RIBONUCLEIC ACID-INDUCED CHANGES IN MAMMALIAN CELLS*

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Communicated by E. L. Tatum, August 1, 1961

Previous experiments have shown that under proper conditions ribonucleic acid (RNA) can indeed initiate differentiation.1, 2 Of particular interest in this work is the development of highly specific structure. That the type of structure developed correlates with the tissue source of RNA indicates a response of the reacting tissue to a message carried directly by RNA or RNA-containing substances of the inducing tissue. Thus, on the cellular level, when a piece of the epidermal ectoderm of urodele gastrula is transplanted into the presumptive kidney area, the ectoderm contributes to the formation of extra kidney tubules3 and develops into muscle in the presumptive muscle area.4 At the molecular level, ribonucleoproteins (RNP) from different tissues were found to be capable of inducing the formation of muscle,5, 6 blood,7 thymus,1 and other tissues.2

Some two years ago, an examination of the effects of normal RNA on mouse ascites cells was initiated. After incubation with liver RNA, only 10 per cent of the transplanted ascites cells gave rise to tumors. The loss of the tumor-forming ability was correlated with induced cellular changes rather than with regression caused by either cell death or immunological response.8 The cellular changes were shown in vivo by the lack of mitotic division and invasiveness. In vitro, they were demonstrated by the acquired capacity of synthesizing the liver protein, serum albumin.9

In this communication, observations which indicated an inhibitory effect of RNA on tumor growth8, 9 are presented in detail. These studies and comparable studies with the Novikoff hepatoma10 appear to support the concept that RNA induces cellular transformation in mammalian cells.

Materials and Methods.—Mice and ascites cells: During 1959 and 1960, white Swiss mice of the Rockefeller Institute, and in 1961, Swiss Webster mice supplied by Pied Piper Farms were used in this study. The ascites tumor cells were developed and maintained through successive transfers in the peritoneal cavity of the Princeton strain by Dr. John B. Nelson of the Rockefeller Institute, whose generosity in supplying ascites fluid biweekly during 1959 and 1960 the authors are pleased to acknowledge. Upon receipt of the cell suspension, our customary procedure was to wash the ascites cells with 0.9% saline. Intramuscular injection of the washed cells give rise to solid tumors,11 and the mice usually died within a month. For the preparation of tumor RNA, solid tumors without necrosis (8 to 10 days) were used as source material. Mice from the same litter were used for control and experimental series. The number in each series was limited to 5 to 8 mice, weighing 10 to 15 grams each.

Isolation of RNA: RNA was prepared from mouse and calf liver (L-RNA) and solid tumor (T-RNA) by a modified Kirby procedure involving low-speed centrifugation.2 Beginning in November 1960, the isolated RNA was centrifuged at 40,000 RPM (Spinco Model-L Rotor #40) for 30 min. The supernatant fluid was subsequently treated with ether and the ether was expelled by nitrogen. The preparations were used immediately or frozen in dry ice-acetone and stored at −20°C for 7 to 10 days. No apparent loss of activity was detected.

Ultraviolet absorption spectra of both preparations of RNA were similar, with the lowest absorption at 230 μμ and the highest at 258 μμ. In each case, the ratio, absorption at 260 μμ/absorption at 230 μμ, exceeded 2.2. The amount of RNA calculated from the orcinol determination, the phosphorus content, and the absorption at 260 μμ agreed, thus indicating that the sub-
stances present in solution were RNA. In addition, routine laboratory tests for protein, DNA, and polysaccharides were negative. However, the RNA hydrolysate gave a ninhydrin color reaction, indicating the presence of some amino acids.

Preparation of ascites cells for study: The ascites cells were washed 5 to 6 times with 30 to 50 volumes of 0.9% saline and divided into aliquots. One served as control, the second and third were mixed well with 10 volumes of L-RNA with an optical density (O.D.) at 260 my of 25/ml and O.D. 100/ml respectively, and the fourth was mixed with T-RNA with O.D. 100/ml. They were incubated for 12 to 20 hr in the cold (5 to 8°C). To insure contact of RNA with the cells, the mixture was stirred at intervals, especially during the first few hours of incubation. Next morning, each sample was centrifuged and resuspended in fresh samples of the incubation medium.

