Changes in Proteolytic Enzyme Systems of Rat Tissues in Response to Heterologous Growth of Human Ovarian Tumors*

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SUMMARY

Studies were made to determine the effect of heterologous growth of a human ovarian tumor on the proteolytic enzyme system of various tissues of weanling rats conditioned with cortisone and x-radiation. Conditioning per se had no effect on the activities of the tissues studied. Significant increases were observed in both aminopeptidase and cathepsin B activities of liver, kidney, and spleen of tumor-bearing animals. Trypsin inhibitors were found in lower concentration in tumor-bearing rats, thus exhibiting an inverse relationship to cathepsin B.

The role of proteolytic enzymes in malignant tumors has been investigated to a lesser degree than that of enzymes associated with nucleic acid metabolism or the glycolytic enzyme system. Proteolytic enzymes, however, have been associated with invasiveness of tumors. Specifically, Sylvén and Malmgren (14) found positive correlations between proteinase activity and invasiveness; Glenner et al. (6) correlated high peptidase activity with invasiveness of the tumors, and Benz and Lehmann (1), using nonspecific substrates, reported that certain catheptic activities were increased in tumor-free organs of rats bearing homologous tumors.

The objective of the present study was to investigate the effects of heterologous tumor growth of human ovarian papillary serous cystadenocarcinoma cells on an aminopeptidase, a trypsin-like cathepsin B, and a trypsin inhibitor in various tissues of the rat as the host.

The use of specific substrates for the study of proteolytic enzymes offers certain advantages, since multi-enzyme patterns and ratios of different tissue enzymes can be obtained in conjunction with other specific substrates. Enzymatic activities determined by using, for example, a non-specific substrate such as hemoglobin are the sum of activities of several enzymes attacking various bonds in the same molecule. Leucine-ß-naphthylamide (L-NA) and benzoyl-arginine-ß-naphthylamide (BA-NA) were used in these studies as specific and representative substrates for an exopeptidase and an endopeptidase, respectively.

The heterologous growth of human tumors in laboratory animals overcomes some of the difficulties inherent in the study of human tumors per se, in that it assures a regular and adequate supply of human tumor material for biological and biochemical studies and permits, besides, studies on aspects of the tumor-host relationship that otherwise could not be attempted with human tumors. Moreover, knowledge of changes in enzymatic activity of non-affected organs of tumor-bearing animals would give some insight into host response to tumor growth.

MATERIALS AND METHODS

Host animals and heterologous tumors.—Weanling Sprague-Dawley rats weighing 40-50 gm. received ad libitum a standard diet of Purina chow and water to which was added Terramycin (Animal Formula, Pfizer). They were conditioned with 400 r of total-body x-radiation and two 5-mg.

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doses of cortisone acetate injected subcutaneously on the 1st and 3rd days after x-radiation. On the 4th day the conditioned rats were given an intraperitoneal injection of 10 million human ovarian papillary serous cystadenocarcinoma cells which had previously been established in tissue culture for over 30 subcultures. The tumor cells grown in tissue culture were kindly supplied by Dr. Harold Tovell and Mrs. Lilian Adams of the Tissue Culture Laboratory of the Department of Obstetrics and Gynecology, Columbia University. These tumor cells were established from a bilateral papillary serous cystadenocarcinoma of a 70-year-old woman. The primary tumor was diagnosed by Dr. M. E. Long as a Grade III papillary serous cystadenocarcinoma with areas of Grade II differentiation (15).

Control and experimental groups were as follows: Group I: normal rats; Group II: rats treated with cortisone; Group III: rats treated with x-radiation; Group IV: rats treated with cortisone and x-radiation; Group V: rats treated with cortisone and x-radiation and given implants of tumor cells; Group VI: Pair-fed controls for Groups II, III, IV, and V—normal rats whose food intake was regulated so that their weight curves were similar to those of Chart 1.

The total weight of each group was determined every 2 days. On days 7, 14, and 21 postirradiation, representatives from each group were sacrificed, and liver, kidney, spleen, and tumor excised, weighed, and analyzed for proteolytic enzymes and inhibitors.

Preparation of homogenates.—The animals were sacrificed by decapitation. The tissues: liver, kidney, spleen, and tumor were excised, weighed, washed briefly in ice-cold normal saline, and dissected free of macroscopically visible blood vessels. Adhering moisture was removed by pressing the tissues lightly between filter paper. The samples were weighed and immediately transferred to glass homogenizing tubes immersed in crushed ice. Homogenization was effected in cold water (100 mg wet tissue/ml), in a polytetrafluoroethylene homogenizer for less than 1 minute. The homogenates were centrifuged 8 minutes at 500 g.

The nitrogen content of each tissue homogenate was established by micro-Kjeldahl determinations (9).

Biochemical procedures.—Aminopeptidase was determined by the Goldbarg and Rutenburg (7) modification of the Bratton-Marshall test (3) with L-leucine-\(\beta\)-naphthylamide (L-NA) used as the substrate. The test was performed with 0.02 ml. of the homogenate for all tissues except kidney, for which 0.01 ml. was used. Colorimetric readings were expressed as activity per 0.02 mg. homogenate nitrogen. Klett readings were used, since they are adequate to show relative values. They could, however, be expressed as optical density or, from a calibration curve, be converted to \(\mu\)g. naphthylamine released (7).

