CARCINOSTATIC LIVER FACTOR
II. DISCRIMINATION FROM LIVER RIBONUCLEIC ACID

Waro Nakahara, Fumiko Fukuoka, and Hiroto Naora
(Cancer Institute, Japanese Foundation for Cancer Research*)

INTRODUCTION

In the first article of this series Nakahara and Fukuoka\(^2\) reported that normal mouse liver contains a potent carcinostatic factor, which, when allowed to act upon Ehrlich mouse ascites carcinoma cells \textit{in vitro}, completely destroys the transplantability of the cells in susceptible mice. No similar effect was demonstrated for extracts from other tissues, normal as well as malignant, at least under the same experimental conditions. Although livers of animals other than mouse were shown to yield extracts apparently as potent as those of homologous liver, the marked thermostability of the liver factor demonstrated it to be unrelated to the well-known oncolytic system of certain heterologous blood sera.

While further work on this liver factor was in progress, two papers of peculiar interest came to notice, both claiming that the ribonucleic acid (RNA) isolated from normal liver is capable of so modifying tumor cells as to greatly reduce their transplantability. The first of the two papers was that of DeCarvalho and Rand,\(^1\) reporting, among other things, that dissociated Novikoff hepatoma cells (rat) suspended in a solution of RNA from normal rat liver and kept at 2\(^\circ\)C for 15 hours, show a very much lower rate of takes (15\%) than control cells (95\%), while RNA from normal rat spleen, kidney, or hepatoma itself exhibits no effect. The result was interpreted as being due to “a certain degree of re-differentiation of the original wild malignant tumor cells by the normal RNA preparation.” The second paper, by Niu, Cordova, and Niu,\(^3\) described experiments showing that the Nelson mouse ascites cells, which normally produce tumors in 96\% of the implanted mice, gave a very markedly reduced rate of tumor development (less than 10\%) when they were treated \textit{in vitro} with RNA from calf liver before implantation. These workers also apparently regard the effect as resulting from RNA-induced change in the tumor cells.

It is of interest to note that while our own work was started with the idea of looking for the possible defensive factor against tumor cell dissemination which the host body may possess and which may be capable of destroying isolated cancer cells

\* Nishi-Sugamo 2-chome, Toshima-ku, Tokyo (中原和郎，福岡文子, 直良博人)
in the circulation, the guiding principle in the experiments of DeCarvalho and Rand\textsuperscript{1}) and of Niu, Cordova, and Niu\textsuperscript{3}) was that RNA from normal cells may incorporate itself into malignant cells without loss of its biological potentiality and may function toward "normalizing" cancer cells, so to speak. In spite of this difference between the two schools of thought there are remarkable similarities in the actual experimental results, which are at one at least in showing a marked suppression of takes of transplanted tumors by the preliminary treatment \textit{in vitro} of tumor cells with liver constituent(s). This fact raises the question of the possible identity of our carcinostatic liver factor and the liver RNA.

Our unpublished data, which have been obtained incidental to our attempts to purify the carcinostatic liver factor, strongly suggest that the properties of this factor are unlike those of RNA. As already recorded,\textsuperscript{2}) this liver factor is extremely thermostable, withstanding autoclaving at 120\textdegree C under 15-lb pressure for 10 minutes. When the autoclaved extract is treated with 2 or 3 volumes of absolute ethanol, the active factor goes into the alcohol solution, precipitate being entirely inactive. In this case, the original liver extract was prepared with normal salt solution and so RNA might be easily precipitated with alcohol. We also know that the factor is soluble in 80\% acetone.

However, more direct evidence seemed necessary in order to settle the question and for that purpose, we have recently tested the effect of pancreatic ribonuclease (RNase) on active liver extracts and found that RNase entirely failed to affect the activity of the extracts, thus excluding RNA from consideration in the study of the carcinostatic liver factor.

