The Growth and Spread of Walker 256 Carcinoma in Pinealectomized Rats*

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SUMMARY

Walker 256 carcinoma was injected into the thighs of control, sham-pinealectomized, and pinealectomized Sprague-Dawley rats weighing between 40 and 50 grams. The pinealectomized group had statistically significant shorter survival times, larger tumor diameters, and more extensive metastases than the control and sham-pinealectomized groups. Pineal cells of rats that had died of tumor had more vesicular nuclei, prominent nucleoli, and more abundant cytoplasm than tumor-free animals. These results are considered to be indicative of a relationship between the pineal gland and the growth and spread of Walker 256 carcinoma in the Sprague-Dawley rat.

Within the past decade the pineal body has been elevated to the status of a possibly active gland (12). It may produce a gonad-inhibiting substance which opposes pituitary gonadotropin-stimulating hormone (17). Extracts from pineals of dogs have stimulated production of aldosterone from the zona glomerulosa of the adrenal glands (3), although pinealectomy has not produced a reverse effect (1). Another substance appears to oppose the melanin-stimulating hormone of the pituitary gland (10). These reports of pineal activity raise the possibility that it may play some role in certain diseases of unknown etiology and pathogenesis. For example, there has been no acceptable explanation for the marked variability in biologic behavior of even similar tumors in different individuals. Consequently, every possibility should be explored, including the pineal gland.

Several observations suggest that there may be a relationship between tumor growth and the pineal. The production of gonadal hypertrophy by pinealectomy (17) may be significant in light of the experimental induction of tumors by estrogen (10) and the hormone dependency of certain tumors (7). Drexler and co-workers (2) have found that the incidence of radiologically apparent pineal calcification is significantly greater in women with malignancy than in those without, in the same age group. This may suggest that individuals with atrophic changes in the pineal are more prone to develop malignant tumors, or it may suggest that pineal atrophy is enhanced by the presence of tumor in the body. However, these considerations are merely speculative, and recourse must be had to the experimental animal before elaboration of any theories.

There have been over a dozen reports on the effect of pineal injection on tumors in man, mice, and rats (9). These papers describe inhibition of tumor growth by pineal extract. Two reports from Japan (8, 11) indicate that pinealectomy accelerates the growth of sarcoma transplants in rats. However, one report from Germany (4) describes inhibition of spontaneous and inoculated carcinoma in mice subjected to pinealectomy. The present experimental project was undertaken in order to elucidate the effect of pinealectomy on growth and metastatic spread of Walker 256 carcinoma and on survival time after tumor inoculation.

MATERIALS AND METHODS

Technic of pinealectomy in the rat.—The rat is particularly suitable for pinealectomy because its pineal is quite close to the surface of the brain, although located between the cerebral and cerebellar hemispheres as in the human (5). With a little experience the procedure can be carried out in only a few minutes so that anesthesia need not be prolonged. A suitable method of anesthesia was...
found to be the intraperitoneal injection of Nembutal, 4 mg/kg. After the animal was anesthetized, the skin over the skull was shaved free of hair. A longitudinal skin incision was made down to the periosteum over the occipital and parietal bones, and a 1-cm. square segment of occipital bone was excised (Fig. 1). In older rats a dental drill was necessary to cut the bone. However, in rats up to several weeks of age scissors were adequate.

It is at this stage of the procedure that complications may ensue in the form of brisk hemorrhage from venous sinuses. The amount of hemorrhage varies considerably but usually can be controlled by gentle sponging pressure. Another method of hemostasis is electrocautery, but this introduces the possibility of damaging other parts of the brain.

After removal of the square segment of occipital bone, the triangular area between the cerebral hemispheres and the cerebellum was examined for the pineal (Fig. 2). It was readily identifiable as a minute, pale body, approximately 1 mm. in diameter and situated in the mid-line. The pineal gland of the rat is generally circular in form but occasionally may be elongated.

It is at this point that the pineal can be removed by an incision through its base; if it is not removed, the animal can be retained for the sham-operated group. After some experience with the method, the mortality rate is now about 5-10 per cent. Death from the operative procedure occurs within 2 days and is invariably due to continuing hemorrhage from the venous sinuses with formation of a large intracranial hematoma. Examination of the operative site in animals which have survived the procedure has revealed no evidence of hemorrhage or damage to brain structures.

**Experimental methods.**—For this project male Sprague-Dawley rats were used. The experiment was begun when they weighed between 40 and 50 grams. They received a standard laboratory diet. Three different groups of animals were established, each with 13 rats. The groups were designated control, sham-pinealectomy, and pinealectomy. The operative procedures on the latter two groups were carried out on the same day. Two weeks later, all three groups were given injections of Walker 256 carcinoma. The tumor was obtained from a donor rat and homogenized with 0.9 per cent NaCl in the proportion of two-thirds saline to one-third tumor mass. Each injection consisted of 0.05 cc. of this homogenate inoculated into the thigh muscle. This amount corresponds to 300,000-400,000 tumor cells.

