Observations on a Liver Mitotic Stimulant Present in Tumor Tissue

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It is well recognized that the presence of a cancerous growth results in certain pathologic changes in host tissues anatomically removed from, and not involved by, the neoplastic process. These systemic alterations in the host include such changes as depression of hepatic catalase activity (7, 9, 17), anemia (22), thymic involution (20), and lymphoid hyperplasia (18, 20). There are also reports indicating the loss of ascorbic acid and cholesterol (10, 19), histologic changes (6), and possible hypofunction associated with adrenal gland enlargement. More recently, Annau et al. (1) have reported an increased mitotic rate in the livers of tumor-bearing rats and mice.

It has been postulated by some that these host changes result primarily from the excessive uptake of some nutritional element(s) by the tumor (14), and this concept appears to be substantiated in part by a number of nutritional and metabolic studies in tumor-bearing hosts (6, 15, 16, 23, 24).

On the other hand, there appears to be equally valid evidence to support the postulate that systemic changes in the host result from the elaboration of some substance(s) by the tumor (2, 7, 8, 21).

In vivo these various systemic changes are most probably related and to a certain extent dependent upon each other. More striking than their possible in vivo relationships, however, is their mutual dependence upon the presence of a tumor for their initiation.

The following study is an attempt to explore more fully certain facets of this tumor-host relationship. Since increased mitotic activity is so closely associated with the malignant process itself, investigation of this aspect of the problem seemed particularly interesting, and the present experiments were designed primarily to study the tumor properties which result in an increased mitotic rate in the liver tissue of cancerous animals.

MATERIALS AND METHODS

Male and female strain C3H/He and C3Hf/He mice, 2–8 months old, were used as both donor and host animals. The tumor used throughout the experiments was the transplantable mammary tumor C3HBA. Both donor and host animals were killed by exsanguination, and all the procedures, except the 6-hour and 12-hour time interval studies, were carried out between 9:00 A.M. and 11:00 A.M. Normal mice of comparable strain, sex, and age, killed and examined in the same manner as the treated groups, served as controls.

In all cases liver tissue for microscopic examination was taken from the left lobe of the liver, placed immediately in 10 per cent formalin fixative, imbedded in paraffin, sectioned at 5 μ, and stained lightly with hematoxylin and eosin. The microscopic procedure consisted of examining 25 different fields at a magnification of 430X. The total number of mitoses observed in the parenchymal cells in these 25 fields was considered the mitotic index of the specimen.

The first experimental group consisted of a number of mice implanted subcutaneously with 0.1 ml. of mammary tumor C3HBA. These animals were killed, and their livers examined as previously described when their tumors attained sizes ranging from 0.3 gm. to 9.9 gm.

For other test series, tissues removed from the donor animals were prepared and injected in the following manner:

Breis.—Tumor breis were prepared by passing the tissue through a tissue press, sieve size, 0.8 mm. Four-tenths ml. of the resulting tissue breis was injected subcutaneously in the back of each recipient. For comparison, separate homogenates of normal tissues (liver, kidney, and embryo) were also prepared and injected in the same manner. The recipient animals were sacrificed at 12, 24, 48, 72, 96, and 120 hours after injection for determination of the liver mitotic index.

In another test series tumor breis were injected subcutaneously in the same manner as the treated groups, served as controls.

TISSUE FRACTIONS.—The saline supernatant fraction of tumors weighing 2–8 gm. was prepared by homogenization for 2 minutes with an equal volume of chilled 0.85 per cent saline solution in a chilled Waring Blender. The brei was centrifuged at 20,000 × g for 10 minutes at 5°C. A plug of sterile cotton was placed over the surface of the brei before centrifugation to help carry down the cells and cellular debris. The resulting supernatant was recentrifuged at the same speed for the same length of time, and 1 ml. of the resulting supernatant was injected subcutaneously into the back of each host animal.
supernatant was examined microscopically for intact cells, and a number of animals receiving injections of 1 ml. of the supernatant were observed for 60 days for tumor incidence.

