The Effect of Steroids on Methylcholanthrene-induced Subcutaneous Tumors in C3H and BALB/c Mice*

PHILIP C. MERKER, TSUNEIO BABA,† AND KENNETH SINGER

(Division of Human Tumor Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York, N.Y.)

SUMMARY

Tumors were induced in mice with 20-methylcholanthrene for the purpose of studying the anti-cancer activity of steroids. Repeated subcutaneous injections of 20-methylcholanthrene (MC) were used to induce sarcomas in two strains of mice, BALB/c males and C3H/Jax females. Over 80 per cent of the BALB/c and 100 per cent of the C3H mice developed tumors within 90 days after the start of carcinogen injections.

When tumor growth and survival time were used as indices for drug efficacy, it was found that cortisol acetate, administered subcutaneously at a dose of 8.0 mg/kg, was ineffective in tests conducted against BALB/c mice bearing well established MC-induced tumors. Prednisone (12.5 mg/kg), prednisolone (3.125 mg/kg), hydrocortisone acetate (25.0 mg/kg), 9a-fluorocortisol (12.5 mg/kg), and 17a-ethynyl-19-nortestosterone (250.0 mg/kg) were found to be ineffective chemotherapeutic agents against well established MC-induced tumors in C3H mice.

In our laboratories, we have been studying the effects of steroids on the growth of a human epidermoid carcinoma (H.Ep. #3) transplanted to the conditioned Swiss mouse (17). In an effort to evaluate the utility of H.Ep. #3 growing in mice as a bioassay procedure for steroids, tests have also been made against a transplantable mouse mammary carcinoma (C3HBA), spontaneous mammary carcinomas of C3H/Jax mice, and methylcholanthrene-induced sarcomas growing in C3H/Jax and BALB/c mice. The purpose of this paper is to present our experiences with the chemical-induced tumors.

Shubik and Hartwell (23) have reviewed the extensive literature on carcinogenic substances. One of the recommended procedures for the production of chemical-induced tumors is the subcutaneous injection of a solution of the compound (31). It is more easily standardized than skin painting and always produces, at the site of injection, a sarcoma. However, investigations have used, in the main, animals in which tumors have been produced by skin paintings or oral feedings (3, 3, 7, 8, 22, 24, 25, 30) of carcinogens.

For the present investigation, therapy has been started after tumors were well established, and drug effects have been evaluated in relation to tumor growth and survival time of the animal.

MATERIALS AND METHODS

Two experimental series of mice were used: the first consisted of 100 BALB/c male mice weighing 20–30 gm. and less than 6 months old at the start; the second series, started approximately 8 months later, consisted of 100 C3H/Jax female mice, also weighing 20–30 gm. but less than 3 months old at the start. Animals were initially housed in groups of ten.

The carcinogen, 20-methylcholanthrene (MC), was made up as a 0.3 per cent suspension in propylene glycol. This preparation was injected subcutaneously in the back, at a dose of 0.1 ml. (0.3 mg/mouse), given twice weekly for 5 weeks (total dose, 3.0 mg/mouse). After the last injection had been given, the animals were examined once weekly for

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† Visiting Research Fellow, Sloan-Kettering Institute, 1957–1958; Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan.

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the appearance of tumors. When tumors were of measurable size, diameters were taken with vernier calipers in two directions, and body weights were recorded. Dead animals were autopsied, and, when possible, representative specimens of tumors and organs were taken for histology.

For chemotherapy experiments, groups of six mice were segregated on the basis of tumor size. An effort was made to have a mixture of equal numbers of large (>3.0 cm.), medium (2.0–3.0 cm.), and small (<2.0 cm.) tumors in each treatment and control cage. Prior to the start of therapy, mice were observed for an additional 7–14 days, during which time body weights and tumor diameter measurements were made 2–3 times a week.

All mice were housed in standard cages, bedded on wood shavings, and fed Purina Laboratory Chow (pellet) and water.

