CARCINOSTATIC LIVER FACTOR

I. EFFECT IN VITRO OF HOMOLOGOUS LIVER EXTRACT ON VIABILITY OF EHRlich ASCITES CARCINOMA CELLS

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Histopathology of human tumors has long since established that there is at the growing edge of malignant tumors frequent liberation into the surrounding tissues of isolated tumor cells, the phenomenon of the so-called disjunction. In recent years clinical studies have come to widely recognize the occurrence of tumor cells in the peripheral blood of cancer patients, often in no negligible numbers. Considering these facts, one may wonder why the actual metastasis formation does not take place more frequently than it does. It would appear that cancer cells liberated into the circulation are largely killed or otherwise disposed of before they establish themselves in some distant organ sites. The host body, at least during early stages of the disease, may be assumed to possess a mechanism by which these isolated cancer cells are destroyed. The present study has been undertaken upon the basis of this assumption.

It is self-evident at the outset that, even if such a mechanism exists, which disposes of isolated cancer cells in body fluid, the direct experimental demonstration of the fact would be extremely difficult. As a matter of fact it is a common knowledge that under ordinary conditions normal mouse blood is without effect on the viability of mouse tumor cells, showing that the concentration of the hypothetical carcinostatic factor, if sufficient to destroy few isolated cells in nature, is too low to affect such number of cells as must be used in any practicable experimental procedure. It can be supposed, however, that the carcinostatic factor in question may by produced somewhere in the body and thence thrown into the circulation, and, if so, it may be possible to obtain the factor from some normal organs in sufficient concentration to be experimentally assayable. The idea is perhaps not too far-fetched since the growth of rat hepatoma cells in tissue culture is known to be somewhat inhibited when liver extracts are added (1). Also, Herbut and Kraemer's (2) recent demonstration of the inhibition in vivo of mouse lymphosarcoma by certain heterologous liver extracts makes it advisable to examine the homologous liver extract-tumor cell interaction.

Our recent experiments showed that the above assumption may be correct. It was discovered that normal liver contains a carcinostatic factor of unexpected potency,

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which is active against homologous tumor cells *in vitro*.

**Experimental Methods**

Preliminary experiments were conducted to determine the most suitable experimental conditions, which are simple and dependable, paying special attention to the concentration and amount of tissue extracts in relation to the amount of tumor cells, to the duration of incubating the mixture and to the method of determining the effect on tumor cells. The experimental procedure finally adopted as the standard method was as follows:

Believing that perhaps tumor cells in isolated state may be more easily affected than solid tumor tissue we used the ascites form of Ehrlich carcinoma cells in our tests. Tumor ascites was aspirated 7 to 10 days after intraperitoneal injections and was divided into 2 cc portions in sterilized centrifuge tubes. Tumor cells in each tube was washed once with saline solution and sedimented.

Normal tissue extracts were prepared, unless otherwise stated, always as follows: 1 g of freshly removed tissue was homogenized with 10 cc of physiological salt solution by means of a Potter-Elvehjem homogenizer and rapidly centrifuged at 3,000 rpm for 5 minutes. The turbid supernatant was used as the tissue extract.

One of the various tissue extracts was added in 2 cc amount to the tumor cell sediment above described, the mixture well stirred to make the cell suspension even, and incubated at 37°C for 1 hour, shaking the mixture once or twice during the time. After the incubation, tumor cells were sedimented, discarding the supernatant, re-suspended in the original volume (2 cc) of normal salt solution, and injected into normal mice subcutaneously at right groin in 0.2 cc doses. Cell counts have been made unnecessary by using the overwhelmingly adequate dose of tumor cell suspension, containing some 20~30 million cells. Subcutaneous implantation in the very large dose was chosen in order to facilitate the early determination of the outcome.

Under these experimental conditions sizable tumors can be definitely recognized in 4 or 5 days after implantation in the cases of positive takes. A group of 5 to 8 mice was used for each test, and the final results were recorded at the end of 3 weeks after tumor implantation by killing all the mice and performing autopsy.

