Altered RNA/protein ratio associated with the induction of differentiation of Friend erythroleukemia cells

(aminonucleoside of puromycin/dimethyl sulfoxide/erythroid differentiation/inosine/sodium butyrate)

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ABSTRACT The synthesis and accumulation of RNA in Friend erythroleukemia (FL) cells induced to differentiate by treatment with the aminonucleoside of puromycin (AMS) or inhibited from differentiating by the addition of inosine to the medium were studied. When FL cells were grown in the presence of AMS, RNA synthesis was substantially inhibited. This effect could not be attributed solely to the inhibition of de novo purine synthesis because the biosynthesis of purine nucleotides from labeled inosine was much less depressed. The ratios of ATP to protein and of GTP to protein were slightly modified as compared to the untreated controls. However, the RNA/protein ratio was decreased. Thus, the RNA content of the cells was reduced 30–40%, but the protein content was not significantly affected. When the cells were treated with AMS together with inosine at a concentration that inhibits AMS-induced differentiation, the RNA/protein ratio was increased as compared with that found in cells treated with AMS alone and approached the level of the ratio in untreated control cells. Adenosine had a similar effect in overriding the inhibition of RNA synthesis by AMS. Because the RNA/protein ratio of FL cells treated with dimethyl sulfoxide or sodium butyrate, two other potent inducers, was decreased by 44%, our results suggest that a correlation exists between the RNA content of the cells and the triggering of differentiation by inducers.

The mechanism of action of the various compounds that stimulate Friend murine erythroleukemic (FL) cells to express a program of erythroid differentiation remains unclear (1). In an effort to gain further understanding, we have been studying the interaction of two closely related compounds that play an agonist/antagonist role in modulating gene expression in FL cells. We have shown that the aminonucleoside of puromycin (AMS), an analogue of adenosine, is a potent inducer of FL cell differentiation and that the induction of differentiation by AMS is inhibited by the addition to the medium of a purine such as inosine or adenosine (2). AMS had been shown to inhibit ribosomal RNA synthesis (3) and to decrease the RNA content of heteroploid cells. These effects were prevented by inosine (4, 5), which has been reported to inhibit the accumulation of globin mRNA in AMS-treated FL cells (6).

In the present study, RNA synthesis in FL cells grown in medium containing AMS with and without inosine was examined and the RNA and protein contents of the cells were determined. Our data show that AMS decreases the RNA content of the cells without having an appreciable effect on protein content. When inosine at a concentration that inhibits AMS-induced differentiation of the cells is added to the medium, the RNA/protein ratio is increased as compared with that of cells treated with AMS alone. Because the RNA-to-protein content of FL cells treated with dimethyl sulfoxide (Me₂SO) and sodium butyrate is also decreased, our results suggest that a correlation exists between the RNA content of the cells and the triggering of differentiation by inducers.

MATERIALS AND METHODS

Cell Strains. FL cells, clone 745A, were maintained in continuous culture and grown in suspension in BHK-21 medium (Eurobio, Paris) supplemented with 10% (vol/vol) fetal calf serum. Cultures were initiated at a concentration of 10⁶ cells per ml of medium. Cells were counted with a Coulter counter. Hemoglobin-containing cells were detected by a modification of the wet benzidine method of Orkin et al. (7). All determinations were performed in triplicate. AMS was obtained from Sigma, Me₂SO and sodium butyrate from Fisher, and inosine and adenosine from P-L Biochemicals. All test compounds were added at the time of seeding the cultures.

Rate of RNA Synthesis. Cells labeled as noted in the figure legends were centrifuged at 250 × g for 5 min and washed twice with 20 ml of phosphate-buffered saline and once with 20 ml of isotonic NaCl solution. The acid-insoluble fraction was separated from the acid-soluble fraction by treatment of the cell pellet with 2 ml of 10% (wt/vol) trichloracetic acid at 0°C for 30 min. The acid-insoluble fraction was washed twice with 10 ml of 10% trichloracetic acid. The radioactivity incorporated in the RNA fraction was determined (i) on the acid-insoluble fraction directly hydrolyzed in 0.3 M KOH when cells were labeled with [³H]uridine or (ii) on the RNA fraction separated from phospholipids, DNA, the phosphate of phosphoproteins when [³²P] was the precursor, as described (8). When inosine was the precursor, the acid-insoluble fraction was solubilized in 0.3 M KOH, and the DNA and proteins were precipitated with pyrogalutamate (final concentration, 10% (wt/vol)). The protein content of the cell pellet was determined by the Lowry method (9) on alkaline hydrolysates of the acid-insoluble fraction as described (9). The radioactivity of the different fractions was measured in a Beckman scintillation spectrometer.

