Morphology and Growth of Subcutaneous Tumors Induced with Carcinogenic Hydrocarbons in Strain C3H Male Mice

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The purposes of this investigation were to study the growth rate of tumors induced in strain C3H male mice by the subcutaneous administration of carcinogenic hydrocarbons, to describe the morphologic features of these neoplasms, and to determine the relations between the growth rate, latent time, the dose and type of carcinogen, and the histologic appearance of the tumors.

EXPERIMENTAL PROCEDURE

The material is provided from an experiment on the response of young C3H male mice to 12 logarithmically spaced doses of 20-methylcholanthrene (1.0 to 0.00024 mg.), 3, 4-benzpyrene and 1, 2, 5, 6-dibenzanthracene (8.0 to 0.00195 mg.) dissolved in 0.25 cc. of tricaprylin and administered as single subcutaneous injections.

The details concerning the grouping of the animals, the preparation and the dosages of the three hydrocarbon solutions, etc., are presented in the original communication (1).

The mice injected with the three hydrocarbons were examined every 4 to 5 days. The latent times were estimated in terms of the interval between the injection of the hydrocarbon and the original diagnosis of tumor by observation and palpation. The tumor-bearing mice were then separated, and the tumors were measured every other day with a pair of calipers. Measurements were made of the two greatest diameters to the nearest millimeter, and approximately constant pressure was used.

Growth rate was expressed in terms of a constant, $K$, which represents the proportionate increase in tumor volume per unit of time. The values of $K$ were derived by the use of equation 12 discussed by Blum (2) and reproduced as follows:

$$3 \left( \frac{\log (a_2 \times b_2) - \log (a_1 \times b_1)}{t_f - t_i} \right) = K$$

where $a$ and $b$ represent the two diameters determined at a given measurement, and $t$ represents the time at which the measurements were made. The subscripts 1 and 2 represent initial and final observations, respectively (or any two sets of measurements taken at different times). In the present study the diameters were expressed in millimeters and the time in weeks. $K$ is therefore the logarithm of the proportionate increase in tumor volume per week. Thus, a $K$ value of 0.301 would indicate a doubling of the tumor mass each 7 days. Since the earlier measurements, taken when the tumors were small, are subject to greater relative errors than the later measurements of larger tumors, two points on the smoothed growth curve for a given tumor were employed for the calculation of $K$, rather than actual initial and final measurements. For this pur-
pose, the diameter products were plotted on semilogarithmic paper against time. The majority of the plots gave an excellent fit to a straight line, but others followed a steplike course. In the latter event a straight line was drawn to represent an over-all average rate of increase, and points on this line were used in determining K. Most of the tumors were followed until they reached a size of approximately 20 by 20 mm., but some of the mice died before this size was reached. Tumors for which there were fewer than four measurements extending over a period of 1 week were not included in the analyses.

At the termination of the observation on growth, or upon the death of the animals, the local tumors were removed, fixed in Zenker's fluid, and embedded in paraffin. At least two, and in the majority of cases three sections were made through the tumor. The sections were stained with haematoxylin and eosin, and approximately half of the sections were also stained with Mallory's phosphotungstic acid-haematoxylin. No sections were

### Table 1. — Summary of data obtained with 3 carcinogenic hydrocarbons in male mice of C3H strain

<table>
<thead>
<tr>
<th>Type of tumor</th>
<th>Dose</th>
<th>Mice with tumors (total)</th>
<th>Mean latent period</th>
<th>Tumors measured for growth</th>
<th>Mean rate of growth (K)</th>
<th>Mice with multiple tumors</th>
<th>Tumors examined histologically</th>
<th>Sarcoma</th>
<th>Sarcoma with muscle-cell elements</th>
<th>Carcinoma</th>
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<th>Mean rate of growth (K)</th>
<th>Mice with multiple tumors</th>
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<td>0.44</td>
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1 See text.
2 Only the first appearing tumors of mice with multiple tumors are included.
3 With or without sarcoma.
made of other tissues except when indicated by the presence of gross abnormalities.