The nucleoprotein fraction, isolated by the streptomycin technic (18), and the nuclear, mitochondrial, and microsomal fractions, isolated by the sucrose method (11), were also prepared from CSHBA mammary tumor tissue. Each host animal received 1.0 ml. of one of the tissue fractions by subcutaneous injection (equivalent to 0.4 cc. of original tissue). All the animals treated with the various tissue fractions were killed at 48 hours for determination of the liver mitotic index.

In a final study, 1.0 ml. of the saline extract previously described was injected subcutaneously into the host animals, which were killed for liver examination on the 1st through the 4th day after injection.

RESULTS

Mitotic counts done on sections of liver tissue obtained from normal adult strain C3H/He and C3Hf/He male and female mice revealed that liver cell mitoses were extremely rare. In contrast, numerous mitotic figures were observed on the liver sections obtained from strain C3H/He and C3Hf/He male and female mice bearing transplanted mammary carcinoma CSHBA. The correlation between tumor size and the number of mitotic figures observed is shown in Table 1.

TABLE 1

<table>
<thead>
<tr>
<th>Tumor size (gm.)</th>
<th>Host liver mitotic index</th>
<th>Av. mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-1</td>
<td>0.0, 5.1, 18.19</td>
<td>10.3</td>
</tr>
<tr>
<td>1-2</td>
<td>25.5, 5.1, 18.19</td>
<td>16.8</td>
</tr>
<tr>
<td>2-3</td>
<td>31.5, 5.1, 18.19</td>
<td>21.2</td>
</tr>
<tr>
<td>3-4</td>
<td>11.4, 5.1, 18.19</td>
<td>6.2</td>
</tr>
<tr>
<td>4-5</td>
<td>0.0, 5.1, 18.19</td>
<td>3.7</td>
</tr>
<tr>
<td>5-6</td>
<td>0.0, 5.1, 18.19</td>
<td>0.3</td>
</tr>
<tr>
<td>6-7</td>
<td>4.0</td>
<td>1.3</td>
</tr>
<tr>
<td>7-8</td>
<td>0.0</td>
<td>0.5</td>
</tr>
<tr>
<td>8-9</td>
<td>1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>9-10</td>
<td>0.0</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Tumors weighing 0-1.0 gm. are capable of eliciting this liver cell response. Their effectiveness, 10.3 mitotic figures per specimen, was, however, considerably less than that of 2.0-3.0-gm. tumors, which resulted in a mean liver mitotic index of 31.2 with a P value of less than 0.01. This ability to increase the liver mitotic index appeared to decline gradually after the tumors reached a weight of 8 gm. or more, since the mean mitotic index for tumors weighing 5-9.9 gm. was approximately 0.26.

The next series of tests was designed primarily to determine whether established tumor growth, or simply the presence of tumor tissue, was implicated in this host response. Following the injection of 0.4 ml. of a mammary tumor brei, examination of liver specimens from the treated recipients obtained 1 through 5 days after treatment revealed a slight increase in the liver mitotic index in 24 hours (mean value, 1.6). A marked rise in the liver mitotic index to a mean value of 18 occurred in 48 hours and was followed by a gradual decline to a mean value of 5.2 from the 9th through the 5th day after injection. The P value of the comparison between the 24- and 48-hour mitotic indices was less than 0.01. In contrast, the subcutaneous injection of a similar amount of normal tissue brei (liver or kidney or embryo) did not produce a significant increase in the liver mitotic index of the treated mice at 48 and 72 hours (Table 2).

Since these results indicated that the observed host liver mitotic response was elicited only by tumor tissue and was due primarily to the presence rather than the establishment and growth of the malignant cells, the next experimental step was designed to determine whether the injection of various concentrations of tumor brei would result in a concomitant variation in the number of mitotic figures in the host liver. For this purpose, 0.4 ml. of a mammary tumor brei diluted with normal saline solution to concentrations of 50, 25, 12.5, and 6.25 per cent was injected subcutaneously into mice which were killed 48 hours after injection. Examination of the liver sections revealed a positive correlation between the concentration of tumor brei and the number of mitotic figures observed in the liver, ranging from a mean value of 3.1 mitoses with a P value of less than 0.01 at the brei concentration of 6.25 per cent to a mean value of 18.0 mitoses at a brei concentration of 100 per cent (Table 3).

