Sensitization of Skin by Carcinogenically Inactive Methylcholanthrene to Subsequent Carcinogenesis*

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The fact described in the preceding paper (9), that methylcholanthrene dissolved in anhydrous lanolin fails to produce a carcinogenic effect and does not even induce early skin changes such as epilation, epidermal hyperplasia, destruction of sebaceous glands, and diminution in the calcium and iron content of the epidermis, has been established in 4 experimental series comprising 312 mice. Since examination in ultraviolet light demonstrated that the skin absorbs methylcholanthrene from its solution in lanolin, the absence of the carcinogenic effect cannot be attributed to inadequate absorption. Two other possible explanations suggest themselves to account for this phenomenon. One is that the carcinogen exists in lanolin in a physically or chemically inactive state, and the second, that the skin has undergone a biological alteration in the sense of having become resistant to the carcinogen.

In order to investigate this question two experiments have been carried out in which the skin of mice, after having been subjected to prolonged treatment with a lanolin solution of methylcholanthrene, was tested subsequently by applying to it a benzene solution of the carcinogen. The results form the subject matter of this paper. Details of the preliminary treatment with the lanolin solution of methylcholanthrene have already been described (9). The experiments designated here as "A" and "C," which supplied the animals for the subsequent treatment with the benzene solution of the carcinogen, are identical with experiments "A" and "C" of our previous paper, so far as the preliminary treatment with methylcholanthrene in lanolin is concerned.

The two experiments were carried out in essentially the same manner. Young female Swiss mice from 2 to 3 months old were subjected to 42 applications of a 0.3 per cent solution of methylcholanthrene in anhydrous lanolin. These applications were given 3 times weekly for a period of 14 weeks. The animals, which, as a result of this treatment, showed no skin changes and were in good condition, as shown by their increase in weight, were then given 5 applications of a 0.6 per cent benzene solution of methylcholanthrene by means of a single brush stroke. The applications were made at intervals of 3 weeks, so that the last application was given 12 weeks after the first one.

With normal mice this discontinuous technic of administering small total doses of the carcinogen dissolved in benzene had been found by Cramer and Stowell (3, 4) to induce skin cancer in from 40 to 50 per cent of the animals during an interval of 9 months after the first application. As pointed out by these authors, such a technic is better suited to reveal any increase or decrease in the sensitivity of the skin to a carcinogen than is the routine method of applying the carcinogen 3 times weekly for a period of 14 weeks, which results in inducing cancer in 100 per cent of the animals.

The two experiments designed to test the effect of a preliminary treatment with a lanolin solution of methylcholanthrene on the sensitivity of the skin to a benzene solution of that carcinogen differed in only one point. In one, experiment C, the benzene solution of methylcholanthrene was applied for the first time 2 weeks after the last application of the lanolin solution of methylcholanthrene had been given; in the other, experiment A, an interval of 3 months was allowed to elapse between the last application of the lanolin solution of the carcinogen and the first application of the benzene solution. Both experiments were terminated 9 months after the first application of the benzene solution of methylcholanthrene.

The two experimental series, if timed from the first application of the lanolin solution, extended, therefore, over 12 months and 15 months respectively. This is a relatively long period of time, taking into consideration that the maximal life span of the mouse is about 2 years. Since there are on record observations indicating that the response of mouse skin to carcinogens

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diminishes with advancing age, it was necessary to use for the control experiments, in which the carcinogen was applied to mice not previously treated with a lanolin solution of methylcholanthrene, animals of the same age as those studied in the two main experimental series. This condition was fulfilled by setting aside, at the beginning of the treatment with the lanolin solution, a reserve of mice of the same age as those subjected to this treatment. The mice used for the controls were taken from this reserve and were subjected to the application of the benzene solution of the carcinogen at the same time, and therefore at the same age, as the group that had received the preliminary treatment with the lanolin solution.

The numbers of effective and of cancerous mice in the two experimental series are given in the following table:

<table>
<thead>
<tr>
<th>Mice</th>
<th>Experiment</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>C</td>
</tr>
<tr>
<td>Effective</td>
<td>25</td>
<td>34</td>
</tr>
<tr>
<td>Cancerous</td>
<td>23</td>
<td>16</td>
</tr>
<tr>
<td>Percentage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with cancer</td>
<td>92</td>
<td>47</td>
</tr>
</tbody>
</table>

From the time of the first application of the benzene solution of methylcholanthrene, the mice were examined regularly and any skin changes that had occurred were noted in the protocols. In this way the development of papillomas and the subsequent malignant change was determined. Eventually all the painted skin areas were examined histologically, including those that were apparently negative to the naked eye at the end of the experiment.