Before and after each incubation, one volume of the ascites cells and 1/4 volume of 1% eosin were mixed thoroughly. Within 5 min, the number of stained and unstained cells were counted. The unstained cells are viable and tumor-producing.12

Each aliquot of cells was subdivided for 1 Intramuscular injection in the thigh with 0.05 ml saline containing 5 X 10⁶ cells. The thigh was subsequently fixed daily up to the 10th day to trace the fate of the injected cells. Quantitative determination of nucleic acid. Both RNA-treated and control series were centrifuged and cells quickly washed twice with saline. The cell suspension was treated with an equal amount of cold 4% perchloric acid (PCA), mixed well, and held at 2°C for 5 min. The acid-soluble substances were then removed by centrifugation. The sediment was washed once with 2% PCA and extracted with 10% PCA at 90°C for 15 min. Nucleic acids were estimated by the ultraviolet absorption at 260 my. Amino acid incorporation. The samples were centrifuged and cells resuspended in 5 to 8 volumes of the incubation mixture. They were placed on crushed ice and 0.1 ml containing 1 μc of C¹⁴-DL-leucine was then added for each ml of cell suspension. The samples were then transferred to a shaker at 37°C, and two minutes were allowed for temperature equilibration. The shaking speed was 100 cycles per min. Incorporation was stopped at intervals by addition of equal volumes of ice-cold 10% trichloroacetic acid (TCA).

Incorporation was also followed on cells cultivated in milk dilution bottles14 for various lengths of time.

Fractionation of protein: The TCA protein was centrifuged in the cold and washed twice with cold 5% TCA. The sediment was extracted with 10 volumes of absolute alcohol and then designated as general protein (G.P.). The alcohol was removed by dialysis against distilled water containing some DL-leucine. On removal of alcohol, precipitates appeared, which were collected by centrifugation and called insoluble protein (I.P.) The substance in solution with serum albumin added as carrier (30 μg/ml) was precipitated by addition of the antiserum against bovine serum albumin. Due to its solubility in absolute alcohol, especially after treatment with 5% TCA, and precipitability by the antiserum, the substance in question appeared to be related to serum albumin (S.A.) and was so designated.

The nucleic acids of G.P. were removed by heating the 5% TCA suspension at 90°C for 15 min. Afterwards, this sample was treated in the same manner as I.P. and S.A. The lipids were removed by three extractions with alcohol-ether (3:1) at 62°C for 5 min each, followed by washing with alcohol-ether-chloroform (2:2:1) and ether. The dried samples were weighed and counted in an automatic gas flow counter.

Methods of treatment with RNAse and DNase: One ml of the L-RNA (O.D. 200/ml) was mixed separately with 1 ml saline containing 1 mg RNase or DNase (Worthington Co.). Together with samples of RNAse and DNase in saline (control series) they were incubated at 37°C for 20 min. These four samples were separately mixed with 0.2 ml of the ascites cells, kept in the refrigerator for 15 to 20 hr with frequent stirring, and each then divided into two equal aliquots. One aliquot was used immediately for a test of its tumor-producing capability. The other was centrifuged and the cells were resuspended respectively in the same fresh enzyme solution. They were kept in 5 to 8°C for another 15 to 20 hr before centrifugation, resuspension, and intramuscular injection.

Preparation of C¹⁴-liver RNA and determination of C¹⁴ distribution in RNA: Mice weighing 25 to 30 grams were starved 12 hr and then given 0.5 ml of C¹⁴-alanine acid (5 μc) and 0.5 ml of C¹⁴ adenosine (5 μc) intraperitonely. Five hr later, livers from 15 animals were pooled and the gall bladders removed. RNA was prepared and found radioactive, presumably due to C¹⁴-labeled bases (adenine, cytosine, and uracil) of the L-RNA.
C\textsuperscript{14}-L-RNA was hydrolyzed by 1N HCl for 1 hr at 100°C. The hydrolysates were subjected to paper chromatography according to Smith and Markham.\textsuperscript{14} The four spots representing guanine, adenine, cytidylic acid, and uridylic acid were dried and counted for radioactivity. Samples were eluted with 0.1N HCl, evaporated until dry, and counted.