**Results**

Unequivocal and clean-cut results were obtained under the above experimental conditions, and repeated experiments consistently yielded identical outcome, which may be summarized in Table 1.

These data showed that normal liver extract alone of all kinds of tissue extracts
exhibited carcinostatic action, which was dramatically clean-cut. Liver extracts from tumor-bearing animals were inactive. We have not made critical studies on the morphological and biochemical changes produced by the oncostatic factor on tumor cells. Desultory examinations of the cells, even after their incubation with liver extract for so long as 3 hours, showed no such obvious changes as swelling or blebbing of the cytoplasm, which are known to rapidly occur in tumor cells exposed to the action of oncolytic heterologous blood serum. Also, there was no evidence of cytolysis within the period of incubation, and erythrocytes contaminating the tumor cells remained without being hemolysed. The mode of action of the carcinostatic liver factor must at the present be said to be unknown.

The demonstration of the carcinostatic liver factor opened up a vast field for further studies, some of which are already underway in this laboratory. For the present, we limit ourselves to recording a few results which may be of sufficient interest to be mentioned in passing.

**Interaction temperature.** The carcinostatic liver factor reacts with Ehrlich cells in vitro even at a low temperature. This fact became apparent when a standard extract-cell mixture was kept at the temperature of 5°C for 24 hours and cells implanted into normal mice in the usual dose. No tumor developed in any of the

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>Tumors in implanted mice</th>
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<tr>
<td>Liver (mouse)</td>
<td>None (30 mice)</td>
</tr>
<tr>
<td>Liver (N-F sarcoma-bearing mouse)</td>
<td>Large tumors in 100% (8 mice)</td>
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<tr>
<td>Kidney (mouse)</td>
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<td>Spleen (†)</td>
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<td>Skeletal muscle (†)</td>
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<tr>
<td>N-F sarcoma (†)</td>
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<tr>
<td>Liver (rat)</td>
<td>None (5 mice)</td>
</tr>
<tr>
<td>Liver (Hepatoma-bearing rat)</td>
<td>Large tumor in 100% (8 mice)</td>
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<tr>
<td>Skeletal muscle (rat)</td>
<td></td>
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<tr>
<td>Hepatoma (†)*</td>
<td></td>
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<tr>
<td>Controls (Incubated in salt solution)</td>
<td>Large tumors in 100% (20 mice)</td>
</tr>
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* Ascites hepatoma, strain AH-66F of the Sasaki Laboratory. 2.5 g (wet weight) of tumor cells was collected from tumor ascites by centrifugation and homogenized in 5 cc of distilled water, and centrifuged supernatant was used, which represented an extract 5 times more concentrated than the standard tissue extract used throughout the present series of experiments.
5 mice at the site of implantation, while control cells kept in normal salt solution at the same temperature for the same length of time produced large tumors at the site of implantation in the same individual mice.

**Incubation time.** Although we adopted the incubation of extract-cell mixture at 37°C for 1 hour in the standard method, we did obtain in one test 100% negative tumor takes after only 30 minutes’ incubation. The interaction between tumor cells and carcinostatic factor may thus be a relatively rapid one, suggesting the possibility that by reducing the number of the tumor cells in the test system the reaction may be shown to complete itself in several minutes.

**Question of bile.** Since bile is the specific product of liver, its possible role in the carcinostatic effect of liver extract cannot be ignored. The difficulty in obtaining mouse bile in sufficient amount for experimental purpose led us to use bovine bile.

25 mg of dried whole bovine bile was dissolved in 10 cc of normal salt solution and 2 cc of it was mixed with 2 cc ascites equivalent of tumor cells and incubated at 37°C for 1 hour, after which the cells were resuspended in 2 cc of salt solution and injected into 5 normal mice in 0.2 cc dose. All the mice rapidly developed tumors. When the concentration of bile was increased to 50 mg per 10 cc of salt solution and experiment conducted as above, 4 out of 5 implanted mice developed tumors.