Fig. 1 shows the development of skin cancer in the two experiments and in the controls. The abscissa gives the time in weeks dated from the first application of the benzene solution of the carcinogen, the ordinate gives the number of mice bearing skin carcinomas expressed in percentage of effective mice. The 5 arrows indicate the applications of methylcholanthrene in benzene. The two control series, AC and CC, show carcinogenic responses that are very similar to each other and very similar, also, to that obtained in previous experiments by Cramer and Stowell and described by them as “incomplete carcinogenesis with discontinuous exposure.” The two series, A and C, in which the mice had been subjected to a preliminary course of painting with a lanolin solution of methylcholanthrene, are also very similar to each other, but show a striking contrast with the control series. In these two series the carcinomas develop much earlier and the number of cancerous animals increases much more rapidly than in the control experiments, so that the cancer incidence at the end of the experiment is about double that of the controls. In experiment A no less than 30 per cent of the sensitized mice and only 4 per cent of the controls (i.e., 1 mouse) had fully developed carcinomas at the end of the 12th week, the time at which the last of the 5 applications of methylcholanthrene in benzene was given; in experiment C the figure at the same period is 10 per cent for the sensitized mice and 0 per cent for the controls. A response as early as that of the sensitized mice has never been observed previously in our experiments with the protracted method of application. Very impressive, also, was the contrast between the rapidity with which the appearance of a papilloma was followed by the development of a carcinoma in the sensitized mice and the very prolonged lapse of time required for this process in the control animals.

Although in experiment A an interval of 3 months had elapsed between the preliminary treatment with the lanolin solution and the subsequent treatment with the benzene solution, the carcinogenic response was not less than in experiment C, indicating that the sensitization by the lanolin solution was not a transient effect. Actually the carcinogenic response was somewhat higher in A than in C, but further experiments are necessary to determine whether this can be interpreted as indicating an increased sensitivity or whether this difference lies within the range of experimental variations.

The concept of “cocarcinogens” has been introduced by Shear to designate substances that, though non-carcinogenic when applied alone, are able to enhance the effect of chemical carcinogens when applied together with them. Croton oil and croton resin, for instance, are cocarcinogens, while xylene and turpentine, though irritants, have no cocarcinogenic effects (1). Since in our experiments a potent carcinogen fails to be carcinogenic in the absence of solvents such as benzene, acetone, or chloroform, generally used in the application of carcinogens to the skin, the question had to be considered whether these vehicles might act not merely as solvents but also as cocarcinogens. In order to test this point, mice were submitted to a preliminary painting with a lanolin solution of methylcholanthrene 3 times weekly for 14 weeks. Four weeks were then allowed to elapse in order to ensure the complete disappearance of methylcholanthrene from the skin. Reagent grade benzene was then applied 3 times a week for 18 weeks to the skin of the back of 27 mice that had been thus sensitized. All these mice remained negative, except one that developed a small papilloma. The papilloma persisted as such and remained unchanged till the end of the experiment at the 36th week after the first application of the lanolin solution of methylcholanthrene. The mouse was then killed for microscopic examination.
of the tumor, which was found to be a small benign papilloma without any indication of precancerous change. It follows that benzene, which does not induce skin cancer in normal mice, is also ineffective in sensitized mice.

The genic response of these treated skins to benzene solutions of methylcholanthrene shows. By elimination, then, we are forced to conclude that the explanation of the lack of carcinogenic effect of the lanolin solution is to be found in the state of the methylcholanthrene itself. Some of the factors that may be involved will be discussed presently.

An additional finding of considerable interest is the fact that the lanolin solution of methylcholanthrene produces, without noticeably affecting the structure of the skin, a striking sensitization to subsequent car-

**DISCUSSION**

As a result of these experiments we may definitely rule out the possibility that the failure of a lanolin solution of methylcholanthrene to induce skin cancer is due to an increased resistance of the skin. The opposite is actually the case, as the accelerated carcinogenic effect of the lanolin solution is to be found in the state of the methylcholanthrene.
Estrinization of mice was found by Gilmour (6) to their genesis as a direct and immediate response to a considerable fraction of the tumors must have had cholanthrene to the sensitized skins suggests that a crease in the number of carcinomas but also a pronounced increase in the skin of mice so far as the number of cancerous tumors was concerned, but the rate of development of malignancy was not increased. Bonser (2) inbred mice to produce a strain that was more sensitive to carcinogens as measured by the number of cancerous skin tumors, but the skin cancers did not develop more rapidly in this strain. Estrinization of mice was found by Gilmour (6) to increase the carcinogenic response of the skin to benzpyrene so far as the number of carcinomas was concerned without, however, increasing the rate of their appearance. Paletta and Max confirmed these findings (7).