C\textsuperscript{14}-L-RNA was employed to treat the washed ascites cells. After 20 hr of incubation, the sample was centrifuged. For the removal of free C\textsuperscript{14}-L-RNA, the cells were quickly washed twice. RNA isolated from these cells was radioactive and thus designated as C\textsuperscript{14}-T-RNA. Radioactivity of its hydrolysates was determined in the same manner as that of C\textsuperscript{14}-L-RNA.

Results.—Viability of the ascites cells: Of prime importance in the present investigation was the viability of the ascites cells, particularly after incubation with RNA. Comparing the two groups of cells, blebs appeared more frequently in the RNA-treated cells than in the untreated. The latter packed more firmly when centrifuged. Neither phase nor electron microscopy could detect any additional difference between them. Staining with eosin or trypan blue revealed that 60 to 95 per cent of the washed ascites cells and 40 to 80 per cent of the RNA-treated counterpart resisted staining. It is pertinent to add that the presence of only 19 per cent viable cells in the RNAse-treated ascites population was sufficient to produce solid tumors in a frequency of approximately 60 per cent at the site of injection (see below). Accordingly, one would expect tumor formation by the RNA-treated cells. The apparent reduction in tumor formation (see below) would therefore suggest that other factors are operating.

The presence of metabolically active cells in the RNA-treated sample was further indicated by the study of amino acid incorporation into proteins (Fig. 1).

![Fig. 1.—Time course of incorporation of DL-leucine-C\textsuperscript{14} into proteins of untreated and L-RNA-treated ascites cells.](image)

It can be seen that all three series were capable of incorporating DL-leucine-C\textsuperscript{14} into TCA proteins. The rate of incorporation by those cells exposed to higher concentrations of RNA was reduced to approximately \(\frac{1}{4}\) of the control. This reduction could result from either cell death or cell change, or both. Since the difference in viability between the L-RNA-treated and untreated cells, as estimated by staining with eosin, was only 15 to 20 per cent, it seems unlikely that cell death
was responsible for the decrease of \( \frac{3}{4} \) in metabolic activity. Besides, cancer cells ordinarily are metabolically more active than normal cells.\(^{17}\)

Rate of tumor formation: Intramuscular injection of the ascites cells and the RNA-treated counterpart resulted in the development of solid tumor at the site of injection. The frequency varied, however, according to the concentration and the tissue source of the RNA employed. As a rule, if L-RNA-treated cells developed into solid tumor, the appearance of the tumor was frequently delayed. Table 1 summarizes the data obtained with the Rockefeller Swiss mice (up to the summer of '60). It can be seen that under our experimental conditions a proper concentration of L-RNA (O.D. 100/ml) indeed affected the development of the ascites tumor. In contrast, treatment with T-RNA had no significant effect. Our protocol revealed further that the inhibition was permanent, because those mice that had developed no tumors within a month gave none in six months or longer. In addition, they did not do so even on subsequent inoculation with untreated ascites cells (25 Swiss Webster mice).

The effect of nucleases on L-RNA: Subsequent to the finding that L-RNA could inhibit tumor growth, enzyme studies were carried out to see if RNA macromolecules were required for this inhibition. Both RNAse and DNAse were used, one serving as control for the other. Since RNAse is reportedly tumor-inhibitory,\(^{18,19}\) our cells were tested separately after the first and the second treatments. This was to insure doubly the adequacy of the enzymatic treatment. Table 2 provides data obtained with the Swiss Webster mice. RNAse treatment of L-RNA led to its inactivity in lowering tumor frequency, whereas treatment with DNAse had no effect. The sensitivity to RNAse and indifference to DNAse provides a strong point in favor of the necessity of macromolecular integrity for the RNA function.