Incorporation of [¹⁴C]inosine or [³²P] in Purine Nucleotides of the Acid-Soluble Fraction. Cells labeled with [³²P] or [¹⁴C]inosine were centrifuged and washed, and the cell pellet was treated with 200 μl of 10% perchloric acid. The acid-soluble fraction was neutralized with 4.0 M KOH and kept at 0°C for 30 min before centrifugation to eliminate the potassium perchlorate. Aliquots (10 μl) of the solution and 3 μg of unlabeled ATP, ADP, AMP, and GTP were spotted on PEI cellulose sheets, and the nucleotides were separated by ascending chromatography in 2.0 M formic acid/2 M LiCl, 1:1 (vol/vol) (10).

Abbreviations: FL, Friend erythroleukemia; AMS, aminonucleoside of puromycin; Me₂SO, dimethyl sulfoxide.
The nucleotides added were detected with an ultraviolet lamp. When \(^{14}\)C]inosine was the precursor, the PEI cellulose was treated with a solution of 2,5-diphenyloxazole in ether (11), and \(^{14}\)C radioactivity was detected by fluorography with Kodak X-Omat film. When \(^{32}\)P was the precursor, the \(^{32}\)P-labeled nucleotides were detected by autoradiography with Kodirex film. Autoradiography allowed us to verify the purity of the ATP and GTP spots before assay in the Beckman scintillation spectrometer.

**Determination of ATP and GTP Content of the Cultures.** 10^9 cells per ml were seeded in the presence of \(^{32}\)PO_4 (1 \(\mu\)Ci/ml; 1 Ci = 3.7 x 10^{10} becquerels). The \(^{32}\)P incorporated into ATP and GTP was determined as described, and the cell content of ATP and GTP was calculated from the specific radioactivity of \(^{32}\)P in the medium by assuming that, 24, 48, or 72 hr after seeding the cells in the presence of \(^{32}\)PO_4, the specific radioactivity of the nucleotide phosphate was similar to the specific radioactivity of phosphate in the medium.

**Determination of the RNA and Protein Content of the Culture.** At different times after seeding, the cells were counted and the cultures were divided into three aliquots and centrifuged at 250 x g for 5 min. The cell pellets were washed with phosphate-buffered saline and isotonic NaCl as described. One of the pellets was resuspended in isotonic buffer, and aliquots were used to determine the cell density and the protein content (9). The other two pellets were treated with 10% trichloroacetic acid (1 ml); the acid-insoluble fraction was washed first with 1 ml of 10% trichloroacetic acid and then with 2 ml of ethanol and was dried before hydrolyzing the nucleic acids in 5% trichloroacetic acid at 70°C for 30 min. After centrifugation at 0°C, the RNA content was determined on the supernatant by a modified Mejbaum method (12), and the protein content was determined by the Lowry method (9) on the acid-insoluble fraction after its solubilization in 0.6 M NaOH. This method allowed a precise determination of the RNA and protein content per cell and per ml of the cultures.

**RESULTS**

**Inhibition by AMS of RNA Synthesis in FL Cells.** RNA synthesis was determined in nontreated or AMS-treated cells by following the incorporation of \(^{3}H\)uridine or \(^{32}\)P into the acid-soluble and RNA fractions of the cells. In the experiments I and II (Table 1), cells grown for 24 hr in medium with or without 5 \(\mu\)g of AMS per ml were labeled with \(^{32}\)PO_4 for 1 hr. In experiment III, the cells were labeled with \(^{3}H\)uridine for 2 hr. As compared with the controls, RNA synthesis was inhibited in AMS-treated cells, and this inhibition was evident even when the rate of RNA synthesis was calculated by the ratio \(^{32}\)P in RNA/\(^{32}\)P in the acid-soluble fraction or \(^{3}H\) in RNA/\(^{3}H\) in the acid-soluble fractions, which takes into account accumulation of the precursors in the cells.