Cathepsin B activity was measured against benzoyl-DL-arginine-\(\beta\)-naphthylamide-HCl (BA-NA) as the substrate by the method of Blackwood and Mandl (2). Naphthylamine released was determined as for aminopeptidase, but 1 ml. homogenate was used for all tissues. Colorimetric readings were expressed per mg. homogenate nitrogen.

Inhibition of trypsin activity of tissue homogenates was determined with BA-NA as the substrate as described by Blackwood and Mandl (9). Corrections were made for (a) the digestion of the substrate by the catheptic activity of the tissue homogenates which is generally low at the pH used and (b) the partial destruction of this catheptic activity by trypsic action.

RESULTS
Chart 1 summarizes the variations in total body weight brought about by conditioning and growth.
of the tumor. Cortisone conditioning had little effect on total body weights of rats. X-radiation, however, or cortisone plus x-radiation produced an initial loss of weight followed by a period of recovery constituting a progressive weight gain up to 21 days post-x-radiation. Tumor-bearing animals after the 10th day post-radiation showed progressive decrease in total body weight.

The weights of liver and kidney of conditioned and tumor-bearing rats, when compared with those of normal controls, were in proportion to the total body weight. Spleen, however, in all rats receiving x-radiation, was proportionally less in weight.

The initial intraperitoneal inoculation of tissue culture cells produced tumors in about 70 per cent of animals that survived 3 weeks post-irradiation with an average of 2.5 gm/animal. The majority of the tumor growth appeared in the epigastrum, porta hepatitis, along the greater and lesser curvature of the stomach and over the pancreas and in the omentum. On day 7 post-irradiation there was no evidence of tumors; on day 14, however, small nodules of tumors weighing about 0.5 gm. were observed dispersed in the peritoneal cavity. The aspect of tumor regression was not studied. Enzyme activities on tumor-bearing animals were determined from the tissues of only those animals with tumors weighing 1–2 gm. No correlation was found between size of tumor and enzyme activity.

Table 1 summarizes the data for aminopeptidase (L-NA-ase) and cathepsin B (BA-NA-ase) activities in tissue of (a) the first five experimental groups on days 7, 14, and 21; (b) the pair-fed rats for Group V; (c) conditioned rats which had been hosts to tumor cells but in which no tumors grew. The data show that conditioning did not affect L-NA-ase and BA-NA-ase activity in liver, kidney, and spleen. Pair-fed rats whose food intake was regulated so that their weight curves simulated those of Group V, as well as pair-fed controls to Groups II, III, and IV (not shown in table), revealed that the loss in body weight did not significantly affect the activity of L-NA-ase and BA-NA-ase. In addition, L-NA-ase and BA-NA-ase in

**TABLE 1**

**L-NA-ASE AND BA-NA-ASE IN TISSUES OF THE RAT**

**AVERAGE KLETT READINGS**

<table>
<thead>
<tr>
<th>Groups</th>
<th>No. Cases</th>
<th>Day</th>
<th>L-NA/0.02 mo N</th>
<th>BA-NA/mo N</th>
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<tr>
<td></td>
<td></td>
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<td>pH 7.0</td>
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<td>14</td>
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<td>10</td>
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<td>115</td>
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<td>X-radiated rats</td>
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<td>378</td>
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<tr>
<td>III</td>
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<td>14</td>
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<td>394</td>
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<tr>
<td>IV</td>
<td>10</td>
<td>14</td>
<td>108</td>
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<tr>
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<td>118</td>
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<tr>
<td>Cortisone+x-rad.+tumor cells (no tumors)</td>
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<tr>
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L: liver; K: kidney; S: spleen; T: tumor.
liver, kidney, and spleen homogenates from conditioned rats which had been hosts to tumor cells but in which no tumors grew showed no difference between this group and other control animals. Definite increases in these proteolytic enzyme activities were, however, observed in the tissues of tumor-bearing rats, on days 14 and 21, and, moreover, were statistically significant for liver and spleen (P < .001) though not for kidney (P > .025).

L-NA-ase activity in tumor-bearing rats was considerably increased in liver, kidney, and spleen 14 days post-x-radiation. On day 21 the activity in each of these tissues, though above that on day 7 was, however, less than on day 14. The tumor also showed higher activities on day 14 than on day 21. On the other hand, BA-NA-ase activity progressively increased from day 7 to day 21 in liver, kidney, and spleen. The activity was greater at pH 4.8 than at pH 6.2 or pH 7.2, whereas in tumor homogenates the highest activity was observed at pH 6.2.

The same homogenates which showed hydrolysis of BA-NA were found to inhibit the tryptic hydrolysis of BA-NA. The relative amounts of the inhibitor in liver and spleen were studied in all the experimental groups. Results are tabulated in Table 2. There was, essentially, no difference in the amount of trypsin inhibitor in any tissue of control animals on any day. On the other hand, in tumor-bearing animals there was a progressive decrease of trypsin inhibitor from day 7 to a minimum on day 21, which showed an inverse relationship to the activity of BA-NA-ase.