### TABLE 1

**Frequency of Solid Tumor Formation**

<table>
<thead>
<tr>
<th>Experimental Conditions</th>
<th>Number of mice</th>
<th>Number with tumors</th>
<th>Number without tumors</th>
<th>Percent with tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated tumor cells</td>
<td>158</td>
<td>153</td>
<td>5</td>
<td>97</td>
</tr>
<tr>
<td>Saline</td>
<td>20</td>
<td>0</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>L-RNA-treated tumor cells (O.D. 25/ml.)</td>
<td>43</td>
<td>33</td>
<td>10</td>
<td>77</td>
</tr>
<tr>
<td>L-RNA-treated tumor cells (O.D. 100/ml.)</td>
<td>166</td>
<td>14</td>
<td>152</td>
<td>8</td>
</tr>
<tr>
<td>T-RNA-treated tumor cells (O.D. 100/ml.)</td>
<td>24</td>
<td>17</td>
<td>7</td>
<td>71</td>
</tr>
</tbody>
</table>

### TABLE 2

**Effect of Enzyme Treatment on Tumor Induction Using Thoroughly Washed Ascites Cells**

<table>
<thead>
<tr>
<th>Type of experiment</th>
<th>Untreated (control)</th>
<th>L-RNA</th>
<th>RNAse treated L-RNA</th>
<th>RNase</th>
<th>DNAse treated L-RNA</th>
<th>DNAse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability after first treatment</td>
<td>79%</td>
<td>62%</td>
<td>80%</td>
<td>87%</td>
<td>70%</td>
<td>75%</td>
</tr>
<tr>
<td>Tumors per 8 mice</td>
<td>8</td>
<td>3</td>
<td>7</td>
<td>5</td>
<td>2</td>
<td>7</td>
</tr>
<tr>
<td>Viability after second treatment</td>
<td>40%</td>
<td>12%</td>
<td>36%</td>
<td>19%</td>
<td>30%</td>
<td>45%</td>
</tr>
<tr>
<td>Tumors per 6 mice</td>
<td>4</td>
<td>0</td>
<td>5</td>
<td>3</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>Per cent of tumors</td>
<td>86</td>
<td>21</td>
<td>86</td>
<td>57</td>
<td>14</td>
<td>86</td>
</tr>
</tbody>
</table>
That RNA was taken up as macromolecules was further supported by analysis of C14-L-RNA and C14-T-RNA (ratio of activities about 10:1). Chromatograms of both C14-L-RNA and C14-T-RNA hydrolysates were made by the techniques used for yeast RNA.16 C14- orotic acid is supposed to be incorporated into the C14-uridylic and C14-cytidylic acids and C14-adenosine into the C14-adenine of the C14-L-RNA. If the macromolecules of C14-L-RNA enter the cells without preliminary degradation, the ratios of radioactivity of C14-adenine and C14-cytidylic and C14-uridylic acids of the C14-T-RNA should be equal or close to those found in the C14-L-RNA. Table 3 shows the distribution and ratios of radioactivity in the RNA components. It can be seen that the ratios of C14-L-RNA and C14-T-RNA are similar within experimental error.

TABLE 3

DISTRIBUTION AND RATIOS OF RADIOACTIVITY BETWEEN THE HYDROLYSATES OF C14-L-RNA AND C14-T-RNA (AVERAGE OF FOUR DETERMINATIONS)

<table>
<thead>
<tr>
<th>Material and counts of radioactivity/min</th>
<th>Guanine (G)</th>
<th>Adenine (A)</th>
<th>Cytidylic acid (C)</th>
<th>Uridylic acid (U)</th>
<th>A/U</th>
<th>A/C</th>
<th>C/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14-L-RNA (200 μg)</td>
<td>24.7</td>
<td>81</td>
<td>133.8</td>
<td>225.3</td>
<td>0.33</td>
<td>0.64</td>
<td>0.51</td>
</tr>
<tr>
<td>C14-T-RNA (200 μg)</td>
<td>2.1</td>
<td>4.9</td>
<td>7.7</td>
<td>15.1</td>
<td>0.36</td>
<td>0.61</td>
<td>0.59</td>
</tr>
</tbody>
</table>

So far as RNA function is concerned, untreated L-RNA lowered the tumor-producing potentiality of the ascites cells to 20 percent in Swiss Webster mice and to 10 percent in the Rockefeller Swiss mice. This is in contrast to the 50 to 62 percent reduction observed in the series in which cells were incubated with RNase (column 5, Table 2). It should be emphasized that the experiment with RNase was repeated many times, and the inhibitory effect of RNase never approached that of the untreated L-RNA.

