Skin Carcinogenesis by a Single Application of 20-Methylcholanthrene

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INTRODUCTION

In our study of the early stages of carcinogenesis (2, 3), it was found that a single external application of the carcinogen to the skin with one brush stroke was sufficient to elicit, after 3 or 4 weeks and in a considerable fraction of the animals, an epithelial hyperplasia sufficiently advanced to merit the term “pre-cancerous.” Most of the animals used in that investigation were killed within 1 month after the application, but 6 mice were allowed to survive. Of these, 3 died in the 3rd month; of the remaining 3 mice, 1 developed a carcinoma at the site of application of the carcinogen after 22 weeks and the other 2 died at about the same time without either a papilloma or a carcinoma. At that time the only observations recording carcinogenesis in the skin by a single application of a carcinogen were those of Mider and Morton (12, 13) on mice belonging to the C57 brown strain after a single application of 20-methylcholanthrene. The same procedure applied by Mider and Morton to mice of the C57 black strain failed to elicit carcinogenesis. Since then Law (7) has recorded the development of carcinomas in the skin of mice of the C57 brown strain in response to a single application of another carcinogen, 9,10-dimethyl-1,2-benzanthracene. These observations will be discussed in detail later.

The present paper records the results obtained in a second experimental series of mice of the Swiss strain the skin of which was exposed to a single application of methylcholanthrene. The fact that positive results had been obtained in this second series was briefly mentioned in our previous papers, published before this experiment was completed.

EXPERIMENTAL PROCEDURE

Methylcholanthrene in 0.6 per cent solution in benzene was applied to the unepilated skin of mice of the Swiss strain with a No. 4 camel’s hair brush. With the technic described in a previous paper (2), a single brush stroke delivers about 0.1 mgm. to an area of skin extending from the nape of the neck to the middle of the back. Two groups of Swiss mice were used. One consisted of 14 females about 3½ to 4 months old obtained directly from Tumblebrook Farms. These received 3 brush strokes simultaneously, the total dose delivered being therefore about 0.3 mgm. The second group consisted of 11 mice, 5 males and 6 females, bred in the laboratory from Swiss mice previously subjected to a treatment with methylcholanthrene over a short period of time, so that only a fraction of the animals developed skin cancer and were killed. The surviving negative mice were used for breeding, the offspring representing our “lab-bred” Swiss mice. These lab-bred Swiss mice were given only 1 brush stroke, thus receiving each about 0.1 mgm. of the carcinogen. This second group gave a negative result, while in the first mentioned group, which had received 0.3 mgm. of the carcinogen, 6 mice developed malignant tumors. It cannot, however, be concluded that this difference between the two groups is entirely due to the difference in dosage of the carcinogen; because, as already mentioned, the first carcinoma obtained by us in the Swiss strain, in the course of our studies on the early skin changes after a single application of methylcholanthrene, resulted from a single brush stroke. The lab-bred mice of the second group were derived from parents that had been relatively resistant to the action of the carcinogen. Hence it may be argued that the lab-bred mice represented a resistant substrain of the Swiss strain and that the failure to elicit carcinogenesis by a single application was due to this factor alone or to a combination of this factor and diminution in dosage.

The following account, therefore, refers exclusively to the 14 female mice belonging to the first group, all of which were alive at the time the first malignant tumor appeared (experiment XL). The data on the 6 mice that developed malignant tumors are given in Table I.

In 5 of the 6 mice with malignant neoplasms, the tumor developed in the skin area exposed to the direct action of the carcinogen. In one mouse (No. 1), the tumor, a sarcoma, was situated behind the right ear;
i.e., just outside the area of the brush stroke but within the area to which the solution had spread by capillarity. In each of the animals the neoplasm appeared as a single tumor. Five of the tumors were carcinomas; one (in No. 3) was associated with a sarcoma.

The process of carcinogenesis differs in some significant respects from that induced by the frequent application of carcinogens, which is the method generally in use. After these multiple treatments carcinomas develop frequently, though by no means always, in association with a heavily keratinized papilloma, which precedes the development of the carcinoma. The papilloma increases in size over a considerable period of time without undergoing a malignant change until eventually a carcinoma develops either at its base or from its side. In our experience, most of the carcinomas induced by the frequent application of methylcholanthrene at short intervals have been squamous cell carcinomas of varying degrees of malignancy. So far as we know this has also been the experience of other workers. Many of these squamous carcinomas have been of a low type of malignancy; i.e., composed of typical squamous cells forming a heavily keratinized solid neoplasm. Basal cell carcinomas develop rarely, if at all, in the mouse.

