Carboxypeptidases and Carboxypeptidase Inhibitor in Tumor-bearing Animals. A Possible Blood Test for Neoplasia*

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Carboxypeptidase is here defined as that enzyme, found in a wide variety of tissues, which hydrolyzes chloracetyl-L-tyrosine, with the production of acid groups and free tyrosine. The distribution and properties of this enzyme were recently described by us (1), and it was pointed out not only that two forms of the enzyme can be detected but that there can also be detected a naturally occurring inhibitor for one of these forms.

We wish to report here deviations from normalcy that we have found in this carboxypeptidase-carboxypeptidase inhibitor system in certain animal tumors and in the blood of tumor-bearing animals. We further wish to suggest the possibility that the blood changes may serve as a qualitative test for the presence of malignancy.

EXPERIMENTAL

Details of the enzyme assay appear in a previous paper (1). In general, the assay involves a 10-minute incubation at 37° C. and pH 5.5 of the tissue in question with 0.02 M chloracetyltyrosine in the presence, where desired, of 0.0088 M cysteine. Immediately after addition of the enzyme source, and again exactly 10 minutes later, aliquots are transferred into 10 volumes of absolute alcohol, and these are titrated with alcoholic KOH, in a manner somewhat modified from the method of Grassmann and Heyde (3).

That enzyme which requires cysteine for activity is designated Carboxypeptidase a (CPa), and that enzyme which is active only in the absence of cysteine is designated carboxypeptidase b (CPb). Because of the frequent simultaneous occurrence of a CPa inhibitor (CPai), the presence of CPa is often masked. In order to separate enzyme and inhibitor, high speed centrifugation in the cold is employed. After 30 minutes' centrifugation at 18,000 X g of normal tissues homogenized with 9 volumes of distilled water, almost all the enzyme is found in the supernatant phase, and almost all the inhibitor is found in the precipitate. Details of the distribution of the four components, CPa, CPb, CPai, and CPbi, in a variety of normal rat tissues are presented in the previous paper (1).

Since it is relevant to what follows, we wish to recapitulate briefly that CPa is found in lung, spleen, liver, testis, stomach, intestine, heart, kidney, and brain, in concentrations ranging from 56 to 143 units/gm. The tissues above are arranged in order of increasing activity. Except for lung, which is essentially free of CPb, the same tissues show CPb activity, ranging from 49 to 171 units/gm. The order of activity does not necessarily parallel the CPa order of activity. In all cases, these enzyme activities are found in the supernatant phase after centrifuging. CPai is found in blood cells in relatively high quantity (ca. 180 units/ml whole blood) and also in the precipitate fraction of all normal tissues listed above, except stomach. As indicated in (2), the source of blood CPai is probably the leukocyte. CPai activity ranges from ca. 50 units/gm of lung to ca. 120 units/gm of heart. No CPbi has been detected in any normal tissues tested. Skeletal muscle has also been assayed and found free of all four factors.

Table 1 summarizes the results obtained in the assay of a variety of animal tumors. Although the CPa and CPb of all normal tissues was in the supernatant of centrifuged homogenates and the CPai was in the precipitate, the possibility that the situation might be somewhat altered in tumor tissue led us to test all fractions for all components. All tumors were assayed at as early a stage as would permit sufficient material available.

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for complete testing. When necrosis was encountered, the necrotic area was removed and not assayed.

In Table 1, approximately 20 units of enzyme inhibitor are considered the limit of accuracy of the method; smaller values are of little significance. It will be noted from the table that the tumors studied differ from the normal tissues studied in two ways. First, tumor tissue is essentially devoid of both enzymes and both inhibitors. Second, such enzyme activity as is erratically observed in tumor tissue is found in the precipitate fraction of centrifuged homogenates, rather than the supernatant fraction as in normal tissues.

Ascitic fluid also presents an interesting picture. In contradistinction to tumor tissue, including ascitic tumor cells, this fluid contains an appreciable amount of CPal (twelve specimens averaged 116 units/ml). As has been noted, this is also found in normal tissues, especially blood, but in all cases it is found in, or attached to, the cellular portion of the tissue, i.e., the precipitate. Further, in two out of twelve mice bearing ascitic tumors, significant amounts of CP6I were found in the ascitic fluid (73 and 87 units/ml, respectively). These are the only cases, out of all tissues tested, where CP6I has been found. Because of the occasional use of malignant ascitic fluid as a growth agent in tissue culture fluid in place of embryo extract, this latter was also tested for the CP components. However, extract of 12-day-old chick embryos was devoid of both enzymes and both inhibitors.

It is of some interest that the kidney, heart, brain, spleen, liver, and intestine of tumor-bearing rats behave exactly like their counterparts from normal animals. The only tissue we have found to behave differently in the case of the tumor-bearing animal is blood.