Another group of animals was given injections of various fractions of tumor tissue in an attempt to determine whether the liver mitosis stimulating factor was associated with a particular cellular component. This test group was also killed at 48
hours for liver cell mitotic counts. As shown in Table 4, the saline extract fraction, which was markedly effective in stimulating host liver cell mitosis, resulted in a mean mitotic value of 12.6, which was significantly greater than the effectiveness of any of the other fractions at a P value of less than 0.01. By comparison, the nuclear, nucleoprotein, mitochondrial, and microsomal fractions did not significantly stimulate cell division in the host liver, and the mean mitotic value of 0.3 to 1.0 for these fractions was approximately equal to the mean mitotic value observed in mice receiving saline extract and tumor brei (Table 2).

On microscopic examination of liver sections obtained from all the test animals bearing tumors weighing 3 gm. or less in the various experimental series, parenchymal cells in all stages of the mitotic cycle were observed. Cell division was confined to the parenchymal cells, and no atypical mitoses were seen. This mitotic activity was not confined to any single anatomical portion of the liver lobe examined, although there appeared to be a greater frequency of mitotic figures around the portal areas. In all other respects the liver appeared normal. Microscopic examination of other organs of the tumor-bearing host, including brain, adrenal, pituitary, kidney, and salivary gland, failed to reveal any mitotic activity.

The liver sections obtained from animals bearing larger tumors (5 gm. and over) rarely showed mitotic activity but were remarkable because of the marked cellular infiltrate observed in the sinusoids and portal areas. This infiltrate was composed primarily of megakaryocytes, cells of the rubricytic series, and numerous lymphocytes and polymorphonuclear leukocytes. The extramedullary hematopoiesis, which was uniformly present in the livers of animals bearing tumors of 5 gm. or larger, was not observed in normal mice or in mice bearing tumors weighing 4 gm. or less.

### TABLE 3
MITOTIC INDEX IN THE HOST LIVER 48 HOURS AFTER THE SUBCUTANEOUS INJECTION OF 0.4 ML. OF TUMOR BREI

<table>
<thead>
<tr>
<th>Per cent brei injected</th>
<th>Host liver mitotic index</th>
<th>Av. mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.25</td>
<td>5.8,6,4,3,2,2,0</td>
<td>5.1</td>
</tr>
<tr>
<td>12.5</td>
<td>3,11,3,8,6,5,8,1</td>
<td>5.7</td>
</tr>
<tr>
<td>25.0</td>
<td>0,15,18,5,0,10,4,1,15</td>
<td>10.3</td>
</tr>
<tr>
<td>50.0</td>
<td>10,15,18,15,5,18,7,4,10</td>
<td>18.4</td>
</tr>
<tr>
<td>100.0</td>
<td>34,35,4,4,4,16,51,1,2,4,0,1,1,1</td>
<td>15.0</td>
</tr>
</tbody>
</table>

### TABLE 4
EFFECT OF INJECTION OF VARIOUS FRACTIONS OF TUMORS ON HOST LIVER MITOTIC INDEX 48 HOURS AFTER INJECTION

<table>
<thead>
<tr>
<th>Fraction injected</th>
<th>Host liver mitotic index</th>
<th>Av. mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>0.0,0,0,0,0,0,0,0,0,0</td>
<td>0.6</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.58</td>
</tr>
<tr>
<td>Nucleoprotein</td>
<td>4.1,1,0,0,3,0,0,0,0,0,0</td>
<td>0.73</td>
</tr>
<tr>
<td>Nuclear</td>
<td>1.1,1,0,0,0,0,0,0,1,0,0,0</td>
<td>1.0</td>
</tr>
<tr>
<td>Saline extract</td>
<td>1.3,4,14,56,10,24,0,0,0,0,1,0,1</td>
<td>18.6</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>0.1,1,0,0,0,0,0,0,1,0,0,0,0</td>
<td>0.31</td>
</tr>
<tr>
<td>Untreated controls</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.47</td>
</tr>
</tbody>
</table>

the mean mitotic value observed in mice receiving normal liver tissue and in untreated control mice. Microscopic examination of the saline extract rarely revealed intact tumor cells, and only 25 per cent (ten out of 40) of the animals treated with this saline extract and observed for 60 days developed tumors. In contrast, 100 per cent (40 out of 40) mice treated with a 0.1 per cent tumor brei developed tumors.

