Protein Content and Enzymatic Assays of Interstitial Fluid from Some Normal Tissues and Transplanted Mouse Tumors*

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SUMMARY
Microscale assays are reported on interstitial fluid samples taken from solid unicentric tumor transplants. Comparison was made of the protein content and enzymatic activity of normal mouse plasma and peritoneal fluid in the same strain of mice. The cell-free tumor fluid had a higher protein content, about 5 per cent, than the peritoneal fluid. The total “over-all” dipeptidase activity per volume was increased about 40–100 times; the arginase and glutathione reductase activities were likewise increased from 5 up to 20 times, and the catheptic activity at pH 4.5 showed on average a three- to fourfold increase in activity over the corresponding normal plasma activities.

The observed increases in enzymatic activity cannot be due to a general protein retention in the tumor compartment. It is suggested that the local enzymatic increase in tumor fluids is brought about mainly by leakage of proteins from the tumor cells; this view is supported by other independent in vitro data. Some implications are mentioned with reference to the utilization of interstitial proteins for cell nutrition. The increased catheptic activity seems further to have a bearing on the extracellular proteolysis postulated to partake in the destructive activity of tumor cells.

The observations provide additional evidence that neoplastic cells are characterized by increased permeability also under in vivo conditions.

Information is scant as to the chemical composition of the interstitial fluid. In the case of the normal tissue fluid, current postulations (8, 26) are based mainly on data obtained from induced edema fluids (2), intracavitary effusions of various kinds, including those due to the presence of tumors, and foremost on data obtained from lymph

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(6, 19, 28). In the course of studies on the quantitative distribution of certain enzymes at the histological microscale (14, 15, 24), it became of interest to ascertain the possible magnitude of the enzymatic activities in the interstitial compartment of tumors. This step forms part of a project to investigate the enzymic mechanisms assumed to be associated with the destructive capacity of neoplastic growth (22–25). Sampling of interstitial tissue fluid from some transplanted human tumors was recently mentioned by Libenson and Jena (12). From electrophoretic studies
mice were thus given one glucose injection 16—18 hours before sampling, either intraperitoneally or subcutaneously at a site distant from the tumors. They concluded that these experiments were evidence of an enzymatic proteolysis. This claim is strengthened and extended by the quantitative assays reported below.

In this report, a comparison is made between quantitative data obtained on blood plasma and interstitial fluid from the peritoneal cavity of normal mice, on the one hand, and, on the other hand, with similar data obtained from "free" tumor fluids.

**MATERIALS AND METHODS**

**Tumor strains.—** Solid unicentric transplants of nonhemorrhagic mammary carcinomas propagated in male and female mice of the ABC and CBA strains, and intramuscular transplants of the hyperdiploid Ehrlich-Landschütz carcinoma grown in inbred and albino stock mice, have been investigated. Progressively growing tumors have been used, most of which were larger than 10 mm. in diameter and had acquired a necrotic center. Three additional cases of the slowly growing Harding-Passey melanoma in CBA mice are presently omitted, although the results have been in conformity with the tumors mentioned above.

As to the nature of these tumor materials, it may seem doubtful whether the mammary carcinomas have a secretory function or not; some of them do present small cysts containing clear fluid which is possibly added to the free interstitial fluid. In the case of the Ehrlich-Landschütz tumors there is no microscopic evidence of any secretory functions on the part of the tumor cells, and no cystic or hemorrhagic changes have been noted in the present cases.

**Sampling.—** Free interstitial fluid has been sampled by means of glass capillaries, 0.1—0.6 mm. in thickness, from different parts of tumors either in living anesthetized mice or, preferably, in animals just bled to death. It was previously noted that invasive and rapidly growing tumors were rich in edema-like interstitial fluid at their periphery (22—24). In such tumors, as well as in other less invasive ones, the amount of fluid can be increased by acceleration of tumor glycolysis; for details on the subsequent pH changes, cf. data by Eden et al. (7). Most of the tumor-bearing mice were thus given one glucose injection 16—18 hours before sampling, either intraperitoneally or subcutaneously at a site distant from the tumors. After exposure of the deep intramuscular side of the tumor periphery by careful blunt dissection, avoiding vascular lesions and bleedings, it was quite easy to produce, still with fine blunt instruments, small pouches in and along the friable tumor periphery. Such pouches, when left alone for a few minutes, fill up with liquid which is then sampled with the glass capillaries. Amounts varying from less than 1 µl. to 2—3 µl. were obtained at each site. A total of 10—70 (average 30) mg. fluid pooled from 20—30 capillaries was obtained from the peripheral region of most tumors previously injected with glucose; less than one-third of this volume was obtained when glucose injection was omitted. These amounts proved to be enough for all enzymic assays with the present microtechnics. Similar amounts of fluid from the necrotic tumor centers were collected by the same technic.