Table 2 presents data on the CPa, CPb, CPal, and CP6I of the blood of normal and tumor-bearing animals. It should here be noted, as before, that the limit of accuracy of the assay method is about 20 units. It will be seen that the contents of CPa, CPb, and CP6I are negligible in all groups of animals. However, while normal blood cells carry a considerable content of CPal, the content of CPal in the blood cells of tumor-bearing rats is quite low, and it is upon this phenomenon that we base our suggestion of a possible blood test for malignancy.

Table 2 contains the results of all assays performed on bloods of tumor-bearing animals, with the exception of five rats in which the results were considered difficult to classify because of unknown factors. Three of these five were rats in which tumors had been implanted (one Flexner-Jobling, one Walker, one Bagg) and in which the blood cells were devoid of inhibitor, but in which no tumor was grossly visible. No explanation is available for these apparent false positives, except the possibility that a more thorough search of the animal might have revealed small points of active tumor growth, perhaps metastases. No complete pathologic search was made at the time. The other two cases difficult to classify were those of two rats in which the Murphy lymphosarcoma had been implanted, had grown and ulcerated to the skin, and in which a small, firm residual piece of tissue remained at the site of implantation. These animals gave "negative" tests—i.e., their blood cells contained a normal quota of CPal. That these apparent "false negative" tests were actually true negative tests is indicated by the histological finding that the residual tissue represented a totally regressed lymphosarcoma. This finding is in line with the known tendency of this particular tumor to regress spontaneously.

It is desirable, in any test such as this, to have an indication of the constancy and duplicability of the assay method. For this purpose, seven healthy rabbits were selected, and blood was withdrawn from each a total of 5 times, over a period of 2 weeks or more. The data of Table 3 indicate not

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The table follows:

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>CARBOXYPEPTIDASE a AND b (CPa AND CPb) AND CARBOXYPEPTIDASE a AND b INHIBITOR (CPal AND CP6I) IN RAT AND MOUSE TUMORS</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. animals</td>
<td>Units/gm</td>
</tr>
<tr>
<td>CPa:</td>
<td></td>
</tr>
<tr>
<td>In supernatant</td>
<td>16</td>
</tr>
<tr>
<td>In precipitate</td>
<td>24</td>
</tr>
<tr>
<td>CPb:</td>
<td></td>
</tr>
<tr>
<td>In supernatant</td>
<td>16</td>
</tr>
<tr>
<td>In precipitate</td>
<td>24</td>
</tr>
<tr>
<td>CPal:</td>
<td></td>
</tr>
<tr>
<td>In supernatant</td>
<td>15</td>
</tr>
<tr>
<td>In precipitate</td>
<td>16</td>
</tr>
<tr>
<td>CP6I:</td>
<td></td>
</tr>
<tr>
<td>In supernatant</td>
<td>16</td>
</tr>
<tr>
<td>In precipitate</td>
<td>16</td>
</tr>
</tbody>
</table>

Activity is expressed as the mean and average deviation from the mean. Tumors represented include the rat Walker carcinoma, the rat Bagg reticulum-cell sarcoma, the rat Murphy lymphosarcoma, the rat Flexner-Jobling carcinoma, a transplanted mouse hepatoma, the mouse Ehrlich carcinoma in ascitic form, and the mouse Gardner lymphosarcoma in ascitic form. "Supernatant" and "precipitate" of the ascitic tumors refer only to the tumor cells themselves. They were centrifuged out, homogenized with water, and then separated centrifugally into the usual two fractions. The cell-free ascitic fluid itself is separately discussed in text.

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only that the assay is readily duplicable, but also that the CPal level of a given healthy animal is relatively constant.

To determine the speed with which the malignant process affects the blood picture and to measure the speed of reversion to normal after extirpation of the tumor, the following experiment was performed: Sixty Sprague-Dawley male rats were selected in the weight range of 200 ± 40 gm. Into each was implanted subcutaneously 0.2 ml. of a 10 per cent lightly ground, gauze-filtered homogenate of an actively growing Walker carcinoma. The animals were then sacrificed at selected times in groups of three, and their blood was assayed for CPal. At 7 days, when the remaining animals bore tumors of approximately 1/2 inch in diameter, all the tumors were extirpated. Again, groups of three animals were sacrificed at selected times, and their blood was assayed for CPal. The remaining 30 rats, with tumors extirpated, were kept for 30 days as a check against the thoroughness of the extirpation technic; seven tumors were observed in these animals, including five which were noted at surgery as probably inoperable. The results of the experiment are shown in Chart 1, which indicates that the blood CPal began to decrease within 48 hours after implantation and began to return to normal within 24 hours after extirpation of the tumor.