In the original study, 957 mice were used, and 433 mice that developed progressively growing subcutaneous masses were included in the dose-response analysis. Of the tumors from these 433 mice, 415 were examined and verified histologically. Eighteen mice, on which histologic sections were not available because the tumor was devoured by other animals or was extremely autolyzed after the death of the mouse, were included on the basis of rapid growth of the local tumor.

For the present study, therefore, 415 mice with tumors are included in the morphologic observations; and 398 mice with tumors large enough and of which measurements were made, are included in the analysis of tumor growth. The mice were distributed throughout the dose and hydrocarbon groups included in the dose-response analysis. The number of mice with tumors and the growth data for each group are given in table 1. Twenty-two of the four hundred and fifteen animals with tumors had more than one distinct tumor; a separate analysis of these animals is given subsequently. Only the tumor that appeared first was considered in the data on latent time and growth rate.

**MORPHOLOGY**

Grossly, the tumors were spheroidal, fairly well circumscribed, subcutaneous masses, of firm to fairly soft consistence. They were usually fixed to the overlying skin, which in the case of the larger tumors often was externally ulcerated. On cut section, the masses were pearl-gray in color, areas of hemorrhage and necrosis being frequently encountered in large tumors. The tumors compressed the thoracic wall and were adherent to the ribs, the humerus, and other adjacent structures. Actual penetration into the thoracic or abdominal cavities was rarely encountered. Grossly, distant metastases were not observed. The regional lymph nodes were usually encompassed and invaded by the tumor, and in one case pulmonary metastases were found upon histologic examination.

The tumors grew progressively, and the cause of death in the majority of animals was attributable to the bulk of the tumor and secondary infection owing to ulceration.

Histologically, the tumors were of the following types: sarcoma, 411, divided into 2 groups; spindle-cell sarcoma 269, and spindle-cell sarcoma with muscle elements 142; carcinoma, 2; and mixed sarcoma and carcinoma, 2.

**Sarcomas**

The sarcomas, which comprised 99 percent of the tumors, were in general spindle-celled growths, the cells varying widely within most of the neoplasms. In many instances the cells were arranged in interlacing bundles or broad sheets. Other tumors or other regions of the same tumor contained shorter and broader cells mixed with round or irregular forms and showed no characteristic structural arrangement. The cells resembled fibroblasts. They were large, elongated, with acidophilic cytoplasm, and hyperchromatic, large, oval, vesicular nuclei, and prominent nucleoli. Fine fibrogia were observed in a considerable number of sections stained with phosphotungstic acid-haematoxylin. In more pleomorphic neoplasms fibrogia could not be demonstrated. Mitoses and giant multinucleated cells were abundant in almost all of the tumors. In some tumors the blood channels were extremely

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3 Numerous illustrations of these tumors are presented by Bonser and Orr (3), Haagensen and Kreplie (4), Lewis (5), and others, and reduplication here is considered superfluous.
prominent and suggested a hemangiomaticous structure.

Necrotic, hemorrhagic, and edematous areas of various sizes were present in almost all tumors that reached 20 by 20 mm. in diameter. Some tumors contained areas of hyaline and of myxomatous degeneration. In a considerable number of tumors appearing soon after the injection of the carcinogens were round cysts lined with compressed spindle cells, probably representing localized deposits of the injected material. Invasion by round cells and polymorphonuclear leukocytes occurred to a noteworthy degree only in tumors in which surface ulceration had occurred.

The tumors were not encapsulated, and they actively invaded and destroyed contiguous structures, such as muscle, lymph nodes, ribs, and long bones. The majority of the neoplasms were fibrosarcomas arising from the fibroblasts of the subcutaneous connective tissue. Bonser and Orr (3) suggested that some of the tumors may arise from the endomysium of the panniculus carnosus or of the deeper muscles. The structural variability of the individual tumors suggests that several types of cell may undergo malignant transformation, although from morphologic evidence alone it is possible that the variants are the result of accidental differences in location, pressure, and other factors that produce alterations in appearance of essentially similar cells.