### DISCUSSION

It was found that conditioning by both cortisone treatment and x-radiation was necessary for the growth of heterologous tumors. About one-third of the spleen is lymphoid tissue (5); the smaller spleen found in all animals that received x-radiation may be due to the high sensitivity of lymphoid tissue to radiation (12).

Conditioning per se had little effect on the proteolytic enzyme system of the host, though there were noticeable effects on the total body weights. Tumor-bearing animals showed progressive decrease in total body weights and a concomitant increase in the proteolytic activity. The weight loss may be due to decreased ingestion of food or to metabolic changes. The fact that tumor-free animals deprived of food to simulate weight curves of tumor-bearing animals showed no increased proteolytic activity eliminates the possibility that decreased ingestion of food is the cause of the increased proteolytic activity in tumor-bearing animals. Since there was no significant increase in activity in conditioned animals that received tumor cells but did not develop tumors, the increased activity of these enzymes in the tissues of tumor-bearing animals must be regarded as a host response to growth of a heterologous tumor. The effect is possibly due to metabolic changes brought about by the presence of the tumor. The increased activity is consistent with the observations of Mavor and Dunn (10), who found that the catheptic activities of spontaneous and induced hepatomas were higher than that of normal mouse liver and indicated that catheptic activity increased when normal cells became neoplastic. Greenstein and Leuthardt (8) also reported that, while the activity of dehydropeptidase I in liver was unaffected by hepatoma, benzoyl-arginine-amidase was more active in hepatoma than in normal liver.

In tumor-bearing rats L-NA-ase activity was highest on day 14 post-x-radiation, whereas BA-NA-ase had its highest activity on day 21. It is possible that both L-NA-ase and BA-NA-ase may continue to increase in tumor-bearing animals approaching an equilibrium; but after 14 days, when the activity of BA-NA-ase was comparatively high, it may have attacked L-NA-ase, so that by the end of the experiment the mouse was probably in equilibrium with the two enzymes.

### TABLE 2

| DAY | NO. CASES | NORMAL I | CONT. II | X-RAD. III | CONT. + X-RAD. IV | PAIR-FED TO GROUP V | CONT. + X-RAD. + TUMOR CELLS (NO TUMOR) | CONT. + X-RAD. + TUMOR CELLS (TUMORS) V
<table>
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<td>77</td>
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</tbody>
</table>

L: liver; S: spleen; T: tumor.
day 21 lower levels for L-NA-ase were measured because the rate of its destruction was higher than the rate of its production.

The observed increase in BA-NA-ase in liver, kidney, and spleen of tumor-bearing animals has to be regarded as part of a system, since the same homogenates also contained proteolytic inhibitors which inhibited not only trypsin but also BA-NA-ase. Since the enzymes are more heat-labile than the inhibitors, exposure of homogenates to 60° C. for 30 minutes inactivated BA-NA-ase, whereas the inhibitors remained active. The enzymatically inactive homogenates added to fresh homogenates inhibited the enzyme activities. The inhibitor system, which may be a mixture of several inhibitors, showed similarities to the inhibitors found by Werle and Appel (16) in bovine liver. Their purified inhibitor inactivated kallikrein, trypsin, chymotrypsin, and fibrinolysin, but not cathepsin; however, the same investigators (17) later reported that, in the crude homogenates of liver from several animals including man, an inhibitor for cathepsins as well as for the above-mentioned enzymes occurred. A similar inhibitor which inactivated cathepsin C was also described by Finkenstaedt (4).

The apparent increase in proteolytic enzymes in tumor-bearing animals may, in fact, be a decrease in the concentration of the inhibitor. This is suggested from the correlation between the striking increase of BA-NA-ase activity in liver, spleen, and tumor and the decrease in proteolytic enzyme inhibitor in these tissues.

Data obtained with specific substrates such as L-NA and BA-NA can be extended to obtain multi-enzyme patterns and ratios of various enzymes in tissues of normal as well as tumor-bearing animals. Establishing complete enzyme patterns of various tissues will have the added advantage of bypassing criticisms of the use of nitrogen as a base-line, since the ratios of enzyme activities are the same whatever base-line is used. Thus, amounts of nitrogen and DNA, or dry weight would cancel out when ratios, rather than absolute values are compared.

Our findings show that one host response to tumor growth is an increased proteolytic activity in noninvolved organs like liver, kidney, and spleen. High stromal or cellular proteolytic activity in many neoplasms (8) must not necessarily be related to invasiveness. Orekhovich (11) reported that tissues of tumor-bearing animals are more susceptible to attack by proteolytic enzymes than normal tissues, and Sylvén (13) demonstrated that tumor invasion is always preceded by transformation of the tissue to be invaded. Where stromal or cellular proteolytic activity is especially low, the transformation of the tissues surrounding tumors may be due to the increased proteolytic enzymes of unaffected organs liberated into the circulatory system.

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REFERENCES

1. BENZ, G. and LEHMANN, F. E. Das Verhalten gewebespezi-
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