
<td>17 ± 1.8</td>
<td>132 ± 4.5</td>
<td>10 ± 0.9</td>
<td>8 (7-9)</td>
</tr>
</tbody>
</table>

* Ranges of variation.
tivity to carcinogens and decreasing density of the hair coat in the order: mice, rabbits, rats, guinea pigs. This correlation of epidermal structure with sensitivity to carcinogens may be explained on the basis of a specific correlation of carcinogens with the differentiation process. A more likely explanation, however, lies in the fact that with reduced epidermal layering—which is always correlated with increased density of pelage—the hair follicles act as germinative centers for the epidermis, and that both react directly to the growth-stimulating action of the carcinogens. This action may be facilitated by the reduction of keratin layers in the sensitive regions of mouse skin. In rat epidermis no evidence of a growth-stimulating action by benzpyrene has been detected. On subcutaneous injections of carcinogens, however, sarcomas are elicited in the rat as well as in the mouse (27). Thus a specific sensitivity of mouse epidermis to the growth-stimulating effect of the carcinogenic hydrocarbons must be assumed.

The continuity of the carcinogenic process.—Though carcinomas may be elicited by a single application of carcinogenic hydrocarbons (19, 37, 46), in most animals repeated paintings are required to produce the same result. Thus of 11 litter mates subjected to 8 benzpyrene paintings at weekly intervals, 4 mice produced tumors and 2 more merely warts. In all these animals specimens taken from the painted area for biopsy show an initial epidermal hyperplasia. Animals dying 9 to 12 months later showed only slight or no epidermal thickening. The warts were observed for 4 months after their first appearance and during that period did not undergo malignant change. In other mice warts were seen to regress. Nonspecific stimulation of the hyperplastic or papillomatous regions, on the other hand, may induce the malignant change. There is, furthermore, no correlation between the rapidity with which warts appear in painted animals and the onset of malignant change (7).

All these findings indicate that, while in some mice the process of carcinogenesis may be continuous, in most animals the induction of malignancy represents a definitely new step in the process. The malignant change is not concerned primarily with the rate of cell proliferation, since neither the mitotic index (6) (see Table II) nor the growth rate is necessarily different from those of benign warts (47-49). Anaplastic changes in the cells are seen as quantitative and qualitative insufficiency and change of differentiation potentialities, and as the acquisition of invasive properties, including the induction of localized inflammatory reaction.

Whether these changes are secondary to further growth-stimulation by specific or nonspecific stimuli, or are the outcome of an entirely separate effect such as the chance mutation of a single cell, is not yet settled. In the former case we must assume that further stimulation of growth would render the cells unable to differentiate properly and lead to their anaplasia, and that this would involve changes in their genetic structure since the anaplasia is inherited by the descendants of these cells. These disturbances in cellular physiology would be paralleled by the nuclear abnormalities and the changes in volume and quantitative relationships of nucleus, nucleolus, chromosomes, and cytoplasm. In favor of such a conception is the occurrence of numerous simultaneous confluent malignant foci in the same animal (Figs. 7, 11, 12, 13, 14) or in the same patient (69).

In the latter case one of many somatic mutations must be assumed to succeed in the malignant conversion of a single cell, the descendants of which would overgrow the precancerous and papillomatous region. Here again the observed abnormalities in nuclear and cellular structure would serve as evidence of a labile state, and would thus appear as the symptom rather than the cause of malignancy (9). Such a possibility cannot be excluded. It should be emphasized, however, that it is in the final stage of carcinogenesis that an irreversible mutation must be assumed. The reversibility of warts and their relationship to the generalized epidermal hyperplasia render the assumption of early (48) or of successive mutational (12) changes unlikely.

SUMMARY

A quantitative histological analysis is given of
(a) The normal postnatal development of mouse epidermis.
(b) The regenerative hyperplasia induced in mouse skin by chemical depilation and by turpentine painting.
(c) The changes following the single and repeated application of benzpyrene to mouse and rat skin.

The development of the first pelage in the mouse is correlated with a reduction of epidermal layering. The hair follicles subsequently act as germinative centers for the epidermis. Stratification of the adult epidermis is not equivalent to differentiation.

Regenerative hyperplasia following chemical depilation is due to cellular migration from, and greater mitotic activity in, the hair follicles, and does not involve changes in epidermal mitotic activity.

Regenerative hyperplasia following repeated turpentine paintings is attributable at first to cellular migration and greater mitotic activity in the hair follicles, and subsequently also to increased proliferation of epidermal cells.

The initial hyperplasia of the epidermis and its appendages following benzpyrene painting is a result of a direct increase of mitotic activity in the epidermis and the hair follicles. These proliferative...
changes are not secondary to primary deleterious effects. Benzpyrene in acetone solution applied to mouse skin causes a slight increase in the number of degenerate cells, but does not lead to early ulceration or to a specific inhibition of growth. Epilation is secondary to increased proliferative activity in hair follicles and hair sheaths.

The primary reactions of mouse epidermis to benzpyrene painting are (a) increase in cell growth (both number and size of cells); (b) delay in the onset of differentiation. These reactions are generalized over the whole treated area, and result in an absolute and relative increase in the number of resting cells. These changes account for the hyperplasia of the epidermis and its appendages and for the formation of papillomas.

Carcinomas develop in hyperplastic or in papillomatous regions. Their appearance is characterized by a further absolute and relative increase of the now anaplastic resting cells, by a quantitative and qualitative disturbance of the differentiation process, and by the induction of a focal inflammatory reaction. The mitotic index of carcinomas may not differ from that of nonmalignant formations.

The bearing of these findings on the interpretation of carcinogenesis in mouse epidermis is discussed.

ACKNOWLEDGMENT

I have pleasure in acknowledging my indebtedness to Dr. Hooton R. Fell for her help in preparing the manuscript; to Mr. G. Lenney for technical assistance and the photomicrographs; to Mr. V. C. Norfield for the charts; and to the British Empire Cancer Campaign for a grant.

REFERENCES

28. FISHER, L. P. Carcinogenic Activity, Structure, and Chemic...
400 Cancer Research

The Histogenesis of Benzpyrene-Induced Epidermal Tumors in the Mouse

A. Glücksmann

Cancer Res 1945;5:385-400.

Updated version  Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/5/7/385.citation

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.