**Sarcomas With Muscle Elements**

About three-fourths of all 411 sarcomas contained occasional large eosinophilic cells with multiple nuclei, which varied in size, length, and number of nuclei. The cells were modified muscle fibers or muscle giant cells. In 142 tumors (34.5 percent) these muscle elements were sufficiently numerous and prominent to deserve separate consideration. All these tumors contained spindle cells that were intimately associated with the muscle elements, and many also contained other areas of sarcoma that were free of muscle elements. The muscle elements usually occupied the deeper portion of the tumor and in several cases could not be certainly distinguished on morphologic grounds from rhabdomyosarcomas, although no cross striations could be demonstrated.

Abnormal muscle elements in tumors induced by carcinogenic hydrocarbons in mice have attracted comment in all descriptions in the literature (3-6). The nature of these cells is not clear. Haagenesen and Krehbiel (4) and Hval (6) considered 3 of 32 tumors studied by them as rhabdomyosarcomas or leiomyosarcomas. Bonser and Orr (3) and Lewis (5), on the other hand, considered them as a reaction of injury to muscle produced by the hydrocarbon. It cannot be denied that a few of these tumors may be of muscle origin, but in the majority of cases the appearance is most probably ascribable to muscle damage. Lewis (5) showed that on transplantation of sarcomas with muscle elements, the modified muscle elements with rare exceptions disappear in later transplant generations. He suggested that either the muscle cells are lost or die out, or that they are malignant but dedifferentiate into spindle cells. In this series, metastases from one sarcoma with muscle-cell elements were found in the lungs and contained only the sarcoma cells (fig. 1, A, and B).

**Carcinomas**

Carcinomas were found in 1 percent of the tumors. In two, the total tumor was composed of carcinoma, and in two, the epithelial tumor was mixed intimately with spindle-cell sarcoma. There was no external ulceration preceding the develop-
ment of these tumors, and grossly they were not distinguishable from sarcomas.

The first tumor was a squamous-cell carcinoma that appeared 64 days after the injection of 0.5 mg. of methylcholanthrene. No sarcoma was present, and the tumor consisted of solid masses of epithelial tumor cells. Keratinization was well-marked.

The second tumor was an adenocarcinoma, consisting of solid alveolar masses of polygonal cells and indistinguishable from a type of spontaneous mammary tumor frequently encountered in females of the C3H strain of mice. No sarcomatous elements were present. The tumor arose at the site of injection 75 days after the administration of 0.5 mg. of methylcholanthrene.

The third tumor was chiefly a fibrosarcoma, but in one location extending from a hair follicle was an area of squamous-cell carcinoma, with extensions penetrating into the fibrosarcoma. This tumor arose 83 days after the administration of 1.0 mg. of methylcholanthrene.

The fourth tumor arose 92 days after the injection of 2.0 mg. of benzpyrene and was composed of spindle-cell sarcoma, in the midst of which was a high grade carcinoma. The carcinoma was not in contact with any epithelial structures. The epithelial tumor was arranged in syncytial cords surrounded by a delicate reticulum. The cords alternated with endothelial-lined blood sinuses (fig. 2).

Neoplastic epithelial tissue was not encountered in the other 411 tumors, nor were squamous cysts seen; the cysts in the tumors were lined with either compressed sarcoma cells or with connective tissue fibroblasts. The skin over the tumor was often hyperplastic and keratotic, probably owing to reaction to the hydrocarbon. Mammary tissue of the male mice, which was included in some of the sections, was often hyperplastic, whether encompassed in the tumor or peripheral to it. The second tumor de-
scribed was the only one that developed from mammary tissue.

**OTHER MASSES**

In four mice, the mass at the site of injection of the hydrocarbon consisted of hemorrhagic and necrotic tissue rather than neoplasm. These small masses showed almost no progressive increase in size and appeared in mice that received the lower doses of the hydrocarbons at a protracted period after injection (methylcholanthrene, 0.00098 mg. at 355 days, benzpyrene, 0.00195 mg. at 355 and at 375 days, and dibenzanthracene, 0.00195 mg. at 312 days).