In the last test group, mice receiving subcutaneous injections of 1.0 ml. of a saline extract of tumor tissue were killed at daily intervals on the 1st through the 5th day after treatment to determine whether the host liver mitotic response would be influenced differently by the extract as compared with the brei form of the mitotic stimulant. As previously observed, the injection of equivalent amounts of saline extract and tumor brei resulted in a maximum increase in the mean value of the liver mitotic index at 48 hours. However, it would appear that the saline extract form of the stimulant resulted in a slightly greater increase in the liver mitotic rate in 24 hours and a more precipitous decline in effectiveness after 48 hours (Table 5) than did the injection of tumor brei (Table 2).

### TABLE 5
LIVER MITOTIC INDEX AT VARIOUS TIME INTERVALS AFTER THE SUBCUTANEOUS INJECTION OF 1.0 ML. OF A SALINE EXTRACT OF TUMOR TISSUE

<table>
<thead>
<tr>
<th>Time after injection</th>
<th>Host liver mitotic index</th>
<th>Av. mitotic index</th>
</tr>
</thead>
<tbody>
<tr>
<td>6 hr.</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.4</td>
</tr>
<tr>
<td>12 hr.</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.3</td>
</tr>
<tr>
<td>1 day</td>
<td>18,1,1,4,0,0,0,0,0,0,0,0</td>
<td>3.0</td>
</tr>
<tr>
<td>2 days</td>
<td>14,1,5,1,5,10,10,14,6,24,0,0,0,1,12,5</td>
<td>12.6</td>
</tr>
<tr>
<td>3 days</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.3</td>
</tr>
<tr>
<td>4 days</td>
<td>0.0,0,0,0,0,0,0,0,0,0,0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

### DISCUSSION
It is well recognized that liver tissue, which has the capacity for regeneration, rarely shows mitotic activity in the normal adult animal (3). As summarized by Paget, the proliferative mechanism of liver tissue has also been stimulated in intact adult animals by the systemic administration of various agents (18). Some of the reports concern-
ing mitotic activity in the regenerating liver indicate that the factors important in inciting the increased rate of cell division are humoral (4). Therefore, the observation that the presence of malignant tissues anatomically remote from the host liver also results in an increase in the host liver mitotic index suggests the interesting possibility that certain humoral factors capable of stimulating liver cell division are associated with neoplastic growth.

As shown by Annau et al. (1) with a variety of tumors in both rats and mice, an increase in the host liver mitotic index was associated with the presence of a malignant growth. When the present study, with the transplanted mammary tumor C3HBA in strain C3H/He and C3H/He mice, confirmed Annau’s report, further investigation was directed primarily toward characterizing the properties associated with malignancy which were responsible for initiating the increase in the host liver mitotic index.

First, consideration was given to the possibility that a correlation between tumor size and the host liver mitotic index might be demonstrable if this phenomenon were initiated by the factors implicated in either of the two hypotheses previously advanced to explain the systemic alterations occurring in cancer-bearing animals; i.e., tumor uptake of nutritional elements or tumor elaboration of some unique substance. It seemed likely that increasing tumor size would result in both increasing tumor uptake and tumor output and that either of these forms of stimuli might be reflected by an increased rate of host liver cell division. Consequently, the positive correlation between host liver mitotic rate and tumor size observed for tumors weighing less than 3 gm. might be interpreted as a direct quantitative relationship between the tumor stimulus and the liver cell response. The absence of this positive correlation in hosts bearing tumors of 3 gm. or larger, however, suggested that this interpretation was perhaps incomplete and that additional and complex factors were involved in the in vivo tumor-host relationship.

In animals bearing large tumors, the absence of liver cell mitosis and the appearance of marked extramedullary hematopoietic activity following the fall in the mitotic rate raises the interesting question whether these two host liver changes are related.

The mitotic figures observed in the livers of all the test animals were in all stages of the mitotic cycle, indicating that the tumor acted as a mitotic stimulant rather than as a mitotic inhibitor, not only because mitosis in the normal adult liver is rare, but also because the evidence for mitotic inhibition is generally arrest at a particular mitotic phase.