Spontaneous tumor-bearing C3H mice were obtained from Jackson Laboratories. Mice were observed for 2–3 weeks prior to therapy; they were used for chemotherapy experiments only if their tumors had doubled in size during the observation period. Then, four to six mice bearing small, medium, and large tumors were housed together. Treatment consisted of daily subcutaneous doses of steroids until death.

Transplanted tumor-bearing mice (C3HBA tumor in C3H/Jax female mice) were started on therapy 5 days after tumor fragments (ca. 15 mg.) had been implanted by trocar, subcutaneously in the axilla. An intermittent therapy regimen was used. Treatment was for 10 days, rest for 5 days, continued treatment for 10 days or until death. Steroids were prepared as suspensions or solutions in sesame oil in a concentration such that a total daily dose was contained in 0.2 ml. Injections were made subcutaneously.

RESULTS

**BALB/c mice.**—Chart 1 illustrates that a small percentage of the BALB/c mice (10 per cent) had measurable tumors at about the 60th day after the start of injections of MC, while less than 20 per cent of the experimental mice had died within this period. By the 88th day, approximately 80 per cent of the mice had developed tumors (10 per cent of these were larger than 3 cm., 25 per cent were 2.0–3.0 cm., and approximately 60 per cent were 1.0–2.0 cm. in diameter). Mortality on the 88th day was slightly less than 20 per cent; thereafter, tumor-bearing animals died more rapidly; by the 86th day, 28 per cent; 90th day, 43 per cent; 100th day, 72 per cent; and by the 114th day, over 95 per cent of the animals had died. Examination of tumor sizes in dead animals revealed that approximately 40 per cent of the tumors were >3.0 cm.; 40 per cent were 2.0–3.0 cm., and 20 per cent were 1.0–2.0 cm. in diameter. When tumor sizes in dead animals were compared with the distribution of tumor sizes in living animals, it was clearly seen that the majority of the animals died with growing tumors of a diameter of 2.0 cm. or larger; slightly less than 20 per cent of the BALB/c tumor-bearing mice died with small tumors (1.0–2.0 cm. in diameter).

In some instances, tumors weighed as much as 20 gm.; they were usually greyish-white or greyish-red in color; the surfaces of some tumors showed thick walls and were black in color. Cut surfaces showed central necrosis. The tumors were irregular in shape, firmly attached to the body wall, hard in consistency, and immobile; in some cases, especially those larger than 3.0 cm. in diameter, ulceration was present. Some tumors were well invaded to the pleural cavity, and those which invaded the peritoneal cavity showed either no contact invasion to liver and kidney, or good invasion to these organs. A complete microscopic study of every tumor was not made; however, a specimen on histological examination revealed a polymorph sarcoma composed mostly of spindle and irregularly shaped round cells. Scattered strap and racquet-type cells in disorderly arrangement were observed with eosinophilic occasional granular and finely vacuolated cytoplasm. Multinucleated giant cells were also seen. The stroma was scanty. Cross striations could not be demonstrated with special stains. The tumor was diagnosed as a rhabdomyosarcoma.

For a chemotherapy experiment, a set of six BALB/c mice bearing, on the average, 2.5–3.0-cm. tumors, was treated with daily subcutaneous in-
jects of cortisone acetate (8.0 mg/kg). Treatment was continued until death. Chart 2 illustrates the effects of therapy over a 14-day period, at which time all treated and control mice had died. In the control series, the decrease in body weight from the 3rd to the 4th days after the start of treatment was associated with a slight decrease in tumor size; during the 4th–8th days, when body weight gain was slight and erratic, tumor growth was relatively rapid. For the treated group, during the 4th–8th days, average body weight was plateaued, while the increase in tumor size fluctuated. Thereafter, body weight and tumor weight increased. In general, the treatment had no appreciable adverse effect on the growth of this tumor, and treated mice did not show an increase in survival time.

C3H/Jax mice.—In the experimental series, with C3H/Jax mice, all 100 animals developed tumorous masses (Chart 3). The first group had tumors as early as the 40th day after the start of MC injections; by the 60th–70th days, slightly less than 50 per cent; and by the 80th day, 100 per cent of the mice had developed tumors.