In the 6 mice belonging to the series reported here only 1 of the squamous carcinomas (in No. 2) can be described as having developed from a papilloma. The other 4 originated in massively hyperplastic epidermal epithelium and appeared macroscopically as ulcers which extended progressively. They showed a high degree of malignancy, the cells being anaplastic in mice 2 and 4, while in mice 3 and 5 small isolated groups of cells were scattered widely through the dermis. The tumor in No. 4 was a very anaplastic carcinoma with extensive lymphatic permeation, in which the cells had a resemblance to the basal cell tumors of man (Fig. 1). The carcinoma that developed in the one surviving animal of the preliminary experimental mice was almost identical with this tumor in appearance. Both developed as shallow ulcers which extended progressively. There are, therefore, indications that the type of carcinoma induced in the skin by the carcinogen is dependent to a certain extent upon its mode of application, in the sense that those developing after a single application are on the average more anaplastic, more malignant, and less heavily keratinized than those occurring after frequently repeated applications over long periods of time.

**Comparison with Observations of Other Workers**

Mider and Morton (12) were the first to record the development of carcinomas in the skin of mice of the C57 brown strain after a single application of methylcholanthrene. They painted a large area of skin with a 0.5 per cent solution of methylcholantrene; thus their experimental conditions were similar to ours. In their first series, 3 of 44 mice developed carcinomas in a period of induction varying from 16 to 32 weeks. In their second, carcinomas developed in 7 of 156 mice in a similar period of induction, varying from 15 to 33 weeks (13). In 28 mice of another strain, the C57 black, the same technic failed to induce any carcinomas.

Law (7), using 9,10-dimethyl-1,2-benzanthracene and applying it in a 0.3 per cent solution to a large area of the skin of mice of the same C57 brown strain used by Mider and Morton, also succeeded in inducing carcinomas. But his results differed in several important aspects both from those of Mider and Morton and from ours. No less than 8 of 10 mice developed carcinomas, the period of induction was longer (32 to 60 weeks), and in only 2 of the mice did the carcinomas develop within the painted area of skin.

Most of the animals had multiple carcinomas outside the painted area, some of the tumors developing even on the legs or on the ventral aspect. Law's results may be due to the fact that the carcinogen used in

<table>
<thead>
<tr>
<th>Mouse No.</th>
<th>Period of induction, weeks</th>
<th>Type of neoplasm</th>
<th>Histologic type</th>
<th>Macroscopic appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13</td>
<td>Sarcoma</td>
<td>Spindle cell</td>
<td>Tumor ulcerating through skin</td>
</tr>
<tr>
<td>2</td>
<td>15</td>
<td>Carcinoma</td>
<td>Squamous carcinoma, anaplastic</td>
<td>Tumor</td>
</tr>
<tr>
<td>3</td>
<td>18</td>
<td>1 carcinoma</td>
<td>Squamous carcinoma mixed with spindle cell sarcoma</td>
<td>Large ulcer with high rolling edge</td>
</tr>
<tr>
<td>4</td>
<td>21</td>
<td>Carcinoma</td>
<td>Anaplastic carcinoma with lymphatic permeation</td>
<td>Shallow ulcer</td>
</tr>
<tr>
<td>5</td>
<td>25</td>
<td>Carcinoma</td>
<td>Squamous carcinoma</td>
<td>Shallow ulcer</td>
</tr>
<tr>
<td>6</td>
<td>42</td>
<td>Carcinoma</td>
<td>Squamous carcinoma</td>
<td>Shallow ulcer</td>
</tr>
</tbody>
</table>
his experiments had a decidedly toxic effect, as he pointed out. That this may induce a general systemic change in addition to the local effect on the skin is indicated by his statement that "the majority of mice surviving the toxic effect of the carcinogen developed a leukemoid condition."

Our own results with mice of the Swiss strain correspond closely to those of Mider and Morton. The length of the induction period is similar, but there is a much higher percentage of carcinogenesis in the Swiss strain. The possibility of inducing the development of a carcinoma by a single application of a
potent carcinogen is, therefore, not restricted to one particular strain. The further fact that in our experiments, as in those of Mider and Morton, the neoplasms developed in the area of skin exposed to the carcinogen makes it unnecessary to postulate a general systemic effect as a contributing factor in the process of carcinogenesis by methylcholanthrene.