It will be seen that in this last experiment the amount of bile contained in the solution is 10 mg per 2 cc. The total solids in 2 cc of the liver extracts of tested and proved potency was found to be around 120 mg including NaCl. If the bile were to account for the carcinostatic action of the liver extract, the bile content of the latter must amount to much over 1/12 of the total solids of the extract.

**Thermostability of the factor.** It is possible to state that the carcinostatic liver factor is highly thermostable and it suffers little loss of its activity after being autoclaved at 120°C under 15 lb pressure for 10 minutes.

The clear solution, only slightly tinged with yellowish brown, obtained by autoclaving liver extract and centrifuging off the coagula, was found to be as active as the original fresh extract, that is, 2 cc of it mixed with 2 cc ascites equivalent of tumor cells and incubated at 37°C for 1 hour completely robbed the cells of tumor producing ability as found when implanted into susceptible mice.

Fractionation of liver extract freed of heat-coagulable matter is in progress.

**DISCUSSION**

It has long been known that blood sera of certain animal species show lytic action on heterologous tumor cells when mixed in test tubes. For instance, tested against mouse tumor cells, human serum is highly cytolysic. This heterologous system of oncolytic reaction in vitro has recently been demonstrated to consist of a natural
antibody and complement by Kuru et al. (3) and by Landy et al. (4). It is as a whole a thermolabile system which is inactivated at 55-60°C in 30 minutes.

The carcinostatic liver factor demonstrated in the present study is totally unrelated to this well known heterologous system.

The fact that the liver extract of the rat is apparently as potent as that of the mouse indicates that there is no species specificity in the carcinostatic liver factor, which suggests that extracts from other animal livers may be equally active against not only mouse tumor cells but also tumor cells of other animals, including those of man. In the use of heterologous liver (Herbut and Kraemer (2)) in experiments of this type, however, due precaution must be taken to exclude the complications resulting from the co-existing serum antibody-complement system, which rapidly cytolyses certain heterologous tumor cells. The rat serum has no such serum system which acts upon mouse tumor cells.

One point which may be mentioned in connection with rat liver is the fact that while normal liver yields the carcinostatic factor, the same cannot be obtained from hepatoma cells, apparently showing that the factor becomes deleted when liver cells become cancerized. This, however, is perhaps to be expected a priori.

The classical postulate of Freund and Kaminer (5) that normal human serum but not serum of cancer patient brings about lysis of human cancer cells has since fallen into poor repute, because of the great individual variation of the activity among the sera. It should be pointed out, however, that the source of the anti-cancer cell factor in normal blood serum was entirely unknown at that time. If, as we are now strongly inclined to believe, the factor is produced in the liver and is liberated into the circulation, variation in the concentration of the factor in blood sera is only to be expected. It is also probable that the capacity of the liver to produce the oncostatic factor may become greatly reduced in cancer-bearing hosts, since we know that the general liver dysfunction is a common phenomenon in malignant diseases. This idea is supported by our experiments in which livers from tumor-bearing animals failed to yield active extract.

The important problem is that of the homologous carcinostatic system, which was first studied by Freund and Kaminer in blood serum of man and which may be closely related to the homologous carcinostatic liver factor demonstrated by us. Our working hypothesis that this latter may be operative in preventing metastasis formation can be proved only by direct experimental tests.

**SUMMARY**

By testing effect *in vitro* of various homologous tissue extracts under accurately defined experimental conditions it was demonstrated that normal liver contains a potent carcinostatic factor, which other tissues failed to yield. The complete failure
of the tumor cells to take, when implanted into susceptible host, was brought about by allowing only 2 cc of centrifuged supernatant of mouse liver homogenate, prepared at the rate of 1 g of liver in 10 cc of normal salt solution, to interact with 2 cc ascites equivalent of Ehrlich carcinoma cells at 37°C for 1 hour.

The distinction of this liver factor from the well known oncolytic system of certain heterologous blood sera was pointed out and the possibility of the hepatogenic carcinostatic factor playing a deciding role in the disposal of isolated cancer cells in body fluid and consequently in preventing the formation of metastasis was considered. The importance of homologous carcinostatic system, contrasted to the heterologous one hitherto studied, was especially emphasized.

References


