Growth and Implantation of Free Tumor Cells in Ascites Tumors of Mice Previously Given Sex Hormones*

HORACE GOLDIE, MATTHEW WALKER, KENNETH CHAMBERS, and VERNEL ROBERTS

(Laboratory for Experimental Oncology and Department of Surgery, Meharry Medical College, Nashville, Tenn.)

Experimental research has firmly established the role of the female sex hormones in the carcinogenesis of mammary tumors (26), and clinical experience has demonstrated the possibility of controlling mammary and prostatic malignancies with both male and female sex hormones (21, 23). However, there is no agreement about the influence of these hormones on implantation and growth of transferable tumors in laboratory animals. Castration has resulted, by eliminating this influence, in a decreased frequency of spontaneous tumors or in a lower rate of implantation and growth of transferred tumors under certain conditions of experiments (29-25); however, the opposite effects or no effect was recorded in experiments with different techniques and different strains of mice (19, 27-30). On the other hand, increased hormonal stimulus (administration of sex hormones) either failed to affect growth characteristics of transferred mouse and rat tumors or produced contradictory results when used by various investigators (1, 2, 32, and Refs. in 5-7). It is conceivable that variations in techniques were responsible for variations in results. According to our experience (8-10, 18), free growth of tumor cells in the peritoneal exudate, their infiltration into the serosa, and growth of infiltrated cells as tissue implants may be estimated quantitatively in mice with "ascites tumors" of various strains. We have used our standard techniques for screening the effects of radioisotopes (10, 17, 18), chemical compounds (16), and hormones (11, 13-15) in ascites tumor-bearing mice. Accordingly, these techniques were applied in ascites tumor-bearing mice previously given high doses of purified sex hormones. The results are recorded below.

* This work was supported by a grant-in-aid No. CH 225B from the American Cancer Society, New York, N.Y., and by Grant No. C2080 from the National Cancer Institute, Bethesda, Md.

Received for publication May 7, 1958.

MATERIALS AND METHODS

Mice and tumor strains.—Sarcoma strains S-180 and S-37 were maintained in our laboratory by serial intraperitoneal transfers; Ehrlich Adenocarcinoma (diploid) and Krebs-2 carcinoma ascites tumor were obtained through the courtesy of Dr. Robert Straube, Argonne Laboratories, Lemont, Ill. Male Swiss Albino and CFW mice weighing 25-27 gm. were used.

Technics.—Methods of tumor cell inoculation, peritoneal fluid withdrawal, and free cell counts have been described elsewhere (8-10). Cell counts of peritoneal exudate were taken on the 5th or 6th day after intraperitoneal inoculation of 5 × 10⁷-10⁸ cells. Mitoses were counted in smears stained with acetocarmine (8). This staining resulted in the detection of early stages of division in more cells than were detected by the use of other staining methods.

Autopsies.—Autopsies of ascites tumor-bearing mice were performed on the 8th or 9th day after inoculation.

The degree of implant growth.—This was estimated by the following scale: no implants = 0; implants 1 or 2 mm. in diameter = +; 3-5 mm. in diameter = ++; 5-10 mm. in diameter = +++; more than 10 mm. = ++++

Extent of tissue invasion by free cells.—We have stated previously (8) that in female mice peritoneal tissue invasion by tumor cells followed a standard pattern of localization which varied only quantitatively. For ascites tumors in male mice we have applied the following scale of tissue invasion, which was based on the extent of tumor cell infiltration into the pancreas and into the loose connective tissue between the pancreas and the ventral surface of the spleen: no infiltration = 0; edematosous connective tissue, groups of tumor cells outside and between pancreas lobules = +; perisplenic tissue partly replaced by infiltrated tumor cells, either free or organized as tumor tissue, widely separating pancreas lobules = ++; perisplenic tissue mostly replaced by tumor encircling pancreas lobules and acini = +++; perisplenic tumor, malignant cells penetrating into pancreatic acini = ++++

Hormones.—Synthetic estrogen (diethylstilbestrol) and purified androgen (testosterone propionate) were given intramuscularly in doses of 0.05-0.25 mg., at intervals of 3 or 3 days (on Monday, Wednesday, and Friday of each week).