The sampling procedure did not allow a detailed localization of the topical origin of the free fluid; that collected at or outside the tumor periphery was mixed with some fluid from the surrounding normal tissues and perhaps also with some fluid sieving through from the tumor centers. The central tumor fluid, on the other hand, was strictly derived from the nonvascularized tumor centers. Further objections to this sampling may be raised. The blunt production of pouches damages some cells by unavoidable compression or disruption. The injection of 0.5 cc. of 10 per cent glucose solution before sampling did not, however, dilute the tissue fluid; the protein content and activity figures per µl. obtained 2—3 hours after the injection were of the same order as those obtained before injection as well as those obtained 16—18 hours after the injection, when water equilibrium should be restored. Admittedly, this point has to be more closely studied in future.

When the free liquid available at different sites had been collected, the group of glass capillaries were sealed at one end and centrifuged for 5 minutes in an International microcapillary centrifuge. The weight of the pooled and now cell-free fluid was determined; then the samples were diluted, again centrifuged, and assayed as follows.

Blood samples were taken from normal mice by heart puncture, and the plasma was separated by centrifugation. Sampling of the intraperitoneal fluid was made in normal, living, anesthetized mice by means of similar capillaries pushed into the peritoneal cavity. In this case, glucose injections were not given before sampling. Fluid from at least 20—30 mice had to be pooled to permit a complete set of assays.

**Methods of assay.**—The protein content was determined, on samples diluted 10 times, by a colorimetric micromethod (17) previously calibrated with the micro-Kjeldahl technic. The titri-
metric microtechnic of Linderström-Lang and Holter (13; cf. 24) was used for the dipeptidase assays (substrate alanyiglycine; MgSO₄ was added to a final concentration of 0.006 M; incubation time, 1 hour at 40°C). The samples had to be diluted 20–40 times to permit assay. The activity is expressed in µl 0.1 M NaOH/hour/µl intersitial fluid. The acid proteinases were assayed against a 2 per cent solution of urea-denatured bovine hemoglobin according to a method recently described (cf. 18; incubation time 90 minutes at 35°C; cysteine added to a final concentration of 0.003 M). The observed extinction increases per µl fluid under the above conditions at pH 4.5 (mixture of cathepsins) are recorded in Table 1. Some examples of pH activity curves (for experimental conditions cf. 18) are given in Chart 1.

The arginase assays were performed according to a microadaptation of Greenberg's method (10) recently described in detail (14). The activity is expressed in per cent hydrolysis of the substrate (arginine) per 10 minutes at 40°C per µl intersitial fluid. Under the experimental conditions (14) an extinction of 0.100 corresponded to 0.18 per cent hydrolysis of the substrate. A linear relationship between enzyme concentration and extinction (E) was found up to at least E = 1.5 under these experimental conditions (cf. 10). For assay of the glutathione (GSSG) reductase activity a microadaptation of Racker's method was applied (21; for details cf. 15). One unit of activity is defined as an extinction change of 0.001 per minute at 20°C. To get arginase and GSSG reductase readings within the linear part of the calibration curves, the samples had to be diluted 10–90 times before assay. All data are expressed per µl of the original test fluids.

Unfortunately, it has not as yet been possible to secure data on the concentration of different ions in these interstitial fluids; the potassium content (1) would be of particular interest.

RESULTS

General characteristics of free interstitial fluid.—The fluid obtained from the normal peritoneal cavity, normal subcutaneous and intramuscular tissue distant from tumor sites, as well as that from the periphery of transplanted tumors, was in all cases clear and hardly opalescent. At the edge of the tumors the cell-free liquid was mostly slightly yellowish, but not brownish as the edematous connective tissue sometimes appears macroscopically. These liquids never clotted, not even if they were left for hours at room temperature; this is possibly owing to lack of fibrinogen, which would not be expected to pass through normal capillary membranes. Fluid from the necrotic tumor centers, on the other hand, was mostly turbid owing to the content of detritus removable by centrifugation.

Protein content.—The total protein content of the normal interstitial fluid has previously been estimated to be about 4.5 per cent (8, 26), whereas others have supposed it to be close to that of lymph, viz., about 2.5–3 per cent (5, 6, 19). It appears from Table 1 that the protein content of normal mouse peritoneal fluid is about 8 per cent and amounts to about 5 per cent in and around the tumor transplants. The total protein figures correspond well with the Kjeldahl nitrogen figures obtained on the same fluids. Further, the nucleotide content of both the peripheral and central tumor fluids seemed negligible, since no significant absorption at 260 mµ was observed.

