The Dependence of the Genesis of Induced Skin Tumors on the Caloric Intake during Different Stages of Carcinogenesis*

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In earlier investigations (11, 12) it has been shown that calorie-restricted diets inhibit the formation of tumors in mice. This effect has been demonstrated for the spontaneous breast tumor, induced skin tumor, induced sarcoma, and primary lung tumor. In those investigations the restricted diet was fed throughout the entire experiment. The experiments with spontaneous breast tumors were carried out on dba female mice, in which tumors normally begin to appear when the mice are 9 to 10 months old; in one of the experiments the restricted diet was instituted when the mice were, on the average, 9 months of age, i.e., when tumors begin to appear, yet there was a pronounced inhibition of tumor formation. This suggested that the inhibitory effect on tumor formation may be dependent chiefly on caloric restriction during the period in which tumors appear, rather than in the previous period of carcinogenic preparation (12).

Tumors may arise long after exposure to the carcinogenic stimulus has ceased. Skin cancer of tar workers and bladder carcinomas of dye workers have often occurred many years after the persons exposed have given up employment in these industries. Results of experimental carcinogenesis are in agreement with these clinical observations. Such a separation of a carcinogenic stimulus and its result in terms of a tumor was shown as early as 1922 by Leitch (7), in experiments in which he tarred mice for a limited period.

The production of skin tumors by means of a limited application of carcinogenic hydrocarbon offers an excellent technic for separating arbitrarily the carcinogenic process into two stages. By terminating the paintings with the carcinogen just before tumors are expected, one may regard the period of carcinogenesis as being divided into:

I. The period of carcinogenic application; followed by
II. The period in which tumors appear. This paper is concerned with:

A. Determining in which of these periods caloric restriction produces its main inhibitory effect on tumor formation, and
B. The possible value of these findings in understanding the mechanism of carcinogenesis.

METHODS

Four groups of mice, each on a different dietary regime, were used. Each group consisted of 50 pure strain dba male mice, which were inbred in our laboratory and born within a span of a few weeks. The groups were equivalent as to age and weight; many of the animals in each group had litter mates in the other groups.

Two diets were employed: an ad libitum diet consisting of Purina dog chow, skimmed milk powder, and cornstarch; and a calorie-restricted diet consisting of the same amounts of dog chow and milk powder, but containing no cornstarch. Thus both diets contained equal quantities of protein, fat, vitamins, and minerals, and differed only in carbohydrate content.

1 Caloric restriction is a relative term. In this communication caloric restriction refers to a calorie intake approximately 60% of the ad libitum diet; only carbohydrate (starch) is restricted.

2 The original stock was obtained from the Roscoe B. Jackson Memorial Laboratory.

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The diets in grams per mouse per day had the following compositions:

<table>
<thead>
<tr>
<th></th>
<th>Ad libitum (A) gm.</th>
<th>Calorie-restricted (R) gm.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dog chow meal</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>Skimmed milk powder</td>
<td>0.9</td>
<td>0.9</td>
</tr>
<tr>
<td>Cornstarch</td>
<td>1.9</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>4.2</td>
<td>2.3</td>
</tr>
<tr>
<td>Average daily food consumption,</td>
<td>3.8-4.1</td>
<td>2.3</td>
</tr>
<tr>
<td>(Computed from manufacturers' analyses)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>0.62</td>
<td>0.62</td>
</tr>
<tr>
<td>Fat</td>
<td>0.08</td>
<td>0.08</td>
</tr>
<tr>
<td>Ash</td>
<td>0.16</td>
<td>0.16</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>2.93</td>
<td>1.22</td>
</tr>
</tbody>
</table>

A week's supply of the weighed dietary constituents was mixed with sufficient water to form an easily molded mash, cut into equal blocks, and stored in a refrigerator. The mice were fed daily. Those on the restricted diet consumed all the food given them. The actual food consumption of the ad libitum group was estimated by weighing back each week the food left in the cages. All animals had free access to water.

The animals consuming the ad libitum diet were housed 5 in a cage. Each group of 5 mice on the restricted diet was kept in 2 cages; at the bi-weekly weighings the lighter animals were placed in one cage, the heavier in the other. Thus the restricted animals competed with others of the same order of weight and, over the long period of restriction, consumed approximately equal quantities of food.

Skin tumors were induced by applying 1 drop of the carcinogen solution with a dropping pipette twice weekly to the skin of the interscapular region. A 0.3 per cent solution of 3,4-benzpyrene in benzene was used, each drop containing approximately 0.05 mgm. of the carcinogen.

At 2 week intervals the animals were inspected for neoplasms and weighed. The tumors were recognized as papillomas or carcinomas by their gross appearance and by palpation. None of the papillomas regressed. About 80 per cent were ultimately converted into carcinomas. Most of the remaining 20 per cent is accounted for by those in mice that died before the conversion took place or by papillomas that arose too late in the experiment for the change to be observed. Since the exact time of conversion is not recognizable the tumor count and time of appearance refer to the first tumor that each mouse developed.