Approximately 10 per cent of the C3H/Jax mice were dead on the 70th day, 30 per cent by the 80th day, and over 60 per cent were dead by the 90th day. After the start of injections of MC, the 50 per cent mortality point was at approximately the 85th day (Chart 3).

Representative growth curves are presented to illustrate tumor growth and its relationship to death (Chart 4). When first detected on the 62d to 70th days, these tumors were 0.7–1.5 cm. in diameter; at death, tumors were larger than 2.5 cm., and in one case above 3.5 cm. The largest tumor was found in the animal that lived the longest, i.e., 60 days after the appearance of its tumors.

An examination of tumors in terms of size categories on the 77th day after the start of MC injections revealed the following distribution: 75 per cent, small tumors (1.0–2.0 cm.); 24 per cent, medium tumors (2.0–3.0 cm.); and no large tumors (>3.0 cm.). At death, the distribution was: 42 per cent, small; 50 per cent, medium; and 8 per cent, large tumors.

One tumor was serially passed in C3H/Jax mice. Table 1 indicates that tumor transplants grew satisfactorily. In the eighth generation, transplants grew to a slightly larger size than in the F1 transplant generation, and the survival of mice had been shortened from an average of 23.7 to 18.0 days. Histological examination of the F4 transplant revealed a rhabdomyosarcoma.

The negative effect of hydrocortisone acetate, at 25 mg/kg, on tumor growth is presented in Chart 4. Tumors were first measured at 1.0 cm. in diameter; during the 7–14 days prior to the start of chemotherapy, one tumor more than doubled in size, while two tumors barely grew. The drug did
not suppress or increase the growth of the slowly growing tumors. The rate of growth of the faster growing tumor was slowed during therapy; however, this tumor displayed a previous plateau during the 65th to 71st days. It is of interest that, prior to the death of this mouse, tumor size slightly increased rather than decreased.

The effect of prednisolone (11β, 17α, 21-trihydroxy-Δ4-pregnadiene-3,20-dione) on tumor growth is presented in Chart 4. During the 4 days prior to chemotherapy, tumors grew slightly, although the beginning of a plateau was seen for two of the tumors on the 74th day. Three types of responses were demonstrated: (a) one static tumor increased its rate of growth when therapy was started as a single daily injection regimen; (b) one tumor did not respond to the once daily dose, but dramatically increased in size when the dosage was doubled, and then plateaued, remaining static for 20 days, when the animal died; and (c) one tumor was relatively unaffected in its rapid rate of growth during the single daily treatment regimen, but was slightly slowed, at least for 5–6 days, immediately after the dose was doubled. These responses demonstrate the great difficulty one may encounter when tumor size is used as an index of drug activity.

Survival time data are presented in Table 2. They indicate that 9α-fluorocortisol (9α-fluoro-11β, 17α, 21-trihydroxy-Δ4-pregnene-3,20-dione); prednisone (17α, 21-dihydroxy-Δ4-pregnadiene-3,11,20-trione); prednisolone; hydrocortisone acetate, and 17α-ethyl-19-nortestosterone were ineffective in prolonging the survival time of MC-induced tumor-bearing mice. Untreated mice for these experiments lived, on an average, 85 days after the start of injections of MC. Average survival times for drug-treated mice were: 9α-fluorocortisol, 86 days; prednisone, 88 days; prednisolone, 95 days; hydrocortisone acetate, 90 days; and 17α-ethyl-19 nortestosterone, 82 days. Inspection of ranges of survival time indicates that one mouse in the prednisolone-treated group lived as long as 110 days. However, there was no indication that tumor growth in this animal was affected. Survival time data in Table 2 have had average survival times of 40 days or more, and individual spontaneous tumor-bearing mice have lived as long as 50–70 days after receipt in our laboratories. Therefore, it would not be valid to state that the four corticoids under test have significantly prolonged survival time of spontaneous tumor-bearing mice, even though a comparison of treated and control values for this experiment indicate that treatment may have been effective.