Ninety-five per cent of the mice survived androgen treatment; 82 per cent of the mice, androgen + estrogen; and 71 per cent, estrogen treatment. Accordingly, after preliminary experiments, more mice were used in estrogen-prepared groups. No placebo treatment was given to controls, since large doses were used in these experiments, and morphological changes of gonads were sufficient evidence of their effect. The mice were given:

1 Albino Farms, Red Bank, N.J.
2 Carworth Farms, New City, N.Y.
caged in groups of five; no fighting was observed and no environmental factors which might influence the results.

**Results**

Analysis of information provided by Table 1 and comparison of different series reveal the following:

1. The pattern of results was modified qualitatively by variations in hormonal preparation and only quantitatively by the intrinsic characteristics of the tumor strains used in this investigation.

2. Administration of six or nine higher doses (0.25–0.25) of hormones during a period of 2 or 3 weeks (series 1 and 2) did not influence consistently the concentration and the mitotic index of free tumor cells inoculated on the 4th or the 8th days of treatment.

### Table 1

**Concentration of Free Tumor Cells, Amount of Their Implant Growth, and Extent of Their Tissue (Pancreas) Infiltration in Ascites Tumors of Male Mice Pretreated**

<table>
<thead>
<tr>
<th>SERIES AND GROUP (NO. AND LETTERS)</th>
<th>STRAIN OF TUMOR</th>
<th>HORMONE, Dose (MU) X No. INJECTIONS, PERIOD OF TREATMENT (WEEKS)</th>
<th>CONCENTRATION OF FREE TUMOR CELLS (THOUSANDS/CM. MM.)</th>
<th>MITOTIC INDEX</th>
<th>AMOUNT OF IMPLANT GROWTH</th>
<th>EXTENT OF TISSUE INVASION</th>
</tr>
</thead>
<tbody>
<tr>
<td>A S-110</td>
<td>Androgen, 0.25 X 6 weeks</td>
<td>38.3 (28.8–44.8)</td>
<td>6.8 (5.8–9.8)</td>
<td>+ (++++)</td>
<td>+ (++) + (++)</td>
<td></td>
</tr>
<tr>
<td>B S-110</td>
<td>Estrogen, 0.25 X 6 weeks</td>
<td>38.6 (28.8–44.8)</td>
<td>7.4 (5.8–9.8)</td>
<td>+ (++) + (++)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C S-110</td>
<td>Alternately, 2 weeks Controls (untreated but inoculated)</td>
<td>37.6 (29.0–41.0)</td>
<td>7.1 (5.8–9.8)</td>
<td>+ (++) + (++)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Tumor inoculation on the day of the 4th injection**

| SERIES A | S-110 | Androgen, 0.25 X 6 weeks | 48.1 (35.4–59.8) | 6.8 (5.8–7.8) | + (++) + (++) |
|----------|-------|-------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation on the day of the 8th injection**

| SERIES A | S-110 | Androgen, 0.1 X 6 weeks | 57.5 (49.1–66.6) | 18.7 (9.9–19.9) | + (++) + (++) |
|----------|-------|-------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation on the 4th day after the last injection**

| SERIES A | S-110 | Androgen, 0.1 X 6 weeks | 48.4 (35.4–49.9) | 7.9 (5.8–8.8) | + (++) + (++) |
|----------|-------|-------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Alternately, 2 weeks Controls | 44.1 (38.8–49.1) | 7.1 (5.8–9.8) | + (++) + (++) |
|----------|-------|-----------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Androgen, in Series 3 | 68.6 (45.7–73.5) | 9.4 (5.7–19.9) | + (++) + (++) |
|----------|-------|------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Androgen, in Series 3 | 55.3 (42.4–69.8) | 8.8 (5.8–11.1) | + (++) + (++) |
|----------|-------|------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Androgen, in Series 3 | 61.8 (45.5–74.5) | 10.5 (5.8–18.9) | + (++) + (++) |
|----------|-------|------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Alternately, 2 weeks Controls | 51.8 (41.1–61.4) | 6.9 (5.8–7.5) | + (++) + (++) |
|----------|-------|-----------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Androgen, in Series 3 | 82.1 (65.4–98.7) | 5.8 (5.8–7.8) | + (++) + (++) |
|----------|-------|------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Androgen, in Series 3 | 74.8 (63.5–82.8) | 4.5 (3.5–6.5) | + (++) + (++) |
|----------|-------|------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Alternately, 2 weeks Controls | 84.5 (62.8–92.5) | 6.8 (4.8–7.7) | + (++) + (++) |
|----------|-------|-----------------------------|------------------|--------------|--------------|---------------------|