All animals were examined post mortem. Histological examinations were made of many lesions selected at random, and of all those about which doubt existed; the results of the histological studies indicated that the gross examinations were reliable. Percentages of tumor formation were computed on the basis of the number of animals alive in the group (effective total) at the time the first tumor appeared in the experiment, and also on the basis of an adjusted total, described by Bryan and Shimkin (3), that accounts for the deaths of nontumor animals during the period in which tumors appear.

EXPERIMENTAL

Two of the 4 equivalent groups of mice, 10 weeks of age, were placed on the ad libitum diet, while the other 2 groups were given the calorie-restricted diet. After 4 weeks, there was applied to all the mice of the 4 groups the first of 19 semi-weekly applications of the carcinogen. No tumors had appeared at the time the application of the carcinogen had been completed.

Two days after the final application of the carcinogen the following dietary changes were made: One of the groups being fed ad libitum was continued on this diet while the other was transferred to the calorie-restricted diet; similarly, one of the groups being fed the calorie-restricted diet was continued on this ration while the other was transferred to the ad libitum diet. The experiment was then continued for 52 weeks, during which period tumors appeared. Thus the 4 groups had different sequences of diet: ad libitum—ad libitum (AA), ad libitum—restricted (AR), restricted—restricted (RR), and restricted—ad libitum (RA). The changes in diet (AR and RA) were made after the application of carcinogen was terminated, and before tumors had appeared.

In Fig. 1 the mean weight curves of the mice in each of the 4 groups are given. After termination of the application of the carcinogen the mean weights of the 2 groups, RA and AR, whose diets were changed, begin to approach the corresponding mean weights of groups AA and RR respectively. The adjustment occurs over a transition period of weeks.

Table I shows the cumulative tumor counts in the 4 groups at various periods of the experiment. It is to be noted that at the termination of the experiment 32 tumors had arisen in the AA group, 16 in the AR, 11 in the RR, and 24 in the RA. "Per cent tumors" are given both as percentages of the effective total and of the adjusted total. Both methods of computing the percentage of mice with tumors give comparable figures, indicating that the differences are not due to unequal distributions of deaths (in time or number) among the groups.

In Fig. 2 the tumor percentages (cumulative) are presented graphically. It can be seen that the RA curve begins somewhat later but tends to approach the AA curve. Likewise the AR curve tends to parallel the RR curve.
The two groups, AA and RA, that were fed \textit{ad libitum} during the period of tumor appearance, have a tumor incidence of about the same order, suggesting that the carcinogenic agent produces the initial fundamental changes regardless of the diet—restricted or \textit{ad libitum}—fed during the period of carcinogenic application. Based on this conclusion it is of interest to make the following comparisons: AA and AR, which shows that caloric restriction in the period of tumor appearance inhibits the genesis of tumors; AR and RR, which indicates that the inhibition of tumor formation observed in the latter group is principally due to caloric restriction during the period of tumor appearance; and RA and RR, which, conversely, reveals that full feeding in the period of tumor appearance favors the genesis of tumors. The results of these experiments are best explained by assuming that the process of carcinogenesis is divided into two phases: (a) an initiatory or pre-neoplastic stage, which under suitable conditions leads to (b) a developmental or neoplastic stage that culminates in a perceptible tumor. Caloric restriction has little or no effect in preventing the initial stage, while it distinctly inhibits the fruition of the neoplastic stage.

**DISCUSSION**

The induced skin tumor was chosen for these studies because its use provided excellent conditions for this particular type of investigation. An exact and equal amount of carcinogen could be applied to the animals of both the \textit{ad libitum} and restricted groups, and discontinued before dietary changes were made. Furthermore, the carcinogen and its conversion products, as judged by fluorescence studies (1; 4-6; 10; 13; page 474), are known to disappear from the skin within a short period after the last application of the carcinogen. These two conditions are not obtained as experiments are usually performed, with either the sarcoma induced by carcinogenic hydrocarbons or the spontaneous breast tumor.

Our experiments permit the inferences: (a) that the initial fundamental changes due to the application of a carcinogen to the skin occur essentially to the same extent regardless of whether the animals are on...
a full or calorie-restricted diet, and (b) that it is the caloric regime during the period in which tumors appear that determines, to a large degree, whether or not a tumor will eventuate. Although the qualitative results are definite, certain aspects of the present experiment should be considered. A particularly important factor in the differences in tumor incidence between AA and RA, and between RR and AR, may be that under the conditions of our experiment there is only a very short interval between the time of the reversal of diets (and termination of carcinogen application) and the appearance of the first tumor. Consequently, during at least part of the period in which tumors appear the weights of the RA mice are less than those of the AA mice; conversely, the weights of the AR animals are higher than those of the RR animals. These relationships can be modified by a proper choice of potency, quantity, and duration of carcinogen application.

One may conclude from the experiment reported in this communication that carcinogenesis can be divided into at least 2 distinct phases: (a) a stage of preparation, latency, initiation, or pre-neoplasm, in which the cells become prepared or biased toward forming a tumor; and (b) a stage of development or formation that eventuates in a perceptible tumor.