On the basis of the time-dose studies, which demonstrated that the maximum increase in the host liver mitotic index occurred 48 hours after the injection of a small amount of tumor brei and was roughly proportional to the concentration of the brei, it seemed apparent that the factors important in the liver cell response were transferred with the tumor tissue. It was of particular interest to note that the brei derived from the larger tumors, which were no longer capable of eliciting an increase in liver cell mitosis in their own hosts, was as effective as the brei from the smaller tumors in stimulating the recipient’s liver mitotic rate 48 hours after injection, further implicating perhaps the complex in vitro mechanism associated with this tumor material and its role in the apparent inactivity of tumors larger than 3 gm. Since vascularization and growth of the implant were undoubtedly at a minimum during the first 24-48 hours, the period of maximum host liver mitotic response, it also seemed likely that this mitotic stimulant was contained by the tumor cells at the time of injection and did not result from the proliferative process of malignant tissue. The observation that the host liver mitotic index declined significantly after reaching a maximum response at 48 hours and did not increase again until the tumor became established in the new host seemed to indicate further that the initial mitotic stimulus resulted from some factor present in the tumor brei at the time of injection. The failure of injections of equivalent amounts of normal tissue brei (liver, kidney, embryo) to elicit an increase in the host liver mitotic index indicated that this stimulating factor was a unique property of the malignant tissue.

As shown by comparisons of the liver mitosis-stimulating ability of various morphologic cell fractions of the tumor tissue, the active factors were almost exclusively confined to the saline extract portion. Since the cell content of this saline extract was minimal and far below that determined to be effective in the tumor brei concentration studies, it would suggest that this liver mitotic stimulant was independent of the intact tumor cell. A comparison of the time interval necessary to achieve the maximum liver mitotic response in animals treated with the cell-freed (extract) as compared with the cell-bound (brei) form of the liver mitotic stimulant also revealed that the saline extract form resulted in a slightly more rapid onset and decline in effectiveness, suggesting that prior release from the tumor tissue
accelerated the rate of action of the liver mitotic stimulant.

Studies on the process of mitosis itself have revealed that it is an extremely complex process, and the rise in the host liver mitotic index resulted from the presence of a variety of carcinomas CSHBA are associated with sarcoma of cancer-bearing hosts are common to a number of organisms. Between tumor size and the liver mitotic index can, at best, be postulated. That these events did, however, result from a unique tumor product, and were not necessarily associated with the presence and growth of tumor cells, has been determined with one tumor in one strain of mice. Annau's observation that an increase in the liver mitotic index resulted from the presence of a variety of tumors in two species of cancer-bearing hosts and a limited number of observations in this laboratory indicating that liver mitosis-stimulating factors similar to those demonstrated for mammary carcinoma CSHBA are associated with sarcoma HE 0071 suggest that the active factors important in producing this increase in the liver mitotic index of cancer-bearing hosts are common to a number of tumors.

**SUMMARY**

1. An increase in the liver mitotic index and a relationship between tumor size and the liver mitotic index was observed in strains CSH/He and CSH/He mice bearing transplanted mammary carcinoma CSHBA.
2. A significant increase in the host liver mitotic index was observed 48 hours after the injection of 0.4 ml. of CSHBA mammary tumor brei. The number of mitotic figures in the host liver increased as the concentration of tumor brei injected was increased.
3. A saline extract of CSHBA mammary tumor brei also resulted in a significant increase in the host liver mitotic index at 48 hours. No increase in the host liver mitotic index was observed following the injection of the nuclear, nucleoprotein, mitochondria, and microsome cell fractions obtained from CSHBA mammary tumor tissue.
4. The subcutaneous injection of 0.4 ml. normal CSH/He and CSH/He tissue brei (liver, kidney, embryo) into normal strain CSH/He and CSH/He mice did not result in an increase in the host liver mitotic index 1 through 3 days after injection.

**REFERENCES**

1. **Annau, E.; Manginelli, A.; and Roth, A. Increased Weight and Mitotic Activity in the Liver of Tumor-bearing Rats and Mice.** Cancer Research, 11:504-7, 1951.
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