After tumor inoculation, measurements of rat weight and the greatest tumor diameter were made every 2 days. The rats were examined in this manner until spontaneous death from tumor. Autopsies were then performed, and observations were made on the length of survival after tumor inoculation, on the number and size of metastatic tumors. Samples of the primary and secondary tumors were placed in 10 per cent formalin for sectioning. In addition, pineal glands were removed from the control and sham-pinealectomy rats, and the operative sites were examined in the sham-pinealectomy and pinealectomy groups.

Microscopic sections were made of the primary and secondary tumors, of pineal glands removed after death, and of lungs. Pineals removed during the initial operative procedures on the pinealectomy group were also examined histologically in order to establish the validity of this group. Because of the minute size of the rat pineal, the entire organ was sectioned. Hematoxylin and eosin stains were used on sections 5 μm thick.

**RESULTS**

All structures removed during the initial operative procedures in the pinealectomy group proved to be pineal glands on histologic examination (Fig. 3). Intracranial hemorrhage or brain damage were not found in either of the operated groups. The terminal event in all rats was hemorrhage from ulceration of skin overlying the expanding primary tumors. In no instance were the lungs the site of pneumonic processes. The microscopic appearance of the Walker 256 carcinoma in all groups and sites corresponded to the standard histological description of this tumor (19).

The mean survival times in days were 39.7 for the control group, 36.6 for the sham-pinealectomy, and 30.8 for the pinealectomy (Table 1). The pinealectomy survival time had a statistically significant difference from both the control (P < 1 per cent) and the sham-pinealectomy (P < 2 per cent). There was no such difference between the control and sham-pinealectomy groups. A study of tumor diameters in the three groups reveals similar results (Table 1). The mean tumor diameters in mm. were 35.5 for the control group, 33.6 for the sham-pinealectomy, and 47.3 for the pinealectomy (P < 1 per cent) groups. The mean diameters in the latter two groups were not significantly different from each other.

Metastatic tumor nodules were found in the lungs of none of the control rats, six (46 per cent) of the sham-pinealectomy rats, and ten (76.9 per cent) of the pinealectomy group (Table 1). Of further significance is the fact that the number of
lungs in any rat of the sham-pinealectomy group was under twelve, whereas in the pinealectomy group all lungs had over twelve tumor nodules. The presence or absence of metastatic masses in para-aortic lymph nodes showed a trend similar to that for the lungs (Table 1). These were present in two (15.4 per cent) of the controls, eight (61.5 per cent) of the sham-pinealectomy rats, and thirteen (100 per cent) of the pinealectomy group. The largest size of involved nodes was found in the pinealectomy group, there being three rats with nodes over 40 mm. in diameter. However, in the other two groups there were only a few rats with nodes in the 30-38 mm. range. Metastatic tumors were not found outside of the lungs and para-aortic lymph nodes.

The total body weights of the rats increased during the 6-7-week experimental period from the initial 40-50 gm. to a range of 200-420 gm. The largest increase occurred in the pinealectomized group, which had an average weight of 349 gm. at the time of death (Table 1). Terminal weights in the other two groups averaged 280 gm. for the control and 259 gm. for the sham-pinealectomy. These increases with age are comparable to those occurring in normal rats.

Striking differences were seen between the pineal glands removed before tumor inoculation and those removed from animals which had died of tumor. The former glands corresponded in size and staining with those examined in preliminary studies on the normal pineal of young rats (Fig. 8). However, pineals removed after death from tumor showed a four- to fivefold increase in size and were paler in staining (Fig. 4). Although the increased size was comparable to that seen in normal rats of the same weight, the decreased intensity of staining was abnormal.

Cells of pineals excised before tumor inoculation had dark, homogeneous nuclei and variable amounts of cytoplasm as seen in normal young and adult rats (Fig. 5). However, cells of pineals removed after death from tumor contained larger and more vesicular nuclei and considerably more cytoplasm (Fig. 6). Only in this group were nucleoli prominent.

**DISCUSSION**

The validity of these experimental results depends on the accuracy of the measurements of the different parameters. Survival time in days could be accurately assessed. Because of the situation of the primary tumors in the hind limbs, diameters could easily be measured. Further validation is provided by histologic proof that pineal bodies were removed from all of the pinealectomy group. The possibility of misinterpretation of effects produced by the operative procedure, aside from the actual removal of the pineal, was obviated by having a sham-operated group and by examining the operative fields after death.

Reduced food intake was probably not a significant factor in death of the rats because of the commensurate increase in weight with increasing age. The greater average terminal weight of the pinealectomized group, as compared with the other two, may have two possible explanations. First, this group had considerably larger masses of primary and secondary tumor. Second, there have been many reports of accelerated body growth in pinealectomized rats (9).