Fate of the injected cells: Viable cells in the liver RNA-treated population varied from 40 to 80 percent. When a half million of these cells were inoculated intramuscularly into the thigh, the frequency of tumors was only 10 to 20 percent. The immediate question is what happens to the majority of the injected cells? One possibility is that the liver RNA contributed something to the cells which invoked an immunological response leading to their elimination. If this were the case, there should be a gradual decrease in the number of cells, particularly no organized structure should appear at the site of injection. Figure 2 shows a low-power view of the site of injection of untreated ascites cells. The luxuriant growth of the ascites tumor cells is conspicuous, and invasion into the muscle bundle has begun. Numerous mitotic figures can easily be seen under higher magnification (Fig. 3a) but are seldom found in the RNA-treated cells. In contrast to Figure 3a, there appears to be some sort of cell grouping (Fig. 3b). Four days later, the number of the identifiable injected cells is greatly reduced, but those remaining have oriented themselves into an organized pattern (Fig. 4). It seems then that some of the RNA-treated cells went through stages of progressive specialization instead of being expelled. A detailed analysis of this differentiation will be reported elsewhere.

Possible mechanism of the tumor inhibition: The findings presented above have demonstrated that the L-RNA induced cellular change. Microscopic examinations do not reveal the nature of the change prior to the injection. There was, however,
a quantitative difference in the nucleic acid content of the treated and untreated cells. The increase of from 18 to 43 per cent in nucleic acid in the RNA-treated cells is presumably due to the addition of RNA to these cells. The manner by which this RNA is associated with the treated cells has been under investigation. Preliminary data of autoradiographic studies suggest that it is located inside the cells. How does it get there, and what is the mechanism whereby the host and the foreign species of RNA can be so coordinated that the tumor-forming potentiality of the ascites cells is reduced by 80 to 90 per cent? Well-documented answers to these questions have yet to come. Observations which indicate a relation between the biological potentiality of RNA and differentiation have been accumulated in recent years.\textsuperscript{1, 2, 5, 6, 20} What has happened to the RNA-treated cells may perhaps be that the exogenous RNA, as in the case of TMV-RNA\textsuperscript{21} has stimulated the synthesis of new proteins. A direct test for this mechanism is the comparative study of protein biosynthesis in the RNA-treated and untreated cells (see Fig. 1). The TCA protein was fractionated into G.P., I.P. (mostly lipoprotein), and S.A. The latter is specific and synthesized only by liver cells. Studies on the incorporation of C\textsuperscript{14}-leucine into S.A. showed that the biosynthetic rate was at first higher in the untreated cells than in the L-RNA-treated cells. As time proceeded, synthesis over a 2\textsuperscript{1/2} hr period gradually decreases in the untreated cells but increased 2 to 7 fold in the treated cells. When a lower concentration of L-RNA was used and the cells were cultivated \textit{in vitro} for a few days, albumin synthesis of the treated cells was 5 to 10 times higher than in the control and 3 to 5 times higher than in the T-RNA series. \textit{In vitro} cultivation for three weeks resulted in an even greater increment. This is demonstrated in Figure 5, in which the synthesized albumin (expressed in radioactivity counts) is plotted against the concentration of RNA employed (expressed in terms of O.D. at 260 m\textmu—0,5,10, and
Fig. 3a.—An area of Figure 2 under higher magnification (1,000 ×). Note the abundance of mitotic division and the presence of invaded cells in between muscle bundles.

20. Under our experimental conditions, the synthesis of serum albumin-like protein was directly related to RNA concentration. If protein were contaminating the RNA used, higher concentrations would carry more protein. Accordingly, the observed increment in C\textsuperscript{14}-albumin might be explained either by a C\textsuperscript{14}-leucine exchange in the contaminating protein\textsuperscript{19} or by the influence of incorporated RNA on protein synthesis. The former, however, is unlikely, because cells treated
with either serum albumin alone or T-RNA plus albumin did not show this increase.