**Tumor inoculation 68 days after the last injection**

| SERIES A | S-110 | Alternately, 2 weeks Controls | 72.7 (63.0–78.9) | 4.2 (3.5–4.8) | + (++) + (++) |

* Twenty mice in each group. For legend to columns 6 and 7, see "Materials and Methods." Average of twenty mice and range (in parentheses) are given for each group.
3. In animals with eighteen doses of androgen (0.1 mg.) alone or alternately with estrogen (0.05 mg.) spread over 6 weeks, the tumor inoculation soon (on the 3d day) after the last injection (8A, 3C, 4A, and 4C) induced more free growth in the fluid (as reflected by tumor cell concentration and mitotic index) and tissues (implant growth and pancreas infiltration) than in groups treated with estrogen (9B and 4B) or untreated (controls, 3D and 4D) of the same series (3 and 4). The differences in the amount and extent of growth implied the increase not only of average values, but also of both variation extremes in groups 3A, 3C, 4A, and 4C as compared with other groups of the same series.

4. In mice prepared as above, the postponement of tumor inoculation to 3 weeks (series 5 and 6) considerably modified the results of inoculation, as follows: the minimum extremes of data on growth and implantation were in androgen and androgen + estrogen groups (5A, 5C, 6A, 6C) at the level of controls (5D and 6D), but the maximum extremes were as high as in series 3 and 4. As a result, the averages in groups 5A, 5C, 6A, and 6C were higher than in controls (5D, 6D) but lower than in corresponding groups of series 3 and 4. In other words, the effect of hormonal pretreatment on late tumor inoculation varied widely, as reflected by averages.

Figures 1–6 illustrate various stages of implant growth and tissue invasion which are graded in our tabulated results (Table 1) (see "Materials and Methods"). On the other hand, Figures 7–9 show trophic changes of the scrotum and the testes (spermiferous tubules) induced by hormonal pretreatment in the majority of mice in series 2–6. Extensive implantation and infiltration of tumor cells as featured by Figures 3 and 6 were found only in the animals prepared during 6 weeks with androgen or alternate androgen and estrogen injections (Groups A and C of the above series). On the contrary, hormonal treatment of inoculated mice during the 2 or 3 weeks after tumor inoculation (series 1 and 2) did not induce either significant anatomical changes in sex organs or more than slight increases in tumor cell growth and implantation, as compared with controls. Thus, in the conditions of our experiments on pretreatment with hormones before tumor inoculation, sizable abnormal findings in the gonads and the scrotum were prerequisite for increase of tumor growth and spread in hormone-prepared mice.

**DISCUSSION**

Androgen treatment, discontinued shortly before tumor inoculation, obviously prepared the host for the growth and spread of peritoneal free tumor cells from the inocula. The possibility of direct stimulation of malignant cell activity by hormones could be eliminated because of lack of tumor cell stimulation by hormonal treatment given after inoculation (series 1 and 2, Table 1) and by close similarity of tumor growth in mice treated by androgen alone or in combination with estrogen. Accordingly, the lack of sex hormone dependency in some tumor strains was not expected to be an obstacle for growth stimulation by hormonal pretreatment; this was confirmed by the results in series 3 and 4. The increase in growth and spread of ascitic free cells and implants after hormonal treatment was associated with changes in seminiferous tubules, but not with changes of a specific kind: it was recorded in cases of hypertrophic (after androgen treatment), dystrophic (after alternate injections of androgen and estrogen), and occasionally, to a lesser extent, of atrophic (estrogen effect) changes. In other words, each type of hormonal treatment had a specific effect on the gonad but a nonspecific effect on the tumor's activity. The only common denominator in the results of various treatments was presumably the disturbance of balance in the whole hormone status. It is conceivable that a synergistic combination of the anabolic effect of androgen with cortisone depression by estrogen was responsible for the high level of the growth and spread of tumor cells in animals treated with both hormones. Thus, we may suppose that hormonal imbalance resulting from the excess of androgen alone or in alternation with estrogen conditioned the peritoneal cavity (and perhaps the whole body of male mice) for ascites tumor growth.