**TABLE 1**

RESULTS AT TIME OF SPONTANEOUS DEATH OF INOCULATION OF WALKER 256 CARCINOMA INTO PINEALECTOMIZED RATS

<table>
<thead>
<tr>
<th>Groups (15 rats in each)</th>
<th>Control</th>
<th>Sham-pinealectomy</th>
<th>Pinealectomy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survival time (days)</td>
<td>39.7±2.3</td>
<td>36.6±1.8</td>
<td>30.8±1.0†</td>
</tr>
<tr>
<td>(mean ± S.E.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor diameter (mm.)</td>
<td>85.5±1.7</td>
<td>83.6±1.5</td>
<td>47.8±3.8†</td>
</tr>
<tr>
<td>(mean ± S.E.)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung metastases (no. of rats)</td>
<td>0</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>Node metastases (no. of rats)</td>
<td>2</td>
<td>8</td>
<td>18</td>
</tr>
<tr>
<td>Av. body wt. (gm.)</td>
<td>280</td>
<td>259</td>
<td>349</td>
</tr>
</tbody>
</table>

* Pinealectomy vs. control, P < 1 per cent; pinealectomy vs. sham-pinealectomy, P < 2 per cent.
† Pinealectomy vs. control, P < 2 per cent; pinealectomy vs. sham-pinealectomy, P < 1 per cent.

The adverse effect of pinealectomy on survival time after tumor transplantation was related to early onset of skin ulceration and hemorrhage produced by accelerated growth of the primary tumor and to extensive pulmonary metastases. Of further significance is the fact that the most frequent and largest metastatic tumors occurred in the absence of the pineal, in spite of the fact that this group had the shortest survival time. Therefore these results provide a clear indication that removal of the pineal, prior to transplantation of Walker 256 carcinoma, enhances tumor growth and spread in Sprague-Dawley rats.

Only three reports describing the effects of pinealectomy on tumor growth could be found in the world literature. The first report was from Germany in 1929 (4). It described regression of spontaneous mammary carcinoma after pinealectomy and failure of transplanted mammary and sebaceous gland carcinomas to grow in pinealecto-
mized animals. The results are contrary to our own. However, different animals (mature mice) and tumor types were used. In addition, descriptions of the experimental procedures and results are cursory, with the major part of the paper being devoted to a depreciatory discussion of evolution.

In 1940 a Japanese paper (11) described marked accentuation of growth of Todo (Fujinawa) sarcoma when transplanted subcutaneously into pinealectomized adult female rats. Another Japanese report, in 1944 (8), presented similar findings. Although the results are similar to our own series, they may be erroneous because sham-operated animals were not used, there were only four rats in each group, observations were not carried on past the 16th post-transplantation day, and autopsies were not recorded. However, the present study has both confirmed and extended the claims of these Japanese workers—that growth of some experimental tumors is enhanced by absence of the pineal gland in rats.

Accentuation of tumor growth and spread by pinealectomy is in agreement with studies that have shown inhibition by injection of pineal extracts (9). In addition, our finding that pineocytes and their nuclei increase in size in the presence of tumor suggests altered pineal activity. Holmgren et al. (6) have used decrease in size of pineocytes, after injection of pineal extracts, as evidence of decreased activity. Similarly, Roth and co-workers (14) have suggested that morphological changes in pineal cells of rats exposed to continuous light or darkness are a reflection of changes in activity. Therefore the increase in size of pineocytic nuclei and cytoplasm in rats dying of tumor is probably a result of increased function. Prominence of nucleoli provides further evidence.

The influence of pinealectomy on tumor growth suggests that this gland may play some role in host resistance to tumor. Both of the Japanese papers (8, 11) postulate the idea that enhancement of tumor growth, in the absence of the pineal, is due to a functional derangement of various endocrine organs. Some support for this supposition is provided by more recent findings that the pineal gland may produce a specific anti-tumor substance. Further investigation of the relationship of the pineal to tumor growth and spread is warranted.

ACKNOWLEDGMENTS

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REFERENCES

Fig. 1.—First stage of pinesectomy operation in the rat. A square segment of occipital bone has been isolated.

Fig. 2.—Location of the pineal after removal of bone flap. Arrow points to the pineal, with cerebral hemispheres above and to the sides, and the cerebellum below, ×16.

Fig. 3.—Section of entire pineal which was removed from normal rat before inoculation of Walker 256 carcinoma (cf. with Fig. 4), ×50.

Fig. 4.—Section of entire pineal which was removed from rat after death due to Walker 256 carcinoma (cf. with Fig. 3). There is increase in size and decrease in staining intensity, ×50.

Fig. 5.—Section of pineal which was removed from normal rat (cf. with Fig. 6). The nuclear chromatin appears dense and the cytoplasm variable, ×500.

Fig. 6.—Section of pineal which was removed from rat after death due to Walker 256 carcinoma (cf. with Fig. 5). The nuclei are vesicular, nucleoli are prominent, and there is abundant cytoplasm, ×500.
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