The carcinogen initiates pre-neoplastic changes regardless of whether the animals are on an ad libitum or calorie-restricted diet. In contrast, the ad libitum diet favors the development of these initial changes into perceptible tumors, while the calorie-restricted diet acts either by reversing these initial changes or preventing their development in many of the animals. These interpretations are given added significance by their agreement with the work reported by Rous and his associates (8, 9), and by Berenblum (2).

Rous and Kidd (9), and MacKenzie and Rous (8), have shown that when a carcinogenic tar is applied to rabbit skin many more epidermal cells are rendered pre-neoplastic than declare themselves by forming tumors. Cells that had been conditioned or biased by tar painting were encouraged to form tumors by wound healing or the application of turpentine. The nonspecific stimulation of wound healing acted as the deciding influence in causing cells that had been "initiated" or "prepared" by tarring to eventuate into perceptible tumors. The authors bring out the need for a sharp distinction between the forces that induce neoplastic change (initiation or inception) and those that determine, or prevent, its realization in terms of a tumor (formation).

Investigations by Berenblum (2) on the augmentation of carcinogenesis by means of noncarcinogenic agents (cocarcinogenic action) have resulted in significant observations and interpretations. This investigator studied the effect exerted on the formation of skin tumors (induced by 3,4-benzpyrene) by croton resin (or turpentine) applied prior to, during, or after the application of the carcinogen. The use of croton resin prior to benzpyrene application had little or no demonstrable effect; its application concurrent with benzpyrene throughout the experiment led to a pronounced increase in the formation of tumors; and its use after a limited period of benzpyrene application also resulted in a pronounced increase in the formation of tumors. This suggests that croton resin produces its principal effect when applied during the second stage (developmental) rather than during the initial (preparatory) stage.

Although the precise cellular changes that eventuate in a tumor are not known, it has been possible to distinguish 2 well-defined stages of carcinogenesis. The first stage of initial changes is brought about by specific carcinogenic agents. A consideration of the 3 distinct and different investigations suggests that these initial changes, once present, may then be favored by certain factors, resulting in tumors; or not favored, resulting in lack of development or possibly regression of these initial changes. Rous and his associates have shown that wound healing favors and encourages tumor formation from these biased cells. Berenblum has shown that croton resin has a similar effect. Both have found that turpentine has comparable properties.

Our own work indicates that an adequate caloric intake favors the formation of tumors from the biased or conditioned cells, while a diet that is calorie-restricted distinctly inhibits the development of tumors. Thus, in the skin of an animal, the changes brought about by a carcinogenic agent can be so altered by three distinct and diverse means (wound healing, croton resin, or a normal caloric intake) that considerably more tumors develop than in the absence of these factors.

All 3 investigations, those of Rous and his associates, Berenblum, and our own, demonstrate that carcinogenesis may be considered to be divided into at least 2 distinct phases: (a) a stage variously designated as initiation, inception, latency, preparation, or pre-neoplasm, produced by the carcinogenic agent; and (b) a stage of development, formation, or epicarcinogenesis, in which these initial changes result in a perceptible tumor even without the continued application of the carcinogenic agent. It is of significance that entirely different means—wound healing, croton resin, and caloric restriction—can affect the developmental stage; whereas it seems likely, though not definitely proved, that these same agents have little or no effect upon the initiation stage.
SUMMARY

In earlier experiments it has been shown that calorie-restricted diets inhibit the formation of various types of tumors in mice. Although the restricted diet had been fed throughout the entire experimental period, there were observations suggesting that the inhibition may have been produced principally during the period in which tumors appeared, rather than in the preceding period of initiation.

The production of skin tumors in mice by means of a limited application of carcinogenic hydrocarbon permitted the arbitrary separation of carcinogenesis into two periods or stages: initiation and development. The results, summarized below, were obtained by feeding various sequences of an ad libitum and calorie-restricted diet to 4 groups of mice that were given equal applications of carcinogen.

<table>
<thead>
<tr>
<th>Group</th>
<th>Diet in period of painting (10 weeks)</th>
<th>Diet in period of tumor formation (52 weeks)</th>
<th>Tumors, per cent (Adjusted)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AA</td>
<td>Ad libitum—Ad libitum</td>
<td></td>
<td>69</td>
</tr>
<tr>
<td>AR</td>
<td>Ad libitum—Restricted</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>RA</td>
<td>Restricted—Ad libitum</td>
<td></td>
<td>34</td>
</tr>
<tr>
<td>RR</td>
<td>Restricted—Restricted</td>
<td></td>
<td>24</td>
</tr>
</tbody>
</table>

These data demonstrate that carcinogenesis can be divided into 2 distinct phases:

I. A stage of preparation or initiation in which the normal cells become prepared or biased toward forming a tumor, and

II. A stage of development or formation that eventuates in a perceptible tumor. Furthermore, it is shown that:

A. The initial fundamental changes due to the application of a carcinogen occur regardless of whether the mice are on a full or calorie-restricted diet, and

B. A full diet promotes the development of tumors from these initial changes, while a calorie-restricted diet inhibits such development.

REFERENCES

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