\textbf{Experimental}

\textbf{Experiment 1}. Pancreatic RNase used was a highly purified preparation obtained from the Worthington Biochemical Corporation. It was dissolved at 2 mg/ml in water. Liver extract was prepared by homogenizing in a Potter-Elvehjem type homogenizer fresh normal mouse liver (with gall bladder removed) in physiological salt solution at the rate of 1 g liver/10 ml and by centrifuging at 3,000 r.p.m. for 5 minutes. The reaction of the extract was adjusted to pH 6.8 with 0.1 N sodium hydroxide solution. Three mixtures were first set up:

1. 3.8 ml of liver extract + 0.2 ml of RNase solution, total volume 4 ml, and final concentration of RNase 0.1 mg/1 ml.
2. 3.8 ml of liver extract + 0.2 ml of water.
3. 1.9 ml of normal salt solution + 0.1 ml of RNase solution, total 2 ml.

The three mixtures were incubated at 37\textdegree C for 60 minutes, after which each of the mixtures was tested against Ehrlich ascites carcinoma cells \textit{in vitro}. To centrifuged cell sediments of 1-ml portions of ascites from the same mouse, 2 ml of one
of the three mixtures pretreated as above was added and made into even suspensions, which were then incubated at 37°C for 75 minutes. A blank control, with the same amount of cell sediment suspended in 2 ml of normal salt solution, was incubated at the same time.

After the incubation, all the mixtures, now four in number, were centrifuged, supernatant discarded, and cell sediments were resuspended in 1 ml of normal salt solution and injected subcutaneously into normal mice in 0.2-ml amounts. Five mice were used for each mixture and all the mice were kept under observation for 3 weeks, at the end of which the results were recorded by killing the mice and performing autopsy. The results were as follows:

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>No. of mice</th>
<th>Tumor production</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver extract+RNase</td>
<td>5</td>
<td>None</td>
</tr>
<tr>
<td>Liver extract only</td>
<td>5</td>
<td>$\ddagger$</td>
</tr>
<tr>
<td>RNase only</td>
<td>5</td>
<td>Large tumor in all the mice</td>
</tr>
<tr>
<td>Blank control</td>
<td>5</td>
<td>$\ddagger$</td>
</tr>
</tbody>
</table>

Absolutely no effect of RNase on the carcinostatic activity of the liver extract was recognized.

**Determination of RNA contents.** RNA content was determined on 2-ml aliquot of each of the two mixtures, consisting respectively of 3.8 ml liver extract +0.2 ml RNase solution, and of 3.8 ml liver extract +0.2 ml water, both after an incubation at 37°C for 60 minutes in Experiment 1.

After cooling in an ice-water bath for 7 minutes, cold trichloroacetic acid was added to each tube to the final concentration of 5%, and the precipitate produced was washed with cold 5% trichloroacetic acid. Nucleic acid fractions were obtained by warming the precipitate in 5% trichloroacetic acid at 90°C for 30 minutes and the quantity of RNA determined colorimetrically by the method of Webb.\(^4\)

The liver extract incubated alone was found to contain 0.647 mg RNA/ml, while the extract incubated in the presence of RNase contained 0.072 mg RNA/ml, showing that 88.9% of RNA in the liver extract was digested by RNase under the conditions of this experiment.

**Experiment 2.** In this experiment was used supernatant solution of autoclaved liver extract, which was prepared by autoclaving the fresh mouse liver extract similar to that used in Experiment 1, at 120°C under 15-lb pressure for 10 minutes and centrifuging. The supernatant was adjusted to pH 6.7 with 0.1N sodium hydroxide solution. The RNase solution was the same as in Experiment 1.

The following three mixtures were prepared and incubated at 37°C for 60 minutes:

1. 4 ml of the liver supernatant+0.2 ml of RNase solution containing 2 mg RNase/ml water (the final concentration of RNase, 0.095 mg/ml).
2. 1 ml of liver supernatant alone.
3. 0.05 ml of RNase solution diluted with 1 ml of normal salt solution.