Total dipeptidase activity.—The observed dipeptidase activities per µl tissue fluid showed in all compartments a marked increase over the corresponding blood plasma level (Table 1). A four-fold activity increase was found in the normal peritoneal fluid. The mammary carcinoma fluid had a 40- to 60-fold increase in activity. The average dipeptidase activity of the Ehrlich-Landshütz tumor fluid was about 100 times higher than that of plasma, viz., about 25 times higher than that of the normal peritoneal fluid. Such high activity readings have previously been observed only in glandular secretions.

The dipeptidase activity of the free interstitial fluid is expected to show great regional variations in different normal tissues; thus, the average figure of the normal peritoneal fluid seems rather low, while that of cellular organs may be considerably higher—cf. the peptidase figures of the interstitial fluid in lymphatic organs as postulated by Praetorius (20) and Doyle (5).

Acid proteinase activity.—In the case of the two groups of acid proteinases (pepsin and cathepsins) the results seem less clear (cf. 18, 25). Interest is presently focused on the catheptic group. The normal peritoneal fluid showed slightly higher activity per µl at pH 4.5 than did the normal blood plasma of the same strain of mice (Table 1). The average catheptic activity was increased 2–4 times in fluid collected from the periphery as well as from the centers of transplanted tumors, but marked individual variations in activity in different tumor cases were noticed (Chart 1, C). Maximum activity readings up to an extinction increase of 1.1 per µl fluid have been obtained from central tumor regions only corresponding to about 5 times that of the pH 4.5 plasma activity of normal mice of the same inbred strain (cf. 18).
### TABLE 1
PROTEIN CONTENT AND ENZYMIC ACTIVITIES PER µL OF EXTRACELLULAR FLUIDS
(Mean values are given, with ranges shown in parentheses)

<table>
<thead>
<tr>
<th>Materials and no. cases</th>
<th>Protein content (per cent)</th>
<th>Total dipeptidase activity (µl NaOH)</th>
<th>Proteinase activity at pH 4.5* (E)</th>
<th>Arginase activity (per cent)</th>
<th>GSSG reductase activity (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse plasma (inbred male mice) (30 cases)</td>
<td>5.5–6‡</td>
<td>0.2–0.3‡</td>
<td>0.15‡</td>
<td>0.1‡</td>
<td>1.9§</td>
</tr>
<tr>
<td>Normal peritoneal fluid #</td>
<td>2.9</td>
<td>1.2 (0.9–1.7)</td>
<td>0.25 (0.30–0.30)</td>
<td>0.2 (0.1–0.8)</td>
<td>1.4</td>
</tr>
<tr>
<td>Transplanted mammary carcinoma</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periphery (7 cases)</td>
<td>5.4 (4.7–6.2)</td>
<td>16.5 (12–25)</td>
<td>0.50 (0.40–0.60)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Centers (6 cases)</td>
<td>5.5 (5.0–6.5)</td>
<td>10.7 (8–14)</td>
<td>0.60 (0.40–1.1)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Ehrlich-Landschütz carcinoma transplants</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Periphery (11 cases)</td>
<td>4.8 (3.5–6.5)</td>
<td>23.8 (12–42)</td>
<td>0.40 (0.30–0.60)</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Centers (6 cases)</td>
<td>4.9 (3.7–6.4)</td>
<td>25.9 (11–36)</td>
<td>0.50 (0.30–0.70)</td>
<td>2</td>
<td></td>
</tr>
</tbody>
</table>

*—SH added.
†—Cf. reference No. 18.
§—Cf. reference No. 15.
# Three samples of fluid pooled from a total of 70 normal inbred mice, not previously injected with glucose solution.
|| Large individual variations.