One of the prime deterrents to any claim of metabolic differences between tumor and normal tissue is the problem of securing the proper normal tissue with which to compare the tumor. This difficulty has been repeatedly emphasized and needs no further comment. In this work, our sole claim to "genuine" differences lies in the wide variety of normal and tumor tissues tested. Actually, all tumors tested, solid and ascitic, a variety of sarcomas and carcinomas, were totally devoid of CPal, a situation which is true of only stomach and skeletal muscle, among all the normal tissues studied. Further, all normal tissues except skeletal muscle contained CPa, and all except lung and skeletal muscle contained CPb; in all cases, these enzymes were to be found in the supernatant of the homogenized tissue. In contradiction, solid tumors had little enzyme activity, and, where present, it was in the precipitate. There seems from these data to be little reason to believe that the differences noted represent differences in embryonic origin, rather than true differences between normal and neoplastic tissue.

A possible explanation for the lack of activity of the tumor tissue is that the amounts of enzyme and inhibitor present may exactly neutralize each...
other and that they may be bound in some manner that prevents their separation upon homogenization and centrifugation. This possibility was tested by shaking a sample of a tumor homogenate with chloroform, a procedure which has been found (1) to destroy CPal without affecting CPA. The CHCl₃ had no effect in this case, however, so it may be concluded that the lack of enzyme and inhibitor in tumor tissue is real.

Similarly, the possibility was considered that the reason for the apparent lack of CPA in the blood cells of tumor-bearing rats is that the cells also contain an equivalent amount of CPa firmly attached to the inhibitor. However, no enzyme activity appeared when a laked solution of blood cells from a tumor-bearing rat was shaken with CHCl₃. It is therefore concluded that the apparent lack of CPAI from such cells is real.

These data are an interesting contrast to the comment of Greenstein (4) that “there seems to be little question that tumors are, in part, characterized as much by high activity of certain proteolytic enzymes as by low activity of certain oxidases.”

Our results indicate that at least one proteolytic system, far from being highly active, is decreased in activity in tumor tissue. At the same time, however, it must be pointed out that the naturally occurring inhibitor of this system is also decreased in activity. It is possible that the increased proteolytic activities in tumor tissue noted by Greenstein were simply because of loss of inhibitor. We have, in fact, some indications of such inhibitors in other proteolytic systems. The implications of such proteolytic enzyme-enzyme inhibitor systems in the cancer problem may well be profound, since such a system represents a control on protein catabolism, at least, and possibly also on synthesis.

The mechanism whereby blood cell inhibitor decreases as a tumor grows is unknown at present, but it is perhaps worthy of some conjecture. The most likely probability is that blood cells, as formed by the hematopoietic tissues, contain a normal quota of inhibitor and that tumors are able in some fashion to inactivate the inhibitor of blood cells passing through. This, of course, implies that the inhibitor decrease is much more an effect, rather than a cause, of the tumor, and so diminishes the possible importance of the growth-control hypothesis suggested above. On the other hand, the fact that other proteolytic enzymes may also have naturally occurring inhibitors leads to the possibility that one may induce, another later aggravate, the neoplastic change.

Two other thoughts for future research may be recorded here. First, the possibility exists that another proteolytic inhibitor than CPal may offer a more satisfactory blood test for malignancy. Second, although the preceding discussion indicates the likelihood that the inhibitor decrease may not be in any way causative, it would still appear worthwhile to attempt to isolate the inhibitor (or other inhibitors) and test them as therapeutic agents. If a tumor-bearing animal (or human) lacks the normal quota of inhibitor, it may be possible, by replacing the inhibitor, to arrest the malignant growth.

The clinical value of such a blood test for malignancy as is here proposed can, of course, be established only by clinical testing. Some results are presented in the succeeding paper. In addition, an attempt is being made to simplify and improve the accuracy of the carboxypeptidase assay. The present method is awkward and requires a good deal of practice before reliable results can be obtained.

SUMMARY

The enzyme-inhibitor system consisting of carboxypeptidase a (requiring cysteine), carboxypeptidase b (inhibited by cysteine), and simultaneously occurring inhibitors (CPal and CPBI) of these two enzymes has been studied in a variety of normal and tumor tissues and in the blood of normal and tumor-bearing animals. Five different solid tumors of the mouse and rat have been found to be completely devoid of both enzymes and of both inhibitors. Two ascitic tumors of the mouse have been found free of both inhibitors but have been found to have both enzymes in the precipitate and none in the supernatant of homogenates of the ascitic cells centrifuged at high-speed. Of ten different normal tissues tested, nine had both enzymes in the supernatant, eight had CPalI in the precipitate, and none had CPBI. The ascitic fluid is the only tissue or fluid in which we have detected appreciable amounts of CPBI, and this was noted only erratically.

In contrast to normal rat and rabbit blood cells, we have found the blood cells of tumor-bearing animals to be essentially free of CPAI. This is suggested as a possible test for the presence of neoplasia.

REFERENCES

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