In our experiments there was not only a great increase in the number of carcinomas but also a pronounced acceleration of the carcinogenic response. In fact, the rate at which carcinomas appeared after the first application of the benzene solution of methylcholanthrene to the sensitized skins suggests that a considerable fraction of the tumors must have had their genesis as a direct and immediate response to that first exposure. Returning now to the question why methylcholanthrene produces no carcinogenic effect when applied in lanolin solution, we find that possible explanations are still numerous, even though enhanced resistance of the skin has been eliminated.

First is the possibility that the carcinogen is inactivated as a result of a direct reaction with some constituent of anhydrous lanolin. On the basis of fluorescence spectrographic data this possibility appears unlikely. Characteristic methylcholanthrene spectra are still obtained from solutions of the carcinogen in lanolin that are over 2 years old, even though this mixture was stored at room temperature and, for the many months during which experiments were in progress, the solution was heated on alternate days to 50° to 60° C. for an average period of an hour or so.

A second consideration may be that frequently advanced to explain the reduction of carcigenic activity of injected carcinogens by lipids, that the inactivity of the lanolin solution results from the rapid absorption and excretion of the carcinogen in this readily absorbable solvent. Against this suggestion can be offered the evidence derived from fluorescence microscopic studies, that each of the 42 triweekly applications persists for at least 18 to 24 hours in the skin. An exposure of this magnitude should effect some morphological change if the methylcholanthrene is biologically active.

The fluorescence microscopic evidence cited above also rules out a third possibility, that the carcinogen, though not reactive with anhydrous lanolin in vitro, is rapidly and completely inactivated in vivo by a reaction with constituents of either the lanolin or of the tissue. The appearance in skin of the characteristic blue-violet fluorescence of methylcholanthrene for many hours after each exposure to lanolin solutions makes such an hypothesis untenable.

If we allow the facts to speak for themselves and dismiss preconceived ideas, a further possible explanation for the failure of a lanolin solution of methylcholanthrene to induce cancer is the assumption that methylcholanthrene is not, per se, carcigenic. Such an assumption involves the separation of the actions usually ascribed to methylcholanthrene into two groups; those due to the unaltered hydrocarbon, and those due to some metabolic derivative. The experiments reported provide, for the first time, clear evidence that the different biological effects of a carcigenic hydrocarbon may be separated. This suggests that the sensitizing action depends upon a chemical substance different from that or those producing the subsequent effects, such as epilation, destruction of sebaceous glands, epidermal hyperplasia, and finally, carcigenesis itself.

Let us consider the last suggestion, that methylcholanthrene is not itself an active carcigenic agent, in the light of these concepts. The role of unaltered methylcholanthrene would be to sensitize epidermal cells to the action of some of its metabolic derivatives, which, for brevity, we shall refer to as MCX. To MCX, which might actually represent a number of compounds, would be ascribed the initiation of all the subsequent activities that lead to cancer.

Several facts favor this explanation of the carcigenic inactivity of lanolin solutions of methylcholanthrene. (a) Methylcholanthrene in lanolin does produce great sensitization of skin in the absence of other morphological effects. This occurs after the skin has been exposed to a great amount of unaltered carcigen over a long period, since each of 42 applications persists for at least 18 to 24 hours. (b) Methylcholanthrene and the recorded constituents of anhydrous lanolin are, in general, relatively inert substances. In addition anhydrous lanolin contains powerful antioxidants, which would tend to prevent the oxidative breakdown of methylcholanthrene dissolved in it. (c) At no time did the skin, after having been painted with the methylcholanthrene-lanolin mixture, ever exhibit any fluorescence attributable to breakdown products of the carcigen. Conversely, such
fluorescences are regularly present in skin treated with the carcinogen in benzene (10).