The masses represented either late reaction to the solvent and the hydrocarbons or were early neoplasms that regressed. The second possibility is suggested by another small mass, obtained with 0.00195 mg. of benzpyrene, which was extremely hemorrhagic and necrotic but in which definite sarcoma was encountered at the periphery. Extensive hemorrhagic and necrotic areas were present in many other tumors, but they were uniformly large and often ulcerated through the skin.

**RELATIONSHIPS BETWEEN MORPHOLOGY, LATENT TIME, GROWTH RATE, AND DOSE AND TYPE OF CARCINOGEN**

The data on the latent time, growth rate, and the histologic appearance of the tumors were examined with respect to the following questions:

1. Is the latent time and/or the growth rate influenced by the histologic appearance of the tumors within each dose and hydrocarbon group?
2. Is the histologic appearance of the tumors influenced by the dose and the type of carcinogen employed?
3. Is the appearance of multiple tumors influenced by the dose or the type of hydrocarbon employed?
4. Is the growth rate of the tumors influenced by the dose and the type of hydrocarbon administered?

**LATENT TIME, GROWTH RATE, AND MORPHOLOGY WITHIN GROUPS**

The analyses pertaining to the last three questions depend upon whether the tumors in a group of animals receiving the same dose of the same hydrocarbon can be considered as a whole, or whether it is necessary to subdivide each such group into histologic types before analyses are made of the relations between the other variables. Tumors of three general histologic types were induced: sarcomas, sarcomas with muscle-cell elements, and carcinomas with or without sarcoma. Since three sections were taken, at the most, through each tumor and since there is great overlap in the designation of sarcomas with reference to the amount of muscle-cell elements they contain, the classification cannot be considered as
highly accurate. Nevertheless, it is important to determine whether these general classes of tumors behave differently with respect to induction time and rate of growth.

The tumors of each dose group of each hydrocarbon were therefore divided into the three general morphologic classes, and comparisons were made between the latent times and growth rates of the different types of tumors. Statistical analyses showed no significant differences between the latent times or between the growth rates of tumors in the two morphologic groups, sarcomas, and sarcomas with muscle-cell elements. The tumors in the third morphologic group, carcinomas, were too few for satisfactory statistical comparison, but all of the growth rates as well as the latent periods fell within the limits observed for the other groups.

A second within-group analysis of importance concerns the possible association between latent periods and rates of growth. The variations in both growth rate (see section entitled "Growth Rate, Dose, and Type of Hydrocarbon," p. 32) and latent time (7) were relatively wide within individual close groups, and no significant correlation could be demonstrated between the length of the latent period and the rate of tumor growth. Brues et al. (7) also failed to find significant differences in growth rate between the early and late appearing tumors of a single series.

It is concluded that within dose groups the histologic variations did not exert significant influence upon the latent time and growth rate, and that the growth rate was not to a significant degree dependent upon the latent period. All tumors in a given dose group, therefore, were considered as belonging to the same population of variates despite differences in the histologic type and in the latent time and growth rate.

### Morphology and Dose and Type of Carcinogen

Analysis of the types of tumor induced with the various doses of the three hydrocarbons (table 1) showed that the percent of sarcomas with prominent muscle elements is higher with the higher doses of the hydrocarbons. If the dose groups of the three hydrocarbons are arbitrarily divided at the mid-point of the effective doses, the incidences of sarcomas with prominent muscle elements are as given in table 2.

<table>
<thead>
<tr>
<th>Hydrocarbon</th>
<th>Dose (mg)</th>
<th>Total Number</th>
<th>Sarcomas with prominent muscle elements</th>
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<td>Methylcholanthrene</td>
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<td>Benzpyrene</td>
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<td>38</td>
<td>26.3%</td>
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<td>Dibenzoanthracene</td>
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It is probable that the larger doses of the hydrocarbons produce greater injury which results in greater alterations in the muscle. The resultant tumors are identical with sarcomas which do not contain muscle elements, as far as latent time and growth rates are concerned.