#### CHART 1
Typical activity curves of the acid proteinases in the pH range 2–6, assayed against urea-denatured bovine hemoglobin (cf. 18 and text). Cysteine added. A.—The average blood plasma activity of albino stock mice (upper curve) and that of inbred male mice of the ABC, CBA, and hybrid (DBA X CSH) strains (lower curve). B.—Examples of the lowest and highest activity curves observed in the normal peritoneal fluid of inbred mice. C.—Typical activity curves of the cell-free tumor fluid of a solid mammary carcinoma and an Ehrlich-Landschütz (ELD) tumor transplant. Fluid pooled from both peripheral and central regions. The extinctions are close to the average figures recorded in Table 1.
The activity between pH 1.8 and 3.5 was quite different. In the normal peritoneal fluid it was subject to great individual variations. In most tumor fluids this activity between pH 1.8 and 3.0 was markedly depressed and in some cases almost extinguished in contrast to the increased activity between pH 3.5 and 5. This led to a considerable change in the shape of the pH activity curve of tumor fluids as compared with normal plasma and interstitial fluid. The activity optimum occurring at pH 3.8 (Chart 1, C) corresponds to the optimum at pH 3.5 in blood plasma and peritoneal fluid. The displacement depends on interference from the increased activity peak at pH 4.5. It may further be stressed that the pH 3.5—3.8 activity optimum in tumor fluid is lacking in homogenates of washed tumor cells, which have only one catheptic optimum at pH 4.5, as previously reported (cf. 25, Fig. 4, p. 557).

Arginase and GSSG reductase activity.—The activities per µl. were of the same magnitude in the peritoneal fluid as in normal mouse plasma. The arginase activity figures of tumor fluids showed large individual variations; on the whole, the average figure was increased about 5—10 times over that of blood plasma. The average GSSG reductase activity of the tumor fluid was increased from 5 to more than 20 times that of blood plasma (Table 1).

DISCUSSION

The marked increases in protein content and enzymatic activity of tumor fluids are considered to illustrate differences in cellular physiology and texture between normal and tumor tissue. A number of factors may be jointly involved, such as differences in cell permeability and dynamics of extra- and intracellular constituents, as well as factors related to the deranged vascular supply of established tumors (9) and impaired transport of the interstitial tumor fluid. Furthermore, the relative ratio of protein and enzymic contributions from the blood plasma and from cellular sources may vary in different parts of the tumor transplants. The capillary permeability is probably increased in the vascularized tumor periphery, thus allowing plasma proteins to diffuse out more freely, while this would not seem to be the case in the tumor centers where the remaining vascular channels are lacking a connection with the circulating blood (9). In the latter region considerable contributions from cellular sources might be expected, together with a general retention of plasma proteins and a selective retardation of large-sized proteins. The enzymatic observations suggest, however, that the major part of the interstitial proteins and enzymes is derived from the tumor cells by way of "secretion," "leakage," and/or lysis (cf. 2, 11, 25). This view is supported by independent in vitro evidence.\(^1\)

A further observation in favor of a local cellular origin of the major part of the increased enzymatic activities is the parallel between the various enzymatic ratios in the tumor cells and in the free tumor fluid, respectively. Thus, the tumor cells have a high content of dipeptidases (16, 24), a moderate arginine (14) and GSSG reductase activity (13), a low content of cathepsins (16, 24, 25), and lack a peptic activity (25). This pattern largely agrees with the distribution of the enzymatic activities in the tumor fluid but not with that of plasma and normal interstitial fluid.

To clarify the significance of present enzymatic observations, it should further be emphasized that one cannot state specifically how much of the observed increases in the different enzymatic activities is due to (a) increased amounts of enzyme and/or (b) changes in the activator-inhibitor balance. It is assumed that most of the increased dipeptidase, arginase, and GSSG reductase activity is due to increased amounts of enzymes liberated from the tumor cells. In the case of the acid proteinases, however, the second explanation may be of major importance (cf. 18, 24, 25).

The remarkably high enzymic activity of the interstitial tumor fluid seems to hold interesting prospects for further comparative studies on the physiology of normal and malignant cells. The implications regarding cellular nutrition, supply and uptake of peptides and amino acids, etc., cannot yet be fully visualized. The observations may furthermore furnish an important clue to the explanation of the destructiveness of malignant cells. The increased protease activity between pH 3.5 and 5 of the interstitial tumor fluid is expected to be operative in the extracellular proteolysis of the fibrous host proteins (cf. 22—25). These problems, as well as other implications with reference to certain stromal reactions, current quantitative enzymic assays at the histological microscale (14, 15, 24), and relating to various histochemical methods for the topical localization of enzymic activity (e.g., aminopeptidases, proteinases) will be considered in more detail elsewhere.

\(^1\) Unpublished results from this laboratory by Mr. B. Holmberg. His comparison refers to cultured strain L fibroblasts and fresh ascites tumor cells incubated in Warburg flasks for 1—4 hours. Tumor cells were found to release more nucleotides than the strain L cells and increasing enzymic activities under both aerobic and anaerobic conditions (cf. 4, 37). The quantitative release of enzymic activity seemed not to be correlated to the rate of cell death as ascertained by dye tests.
ACKNOWLEDGMENTS
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REFERENCES
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