In the preceding discussion an attempt has been made to analyze the remarkable lack of carcinogenic action of methylcholanthrene in anhydrous lanolin. The experiments have achieved their purpose in differentiating clearly between the skin and the carcinogen solution as the important factor in this phenomenon, but merely open up a larger vista of problems when it comes to the attempt to explain the specific nature of the state of methylcholanthrene. Some of the possibilities that may be involved have been suggested, and evidence that makes some of them less tenable than others has been presented. No final conclusions are permissible on the absolute basis of the available evidence. As a working hypothesis, however, the concept is advanced that unaltered methylcholanthrene is a sensitizing agent, itself non-carcinogenic, that prepares the skin for subsequent action by metabolic derivatives of the carcinogen, or by substances formed in the tissue as a result of exposure to these metabolic products. Attempts to test this hypothesis by animal experiments are now in progress. More direct evidence on the effect of metabolites of the carcinogenic hydrocarbons following the action of the unaltered carcinogen might be derived from a less complex situation in which a malignant change can be produced, such as has been so effectively used by Earle (5), but facilities are not available to us for this approach to the problem.

This concept of the mode of action of a carcinogenic hydrocarbon is not entirely new, nor does its sole support come from the evidence presented here. Earlier reports from this laboratory (3) have suggested that the direct effect of methylcholanthrene is a short toxic one, and that "subsequent epithelial proliferation can be accounted for adequately by assuming as a working hypothesis the formation in the skin of substances stimulating the cells to multiply over a prolonged period." These substances were designated as "proliferin." The results reported here support and expand such an hypothesis. It is now apparent that, at least under some conditions, methylcholanthrene has a sensitizing action and that its toxicity varies with the solvent. Possibly all the toxic effects are attributable to its decomposition products. Strength is given the major thesis, that methylcholanthrene is not, per se, responsible for inducing the specific cellular changes that lead, after a long interval, to the development of cancer.

For such a skin carcinogen as methylcholanthrene, and probably also for allied carcinogenic substances that are soluble in lipids and in lipid solvents, the manifestation of the carcinogenic effect, including the earliest changes initiating that effect, depends on the nature of the solvent. For convenience of description the group of solvents such as benzene, chloroform, and acetone, in which methylcholanthrene is carcinogenically active, will be described as forming "active" solutions; those in which methylcholanthrene is carcinogenically inactive will be referred to as making "inactive" solutions.

It appears, then, that a carcinogen in an inactive solution renders the skin more sensitive to the subsequent action of the same carcinogen when in an active solution. Forming one such inactive solution is sebum, a product of the skin that covers its outermost surface. The first effect of a carcinogen when applied in an active solution is to destroy the sebaceous glands. The glands remain intact when the carcinogen is applied in an inactive solution (8).

The existence of a chemical carcinogen for the skin in an inactive solution when dissolved in a secretion of the skin, and the sensitizing effect of this inactive solution, represent new factors in the etiology of skin cancer. They have some resemblance to allergic phenomena. Further investigations are necessary for the analysis of the significance of this phenomenon. But it may be pointed out that in the etiology of human skin cancer due to such agents as soot and tar, carcinogenic agents come into contact with the skin under conditions that do not necessarily involve removal of the sebum. These agents might, therefore, be dissolved in the sebum covering the surface of the skin and become inactive. But they might also sensitize the skin to the effects of a subsequent contact with a chemical carcinogen under conditions in which the protective action of the sebum is weakened or absent.

SUMMARY AND CONCLUSIONS

Methylcholanthrene, when dissolved in anhydrous lanolin, is rendered inactive in the sense that a prolonged administration of such a solution to mouse skin fails to induce those specific morphological and chemical changes that initiate the carcinogenic process, and regularly follow the application of methylcholanthrene dissolved in a lipid solvent such as benzene. A relatively simple chemical substance can, therefore, exist in a biologically active or inactive state according to the medium in which it is dissolved.

The inactive state is not due to lack of absorption of the methylcholanthrene by the skin. Nor can it be accounted for by postulating an increased resistance of the skin to the action of the carcinogen, for treatment with "inactive" methylcholanthrene greatly increases the sensitivity of the skin to a subsequent application of the carcinogen in the "active" state; i.e., when dissolved in benzene. This increased sensitivity manifests itself in a great increase in the incidence of...
skin cancer, and a great shortening of its induction period.

The significance of these findings in relation to a concept of a mode of carcinogenic action of methylcholanthrene is discussed.

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