The four tumors consisting of, or containing carcinoma were induced with the larger doses of the more rapidly acting hydrocarbons. The latent periods were relatively short. They fall, however, within the range of variability of the latent period of sarcomas induced with the particular dose of the hydrocarbons.

In two previous series (8, 9) in which 0.1 to 3.0 mg. of the three hydrocarbons were injected in tricaprylin, the incidence of carcinomas or mixed carcinoma-sarcomas was 4 of 359 tumors (1.1 percent).
The present incidence, 0.96 percent, is not significantly different. This incidence of epithelial tumors in strain C3H males probably is dependent upon the peculiar biologic constitution of this particular inbred stock of mice. In male and female mice of NH strain studied by Strong (10) the distribution of 348 tumors induced by subcutaneous injection of 1.0 mg. of methylcholanthrene in 0.1 cc. of sesame oil was as follows: sarcomas, 209; carcinomas, 83; and mixed tumors involving both carcinoma and sarcoma, 56. Thus, the incidence of tumors consisting of, or containing carcinoma in the NH strain was 40 percent, as contrasted with 1 percent in the C3H strain.

**Multiple Tumors**

Twenty-two of the four hundred and fifteen animals with tumors had more than one separate tumor. Twenty mice had two distinct masses. Two had three masses each; of these, one was injected with 0.25 mg. and the other with 0.06125 mg. of methylcholanthrene. The distribution of the multiple subcutaneous tumors is given in table 1. All of the tumors were sarcomas, and approximately half contained muscle elements.

The data are not sufficient to establish statistically whether there is a correlation between the occurrence of multiple tumors and the dose or the type of hydrocarbon. From inspection, it is apparent that there is no significant correlation between dose and the incidence of multiple tumors in any of the hydrocarbon groups.

The incidences of mice with multiple tumors as the percent of the total number that developed tumors with each hydrocarbon are as follows:

- Methylcholanthrene: 8 of 122, or 6.5 percent
- Benzpyrene: 8 of 127, or 6.3 percent
- Dibenzanthracene: 6 of 184, or 3.3 percent

The differences are not significant statistically, but it is of interest that the trend is in order of the speed of action of the hydrocarbon. Furthermore, methylcholanthrene, the most rapidly acting compound, was the only one to produce three tumors in a given animal.

It is probable that the appearance of multiple tumors is due in part to the area with which the injected carcinogen comes in contact. This depends to a great extent upon the amount of solvent used. In this series, 0.25 cc. of tricaprylin was used in all instances except for the highest doses of methylcholanthrene and dibenzanthracene, in which 0.5 cc. was used. The incidence of multiple tumors in the whole series was 5.1 percent. In a previous study (9), 63 mice were injected with methylcholanthrene or dibenzanthracene, 0.5 and 0.1 mg. dissolved in 0.1 cc. of tricaprylin. The incidence of multiple subcutaneous tumors was 3.2 percent (2 mice).

**Growth Rate, Dose, and Type of Hydrocarbon**

Preceding analyses showed that despite variations in histologic type and latent periods between individual tumors, the tumors within each group can be considered as belonging to the same population of variates with respect to growth. The data were, therefore, examined for correlation between the growth rate of the tumors and the type and dose of hydrocarbon.

The average growth rates, in terms of $K$, are given in table 1. Figures 3, 4, and 5 show the individual tumor growth rates plotted against dose. The range of doses was wide, and a logarithmic scale was used for convenience in plotting the figures.

In the instance of dibenzanthracene (fig. 5) the variation in growth rate between dose groups is not significant as compared with the variation within groups.
The growth rates for methylcholanthrene and benzpyrene (figs. 3 and 4), however, vary significantly among the respective groups (P<0.01). The data are too variable to permit conclusions regarding the nature of the growth rate-dose regression curves, but the general trends of the data are shown in the figures by the lines connecting the group means. The trend of the methylcholanthrene data is clear cut and indicates that on the average tumors produced with the higher doses grew faster than did those produced with the lower doses. The trend of the benzpy-
rene data is not so obvious. The maximum difference between group means is much less than that found with methylcholanthrene.