After incubation, each of the three mixtures was again autoclaved at 120°C under 15-lb pressure for 10 minutes, added in 1-ml amounts to centrifuged cell sediments of 1 ml of Ehrlich ascites from the same mouse, well stirred to make even suspensions, and incubated at 37°C for 75 minutes, a similar cell suspension in normal salt solution serving as blank control.

The incubated mixtures were then centrifuged, supernatant discarded, cell sediments resuspended each in 1 ml of normal salt solution, and injected subcutaneously into normal mice in 0.2-ml doses, with the following results:

<table>
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<th>Tumor development</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver supernatant + RNase</td>
<td>5</td>
<td>None</td>
</tr>
<tr>
<td>Liver supernatant alone</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>RNase alone</td>
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<td></td>
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</table>

These results show that no doubt can be entertained as to the complete failure of RNase to inactivate the carcinostatic liver factor.

**DISCUSSION**

In spite of the apparent similarity in the final results, actually the experiments of DeCarvalho and Rand, and of Niu, Cordovo, and Niu may be fundamentally different from those of our own, since the data presented in this paper seem conclusive in demonstrating that the carcinostatic liver factor cannot be identified with RNA.

In this connection it may be important to compare the concentrations in which liver RNA was used in the experiments. DeCarvalho and Rand give the ratio of 2.5 mg RNA per 10 million cells. In our experiment, fresh liver extract is estimated to contain 0.647 mg RNA per 1 ml and this extract is allowed to act on 1 ml cell equivalent of tumor ascites, which means on an average 10 to 15 million cells. The concentration of RNA in the DeCarvalho and Rand experiments may be said to be about four times that in our experiment. Niu *et al.* give the RNA concentration in the term of optical density (E₂₆₀), active at 100 and inactive at 25. Since E₂₆₀ 1.00 = 0.033 mg RNA/ml, E₂₆₀ 100 = 3.3 mg RNA/ml, which is about five times the concentration used by us. It is conceivable that the samples of RNA used by DeCarvalho and Niu group were impure and contained as an impurity the carcinostatic factor in our sense, or that RNA in a high concentration may adversely affect the viability of cancer cells exposed to it in vitro, quite apart from the effect of the carcinostatic
factor. Neither of these two possibilities cannot explain the inactivating effect of RNase claimed by Niu et al. Our results are conclusive in showing that the carcinostatic factor is not inactivated in the least by RNase, while RNA in liver extract is demonstrated to be decomposed to a very great extent by RNase, without affecting the carcinostatic activity of the extract.

It is of interest to point out that when used at the optical density equivalent of 25 (\(\approx 0.825 \text{mg/ml}\)), RNA was practically without effect in the experiments of Niu et al., while our fresh liver extract, containing only 0.647 mg RNA/ml was highly active.

Thus, we utterly fail to account for the discrepancy between the results of the two groups of experiments. It seems certain, however, that RNA action is not involved in our carcinostatic liver factor, which may now be considered without reference to the RNA question.

The mode of the carcinostatic liver factor remains to be elucidated. Having eliminated RNA from consideration, however, the idea that the liver factor may enter into the genome of cancer cells and so produce a genetic modification in them is scarcely to be entertained. It is far more likely that the liver factor is in fact simply damaging to cancer cells, even though evidence of the injury may not be morphologically detectable.

When a sufficient degree of purification of the liver factor is accomplished, we shall be in a position to clarify many problems connected with this very interesting liver constituent.

**SUMMARY**

The alleged modification of tumor cells by treatment in vitro with normal liver RNA, resulting in the greatly diminished cell transplantability (DeCarvalho and Rand, and Niu, Cordova, and Niu), was investigated in relation to the carcinostatic liver factor, which we previously reported.

It was found that the anti-tumor activity of our liver extracts was not affected in the least by digestion with pancreatic RNase, in spite of the fact that the RNA content of the extract was reduced almost to 10\% by the RNase treatment. Without questioning the validity of the RNA effect claimed by other workers, therefore, we conclude that our carcinostatic liver factor may be totally unrelated to RNA.

**REFERENCES**