**DISCUSSION**

Two strains of mice, BALB/c males and C3H/Jax females, were given repeated injections of 20-methylcholanthrene to induce tumors. Although there are many reports in the literature on the induction of tumors in animals (23), relatively few contain sufficient detailed data to permit one to use one or the other in a standard testing program. Since the ultimate objective was to use induced tumors for chemotherapy, it was decided to use a procedure which would ensure that all mice develop a relatively uniform type of neoplasm. Therefore, an intensive treatment schedule of repeated injections of 20-methylcholanthrene, given twice
weekly over a period of 5 weeks, was used. The choice of multiple injections of carcinogen for tumor induction was completely arbitrary, and since essentially similar tumor incidence results were obtained by other investigators (1, 19) using a single injection of carcinogen, less vigorous tumor induction methods can also be recommended. In the studies of Burdette and Strong (1) and Saxén (19), the predominant tumor type was rhabdomyosarcoma. We consider the several histological specimens taken from both the BALB/c and C3H animals of the present study to be rhabdomyosarcomas. However, cross-striations could not be demonstrated in the tumor cells, and metastases were not available for study to support the contention that the tumors were rhabdomyosarcomas. One of the tumors arising in the C3H strain was passed serially in C3H mice, and the histological characteristics of the original tissue were still present in the fourth transplant generation. The problem of properly classifying induced sarcomas as rhabdomyosarcoma has been discussed by Lewis (16).

### TABLE 1

**THE TRANSPANTABILITY OF A METHYCHOLANTHRENE-INDUCED TUMOR ARISING IN C3H/JAX TO C3H/JAX MICE**

<table>
<thead>
<tr>
<th>GENERATION</th>
<th>NO. ANIMALS</th>
<th>SURVIVAL</th>
<th>AV. BODY WEIGHT (GM.)</th>
<th>TUMOR TAKES*</th>
<th>AV. TUMOR DIAM. (CM.) (10 DAYS)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>AV. span (days)</td>
<td>Range (days)</td>
<td>Day of implant</td>
<td>On 10th day</td>
</tr>
<tr>
<td>F1</td>
<td>10</td>
<td>23.7</td>
<td>19-33</td>
<td>17.1</td>
<td>18.4</td>
</tr>
<tr>
<td>F2</td>
<td>5</td>
<td>32.0</td>
<td>18-38</td>
<td>18.5</td>
<td>18.0</td>
</tr>
<tr>
<td>F3</td>
<td>4</td>
<td>29.0</td>
<td>26-33</td>
<td>16.4</td>
<td>18.9</td>
</tr>
<tr>
<td>F4</td>
<td>4</td>
<td>27.5</td>
<td>27-28</td>
<td>15.4</td>
<td>18.9</td>
</tr>
<tr>
<td>F5</td>
<td>5</td>
<td>18.0</td>
<td>16-20</td>
<td>16.3</td>
<td>17.5</td>
</tr>
</tbody>
</table>

* = Number of mice with tumors on 10th day/total number of mice transplanted.

### TABLE 2

**EFFECT OF STEROIDS ON SURVIVAL TIME OF C3H/JAX MICE BEARING EITHER TRANSPLANTED, SPONTANEOUS, OR METHYCHOLANTHRENE-INDUCED TUMORS**

Route of therapy: subcutaneous

<table>
<thead>
<tr>
<th>STEROID</th>
<th>DOSE (MG/KG)</th>
<th>SUBCUTANEOUS C3H/BA TUMOR [†]</th>
<th>SPONTANEOUS SUBCUTANEOUS C3H/JAX</th>
<th>METHYCHOLANTHRENE-INDUCED SUBCUTANEOUS C3H/JAX</th>
</tr>
</thead>
<tbody>
<tr>
<td>9α-Fluorocortisol</td>
<td>12.5</td>
<td>25 (20-29)</td>
<td>33 (14-67)</td>
<td>86 (76-98)</td>
</tr>
<tr>
<td>Prednisone</td>
<td>12.5</td>
<td>25 (17-28)</td>
<td>37 (31-45)</td>
<td>88 (76-98)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>25.0</td>
<td>20 (19-35)</td>
<td>28 (11-61)§</td>
<td>95 (78-110)§</td>
</tr>
<tr>
<td>Hydrocortisone acetate</td>
<td>25.0</td>
<td>20 (14-31)</td>
<td>13 (9-22)</td>
<td>90 (76-95)</td>
</tr>
<tr>
<td>17α-Ethynyl-19-nortestosterone</td>
<td>230.0</td>
<td>20 (14-31)</td>
<td>14 (2-30)</td>
<td>85 (76-99)</td>
</tr>
</tbody>
</table>