The conception of tumor growth "conditioning" by systemic factors resulted from the work by H. S. Greene (20) on the role of systemic factors in determining the viability and characteristics of tumors. Data provided by recent research have been reviewed by Foulds (4). It was the purpose of our investigation to study this "conditioning" in special types of malignant growth designated as "ascites tumors."

**SUMMARY**

1. Two series of CFW or Swiss albino mice, each consisting of four groups with twenty animals per group, were given, 3 times weekly, either 1 mg. of testosterone, or 0.05 mg. of diethylstilbestrol, or alternate injections of both the androgen and the estrogen. Three days after the eighteenth injection (i.e., after 6 weeks of treatment), all these mice and controls (not pretreated) were
given inoculations intraperitoneally of S-180 or Krebs-2 carcinoma. On the 5th day of ascites tumor growth, the concentration of free tumor cells in the fluid and the amount of peritoneal implant growth were found to be higher (averages and ranges in the groups prepared with androgen alone or with androgen alternated with estrogen) than in the estrogen-prepared and control groups. Autopsies revealed hypertrophic changes in the scrotum and the testes (size and structure of seminiferous tubules) of the testosterone-treated animals; they were atrophic in the estrogen-treated group and dysmorphic in the group that received both hormones.

2. Only slight or no increase of growth was recorded for tumors inoculated before or after (3 weeks) completion of hormonal preparation.

3. It was concluded that extended preparation with high doses of androgen alone or of androgen alternated with estrogen induced in male mice a hormonal imbalance which conditioned, for a limited period, the peritoneal cavity for increased growth and spread of ascites tumors.

REFERENCES


Figs. 1-9.—Pretreatment of male mice with sex hormones before inoculation of ascites tumors. (See "Material and Methods.")

Figs. 1-8.—Peritoneal implants.

Fig. 1.—Control (Table 1, Series A, Group D). Nodules only (grade +).

Fig. 2.—Pretreatment with androgen (Table 1, Series B, Group A); large tumors diffusely spreading over peritoneum and liver (grade + + +).

Fig. 3.—Pretreatment with androgen and estrogen (Table 1, Series C, Group C); abundant infiltration of perichorium (grade + + + +).

Figs. 4-6.—Infiltration of pancreatic tissue with tumor cells from ascites fluid.

Fig. 4.—Infiltration of interlobular connective tissue with free tumor cells (grade +). ×120.

Fig. 5.—Intraacinar infiltration and partial disintegration of acini (grade + + +). ×120.

Fig. 6.—Complete disintegration of acini (grade + + + +). ×120.

Figs. 7-9.—Changes in seminiferous tubules. ×100.

Fig. 7.—Atrophy of spermatogenesis after estrogen treatment. ×100.

Fig. 8.—Dystrophic changes (pyknosis, atrophy of interstitial tissue); same mouse as in Figure 3. ×100.

Fig. 9.—Vigorous spermatogenesis after androgen treatment; same mouse as in Figure 2. ×100.
of the tumor systems. The regeneration test in-
phases biochemically and temporally may prove
chemical inhibition. The ability to separate these
involved two overlapping phases, dedifferentiation
those previously reported for the developing frog
recently studied in an extensive survey of biologi-
and differentiation in regenerating tissues.
A series of tumor-inhibitors and their congeners,
Worthy of note is the agreement between the
"Gellhorn" survey (8) (Table 2). Of the three
age response of the tumor systems studied in the
Amphibia, have been tested for their effects
on proper pituitary-adrenal relationships, as dem-
ing system on synthesis
do their effects
DEOXYPYRIDOXINE, and DL-ETHIONINE. On
its greater sensitivity to such antimetabolites as
azaserine, deoxypyridoxine, and DL-ethionine. On
the difference between s
on April 13, 2017. © 1959 American Association for Cancer Research.
Growth and Implantation of Free Tumor Cells in Ascites Tumors of Mice Previously Given Sex Hormones