Discussion.—It is known that protein synthesis requires the participation of transfer or carrier RNA, of "messenger" RNA, and of microsomal or structural RNA. However, the chemical identification and the relationships among these RNAs are poorly understood at the present time. With this in mind, our experiments on the biological potentiality of RNA have utilized RNA isolated from the whole
Fig. 4.—A high-power view (1,000 x) of the L-RNA-treated cells, eight days after injection. Note particularly the highly organized structure.

tissue. The activity of this RNA depends not only on the freshness but also on the concentration employed. It has been demonstrated in the foregoing experiments that under proper conditions the biological potentiality of isolated RNA is specific. While T-RNA has no apparent effect on the ascites cells, mouse L-RNA has produced changes. These cellular changes can be seen both in vitro and in vivo. Morphological deviation was undetectable in vitro, but biochemically the L-RNA-
treated cells had acquired machinery for synthesizing serum albumin (Fig. 5), a liver specific protein. It appears that L-RNA carries the code of the liver cells and thus dictates the type of protein to be synthesized.

In *vivo*, the untreated ascites cells resumed active mitotic division shortly after inoculation. On the third or fourth day, they began to invade the neighboring muscle bundles and reached the size of a chestnut by the tenth day. The host died within three to four weeks. In contrast, 80 to 90 per cent of the injections with L-RNA-treated cells did not give rise to tumors. The failure was correlated
with the loss of two biological markers for cancer cells, namely, a high rate of mitotic division and invasiveness. Since cell growth is inversely proportional to cell differentiation, the loss of these properties suggests that differentiation might have occurred. This appears true of cells (Fig. 4) which still remain in the muscle tissue eight days after injection. Apparently most of them are gone. The disappearance may possibly be due to one or a combination of three factors: (1) non-viable injected cells may have been removed by phagocytes, (2) the viable cells that have acquired the capacity to synthesize serum albumin may have been relocated, and (3) some cells may even have been "assimilated" by the neighboring muscle tissue. Points (2) and (3) will constitute the subject of another publication.

The concept of "stopping tumor growth through differentiation" is an old one. The present work is actually the beginning of a new approach. This approach has recently been extended to rat hepatoma in experiments by DeCarvalho and Rand, in which the L-RNA-treated hepatoma cells gave rise to slightly haemorrhagic nodules scattered in the mesentery in 85 per cent of the injected rats and in 15 per cent formed a solid tumor. Histologically, the nodules showed a more orderly cordonal organization with the tendency to radiate around a vein. The cells contained glycogen in a polarized distribution resembling normal livers.

The manner by which RNA acts upon the cells is not clearly known. In view of the induced biosynthesis of specific protein, however, it may be assumed that it enters the cells without loss of biological potentiality. Evidence supporting this hypothesis comes from our enzymatic studies showing the requirement of macromolecular integrity for RNA function, the correspondence in radioactivity ratios of the hydrolytic components between C\textsuperscript{14}-L-RNA and C\textsuperscript{14}-L-RNA isolated from the C\textsuperscript{14}-L-RNA-treated tumor cells, and the autoradiographic demonstration of radioactivity within the treated cells. Once in the cells, the exogenous RNA would perhaps be functioning in a fashion similar to infectious RNA. The synthesis of new protein is correlated with the appearance of new morphological entities.

**Summary.**—It has been shown that the treatment of the Nelson mouse ascites cells with ribonucleic acid in suitable concentrations reduced tumor formation from 97 per cent to 10-20 per cent. This reduction was correlated with induced cellular change rather than regression caused by cell death and/or immunological response. The change was found to be accompanied by an induced biosynthesis of specific protein. Apparently hepatoma cells under proper influence of liver RNA gave rise to an organoid structure resembling normal liver.

That the effect of L-RNA was specific was demonstrated by its sensitivity to RNase and indifference to DNase and by the inactivity of T-RNA. The entrance of macromolecular RNA into the treated cells was indicated by the presence of C\textsuperscript{14} in cells treated with C\textsuperscript{14}-L-RNA and by the correspondence of the ratios of labelled bases in the administered C\textsuperscript{14}-L-RNA and in the recovered C\textsuperscript{14}-T-RNA.

* This investigation was supported in part by research grants G-14311 and G-14755 from the National Science Foundation and C-5344 from the National Cancer Institute, U. S. Public Health Service.

Some of these results were reported to the Tenth International Congress of Cell Biology, held in Paris, September 4-9, 1960.
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