**Relation of Single Application of the Carcinogen to a Short Exposure**

Consideration must be given to the question: Does a single application signify a short exposure to the carcinogen? When a carcinogen is injected subcutaneously and a sarcoma develops, there has been a single application of the carcinogen but there has also been a continuous exposure of the tissue to it, since the carcinogen persists at the site of injection in demonstrable amounts. The same consideration applies to the development of carcinomas in the ears of sheep branded with hot tar as observed in Australia, or in the skin of mice treated with a drop of hot tar (6). In these two conditions the hot tar produces a burn. In the process of healing the tar becomes enclosed and microscopically demonstrable amounts of it remain in the skin for a long period. This mode of single application represents, therefore, an intracutaneous application with a continuous exposure to demonstrable amounts of the carcinogen. It differs essentially from the single extracutaneous application in which cancer develops in the absence of demonstrable amounts of the carcinogen. It is generally accepted that under this condition the carcinogen is rapidly absorbed by the skin and that its presence either on the surface or within the skin cannot be demonstrated after a short interval, variously estimated to be a matter of a few days or of 2 weeks.

**Mode of Action of Methylcholanthrene**

Until recently it has been considered essential for the development of malignancy to have the tissue subjected to an unremitting exposure to the carcinogen. Thus Fieser (5) stated in 1938 that “in order to produce skin tumors in mice with even a highly potent carcinogen in benzene solution some 30 deliberate applications must be made.” The universal practice in experimental carcinogenesis, of submitting the skin to applications of a carcinogen repeated at very short intervals and continued for several months, was based on the assumption that the carcinogen elicits epithelial proliferation by maintaining, over a long period, a direct, stimulating effect on the cells. This conception appeared to be supported by the fact that with this technic the carcinogenic effect increased with increasing concentrations of the carcinogen in the solvent. From this point of view, it is interesting to compare the carcinogenic effect of a single extracutaneous application involving a short exposure with the carcinogenic effect of extracutaneous applications repeated 3 times weekly over periods of 2 and 4 weeks, respectively, as observed in two additional experiments on 20 female Swiss mice in each group. The differences in the number of applications, the total amounts of methylcholanthrene applied, and the length of time over which the skin was exposed to the action of the carcinogen are given in Table II. In this table the dose of carcinogen delivered at each brush stroke is taken to be 0.1 mgm., and it must be remembered that in the experiment with a single application 3 brush strokes were made at the same

<table>
<thead>
<tr>
<th>Number of applications</th>
<th>Total amount of methylcholanthrene applied to skin, mgm.</th>
<th>Time of continued exposure, weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>0.6</td>
<td>3</td>
</tr>
<tr>
<td>12</td>
<td>1.2</td>
<td>5</td>
</tr>
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**Table III: Percentages of Mice with Methylcholanthrene-Induced Malignant Skin Tumors**

<table>
<thead>
<tr>
<th>Month of experiment</th>
<th>After 1 painting only</th>
<th>After 6 paintings in 2 weeks</th>
<th>After 12 paintings in 4 weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>14</td>
<td>16</td>
<td>19</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>21</td>
<td>19</td>
</tr>
<tr>
<td>6</td>
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<td>21</td>
<td>37</td>
</tr>
<tr>
<td>7</td>
<td>36</td>
<td>26</td>
<td>43</td>
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<td>8</td>
<td>36</td>
<td>42</td>
<td>50</td>
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<tr>
<td>9</td>
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<td>50</td>
</tr>
<tr>
<td>11</td>
<td>45</td>
<td>47</td>
<td>50</td>
</tr>
</tbody>
</table>
4 weeks. But even in this series 50 per cent of the mice remained free from cancer, indicating that the individual mice resistant to a single application of the carcinogen retain their resistance even after 12 applications repeated every second day for 4 weeks.

We again draw attention to the fact, recorded in a previous paper (4), that a single application of methylcholanthrene will produce a massive hyperplasia within 4 weeks in some mice (Fig. 2), while in others applications repeated 3 times weekly for 2 months or more will fail to do so. It is possible, therefore, to distinguish even in mice belonging to the same strain two distinct groups: (a) animals which react to a single application of the carcinogen with the production of a massive hyperplasia which may or may not lead to cancer—the "susceptible group"; and (b) animals in which the skin, though suffering the initial injury inflicted by a single application of the carcinogen, merely regenerates without developing subsequently a massive hyperplasia—the "resistant group." The experiments just mentioned show that the resistant group fails to react even to an application of the carcinogen continued over 4 weeks. The existence of such differences of susceptibility and resistance in the process of carcinogenesis in the skin was recognized in the early experimental work on tar cancer, in which the carcinogenic agent was much less potent than methylcholanthrene. But we can now identify the vague terms "resistance" and "susceptibility," which may be assumed to be reciprocal, with the visible reaction to a single application of a potent carcinogen. The questions now arise whether the factors determining susceptibility are inherited and fixed in the individual organism, or whether they are capable of being varied. We already know that the resistance of the skin can be broken down by the application of a potent carcinogen over a sufficiently long period, especially if a large area of skin is exposed to its action. In this prolonged application the carcinogen is more effective, if efficiency is measured by the dose necessary to induce cancer, when it is applied at long intervals of time than when applied unremittingly at intervals of 2 or 3 days (4). With less potent carcinogens, however, the resistant animals remain free from cancer even after a prolonged application to the skin. But we have as yet no information whether the reverse process of making a susceptible skin resistant can also be induced. The
method of carcinogenesis by a single application of a potent carcinogen puts at our disposal an experimental approach to this important problem.