It is apparent from the data on the individual observations, however, that there is great variation in the growth rate, and that many tumors induced with smaller doses of methylcholanthrene and benzpyrene grew more rapidly than did others induced with larger doses.

Brues et al. (7), who first recorded that tumors induced with higher doses of carcinogenic hydrocarbons grew more rapidly than those induced with lower doses, correlated the growth rate with the speed of response. They postulated that the growth rate of a tumor in the microscopic stage may be the main factor in determining the latent period in carcinogenesis, and that variations in latent period might be owing to the number of malignant cells originally produced. Blum (12), studying the induction of tumors by ultraviolet radiation, pointed out that both rate of tumor cell proliferation and initial number of tumor cells must affect the time required for appearance of tumors. He found it necessary to postulate an additional factor regulating the proportionality between number of cells present at a given time and number of cells produced.

The induction of tumors, both from the standpoint of incidence and the length of the latent period, is probably primarily dependent upon the magnitude of the stimulus (dosage) and upon the length of time the stimulus acts. The factor of dosage is brought out in the present investigations. The importance of the length of action of the stimulus has been reported by Andervont and Shear (13), who showed that when methylcholanthrene-cholesterol pellets are implanted in C3H male mice the percentage of tumors elicited and the latent time are directly proportional to the length of time such pellets are permitted to remain in situ. The number of malignant cells originally produced is probably of importance in determining the incidence of tumors as well as the latent period. A small number of malignant cells may not become established and develop into a gross tumor, or the local or general resistance of the host may overcome the process. The latter is suggested by the appearance of necrotic, hemorrhagic masses at the site of injection at protracted periods after the administration of extremely low doses. The masses may well be regressed tumors.

In both this series and the investigations of Brues et al. (7), the growth rate of tumors was performed measured after the gross appearance of the masses. Therefore, measurements were commenced when the tumors had reached approximately the same size. Despite this, the tumors induced with larger doses of the hydrocarbons grew more rapidly. Brues showed that the percentage of cells in mitoses was higher in these more rapidly growing tumors. This suggests that the carcinogen exerted a stimulating effect upon the tumor. It is possible, however, that the more rapid increase in tumor size with the high doses is in a larger measure owing to the addition of newly formed tumor cells as a result of the continual action of the carcinogen on surrounding tissues.

A previous study showed that the mean length of time between the detection of subcutaneous tumors by palpation and death of C3H mice, 4.36 ± 0.10 weeks, was the same whether methylcholanthrene or dibenzanthracene was used and whether the dose of the carcinogen was 0.1 or 0.5 mg. With a wider spread of dosage a trend toward longer survival may be anticipated with tumors induced with lower doses. However, the data are sufficient, if gaged against the present observations on growth, to suggest tentatively that the
death of an animal with a tumor is dependent not only upon the size of the tumor but also upon the length of time such tumor has been present in the animal. The death of the animal may be ascribable to the toxic effects of the neoplasm as well as its mechanical interference with motion and other functions.

SUMMARY

Of 415 subcutaneous tumors induced by 8.0 to 0.00195 mg. of methylcholanthrene, dibenzanthracene, and benzpyrene in male mice of the C3H strain, 411 were spindle-cell sarcomas; 2 were carcinomas, and 2 consisted of mixed carcinoma and sarcoma. One-third of the sarcomas had prominent muscle-cell elements.

Histologic variations did not exert a significant influence upon the latent time and growth rate of the tumors.

Prominent muscle-cell elements appeared more frequently in tumors elicited with the higher dose of the carcinogens. The four carcinomas appeared relatively early after the injection of the larger doses of methylcholanthrene and benzpyrene.

Subcutaneous sarcomas that were induced with larger doses of methylcholanthrene and benzpyrene grew more rapidly, on the average, than did tumors induced with smaller doses.

REFERENCES