* Ranges are given in parentheses.
† 15-20 mg. tumor tissue, trocar. Therapy started 5 days after transplant.
‡ Therapy started 75 days after first injection of methylcholanthrene.
§ 3.125 mg/kg dose.
Stewart (27) described a spontaneous tumor arising in BALB/c mice as a rhabdomyosarcoma. However, induced tumors may be of multiple-cell origin because a variety of different types of cells may be directly exposed to the action of the carcinogen (26).

Examination of tumor incidence and cumulative mortality rates for both strains revealed that the differential between the time when most of the animals developed tumors and the time when most of the tumor-bearers were dead is 20–25 days. Since mice were observed for 7–10 days prior to therapy, a "terminal" chemotherapy period of approximately 10–14 days was available for drug testing. Although the majority of palpable tumors were increasing in size during this period, at times it was difficult to interpret drug effects on the basis of tumor size because of the erratic nature of the growth process. Since approximately 43 per cent of the C3H and 20 per cent of the BALB/c mice died with "small" (1.0–2.0 cm.) tumors, the nonmeasurable portions of the tumor which were growing by extension and invasion may be more important in terms of chemotherapy than the palpable masses. Although it is tempting to ascribe death only to tumor growth and invasion, it is possible that other causes, such as infection, may be contributing to the lethality (4). It is realized that an unfair burden may be placed on drugs when "terminal" mice are used in "screening" studies. Nevertheless, an experimental chemotherapy system has been developed recently which knowingly uses "terminal" mice (11). Long-term observations, extending over 60 days or more, could be made on approximately 10 per cent of the mice originally started on injections of MC.

The steroids used in the present study have been tested against a variety of animal tumors (5, 18, 28) and human cancer patients (6, 12, 13). For the present study, doses of steroids were used which are well above the usually physiological level and which have produced in our laboratories significant antitumor effects against 24-hour C3HBA transplants growing in C3H/Jax female mice. However, when therapy was started 5 days after tumor implantation and survival time was used as an index of drug activity, these doses of steroids were judged to be ineffective. Negative findings were also obtained in tests conducted against C3H mice bearing well established spontaneous tumors. Similarly, the steroids did not significantly suppress methylcholanthrene-induced tumor growth, cause tumor regression, or prolong the life of induced tumor-bearing mice. With the exception of one animal, all MC-treated tumor-bearing mice gained weight during therapy. Since full dose titrations were not performed, it is not known whether higher and more toxic doses would have been effective.

The shortcomings of using spontaneous tumors in a chemotherapy program have been adequately discussed by Scholler (20), and the pitfalls of transplanted tumors have been recently presented in separate publications by Furth (10) and Klein (15). As for carcinogen-induced tumors, it has been stated that they are not necessarily similar to spontaneous tumors on the basis of immunological (9), transplantation (29), and histological (14) studies. It has been demonstrated that well established first-generation transplants are more sensitive to chemotherapeutic agents than are spontaneous tumors (21); in the present study, with survival time used as an index of therapeutic activity, mice bearing induced tumors did not respond any differently to corticoids than mice bearing well established transplantable or spontaneous tumors.

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Prednisone, prednisolone, hydrocortisone acetate and 9α-fluorocortisol, and 17α-ethynyl-19-nortestosterone were supplied by the Endocrinology Section, Cancer Chemotherapy National Service Center, Silver Spring, Maryland.

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The Effect of Steroids on Methylcholanthrene-induced Subcutaneous Tumors in C3H and BALB/c Mice

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