These considerations have a bearing on the etiology of skin cancer in man. In man it is probably rare for the skin to be exposed to a more or less continuous succession of potent carcinogenic stimuli, corresponding to an application of a highly effective carcinogen such as methylcholanthrene, extending over a period of several years. The accidental exposure over several years of the early workers with roentgen rays and radium rays was probably an analogous condition. But the majority of skin cancers in man may be assumed to be restricted to persons belonging to the group with a skin susceptible to carcinogenic agents.

In the light of the results presented in this and in previous papers, the concept that carcinogenesis depends on prolonged exposure to a carcinogen that directly stimulates epithelial proliferation requires revision. Our previous findings show that the first application of methylcholanthrene, when this is used in concentrations eliciting an optimal carcinogenic effect by the standard technic, produces injury to the epithelial layer of the skin, including the hair follicles, and not a direct stimulating effect. This injury elicits an epithelial regeneration. The regenerated epithelium is altered in its reaction to subsequent applications of the carcinogen, in the sense of having become more resistant to the toxic effects of the carcinogen. Chemical investigations on the regenerated epithelium, carried out in this hospital by Drs. L. F. Wicks, C. Carruthers, and V. Suntzeff as another part of a group investigation directed by Dr. E. V. Cowdry, show that the alteration in biologic behavior is accompanied by significant and specific chemical changes in the epidermal epithelium (1, 14). The experiments in this paper show that in a considerable fraction of the animals the regenerated epithelium proceeds, without any further intervention and in the absence of the carcinogen in amounts demonstrable by methods available at present, to the development of a massive epithelial hyperplasia which forms the basis for the subsequent development of cancer.

This change in the reaction of the epidermal epithelium to a toxic chemical substance, after an injury has been inflicted by a first application of this chemical substance, is a biologic phenomenon for which an analogy can be found in the observations of MacNider (8-11) on the epithelium of the kidney and liver that has regenerated after the administration of uranium salts and other toxic agents. But the subsequent excessive epithelial proliferation of the regenerated epithelium without any further intervention is a biologic phenomenon for which there is no analogy.

It may be argued that while the bulk of the carcinogen applied extracutaneously disappears a very small amount, not readily demonstrable by the methods available at present, remains and is responsible for the stimulating effect on the epithelium. It is possible, on general biologic conceptions, that a substance which has a toxic effect in large doses may have a stimulating effect in small doses. This possibility cannot be excluded at present. But it must be remembered that the carcinogenic effect of any chemical carcinogen tested so far by continued application diminishes with diminution of the dose applied to the skin. This means that any stimulating effect which may be attributed to very small doses of a carcinogen could become effective only on epithelial cells which have regenerated after a previous specific injury has been inflicted on the epithelium by a more massive dose.

CONCLUSIONS

Cancer can arise in the skin of mice in response to a single exposure of the skin to a potent carcinogen, such as methylcholanthrene. From this result and our previous observations, as well as from those of Mider and Morton, it appears that the conditions required to produce this response are a considerable total dose of a potent carcinogen applied to a large area of skin. Under such conditions the carcinogen injures the epithelial elements of the skin, which respond by regeneration. This regenerated epithelium is altered in its reaction to subsequent applications of the carcinogen, having become more resistant to its toxic effects. In a fraction of the animals the regenerated epithelium proceeds without further applications of the carcinogen to a massive epithelial hyperplasia culminating in the localized development of a skin carcinoma. The reaction of the skin to the carcinogen in this group of animals, the susceptible group, contrasts strongly with that of the resistant group. In resistant animals the carcinogen also produces an injury of the skin followed by regeneration. But in this group the regenerated epithelium does not proceed automatically to a massive epithelial hyperplasia; it even fails to do so when subjected to an unremitting application of the carcinogen extending over several weeks. The bearing of these observations on our conceptions of the mode of action of chemical carcinogens is discussed.

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