**Inosine Metabolism in AMS-Treated Cells.** In order to compare inosine metabolism in treated and untreated cells, cells were labeled with \(^{14}\)C]inosine 24 hr after seeding the cultures in the presence or absence of AMS, and the different nucleotides of the acid-soluble pool were separated. Most of the radioactivity of the acid-soluble fraction was found on ATP and GTP spots (Table 2). Synthesis of ATP and GTP from inosine was about 50% decreased in cells treated with AMS, and the inhibition of the incorporation of the radioactivity into RNA was even greater (over 60%). This is reflected in the decrease obtained when the data were expressed as the ratio of the \(^{14}\)C in RNA to the \(^{14}\)C in the acid-soluble fraction.

Because these results suggested that inosine may act by increasing the RNA, ATP, and GTP content of the cells, we next investigated the effect of the addition of inosine at a concentration that prevents AMS-induced differentiation on the content of these molecules in the cells.

**Effect of AMS and Inosine on the RNA Content of the Cultures.** At different times after seeding the cultures in the presence or absence of AMS and AMS with inosine, the cell concentration, protein, and RNA content of the cultures were determined. No significant differences were found between the control cells and cells treated with inosine alone (data not shown).

The rates of cell growth and protein synthesis in cells treated with AMS or AMS with inosine were decreased as compared with those in controls, but because the level of inhibition was similar, the protein content per cell was not appreciably changed as compared with that of the untreated controls (Fig. 1a and b). However, the RNA content of the cultures treated with AMS (Fig. 1c) was significantly decreased. When inosine had been added to the cultures with AMS, the RNA content was increased after 48 and 72 hr of treatment as compared with that in cultures treated with AMS alone.

Calculation of the RNA/protein ratios showed a decrease in cells grown in the presence of AMS as compared with nontreated cells (Fig. 1d). Inosine could overcome much of the inhibitory effect of AMS on RNA accumulation. In cells cultured with a combination of inosine and AMS, the RNA/protein ratio was increased as compared with ratios in cells treated with AMS alone and approached the level of the untreated controls.

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**Table 1. Inhibition by AMS of RNA synthesis**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>With AMS</th>
<th>% inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(10^6)P in RNA/mg of protein</td>
<td>3.9 ± 0.5</td>
<td>2.1 ± 0.1</td>
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<tr>
<td>(32)P into RNA</td>
<td>9.7</td>
<td>5.85 ± 0.05</td>
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<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(32)P into AS</td>
<td>11.8 ± 0.2</td>
<td>5.2 ± 0.0</td>
</tr>
<tr>
<td>I</td>
<td></td>
<td></td>
</tr>
<tr>
<td>nM ([H])uridine</td>
<td>5.2 ± 0.3</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>RNA/mg of protein</td>
<td>36.5 ± 0.05</td>
<td>19.5 ± 0.05</td>
</tr>
</tbody>
</table>

**Table 2. Inosine metabolism in AMS-treated cells**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>(^{14})C incorporation, nmol/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>GTP</td>
</tr>
<tr>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.7</td>
</tr>
<tr>
<td>With AMS</td>
<td>9.0</td>
</tr>
<tr>
<td>% inhibition</td>
<td>54</td>
</tr>
<tr>
<td>II</td>
<td></td>
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<tr>
<td>Control</td>
<td>17.3</td>
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<tr>
<td>With AMS</td>
<td>8.5</td>
</tr>
<tr>
<td>% inhibition</td>
<td>52</td>
</tr>
</tbody>
</table>

Inosine metabolism in cells grown for 24 hr in the absence (control) or presence of AMS (5 \(\mu\)g/ml). \(^{14}\)C]Inosine (0.5 \(\mu\)Ci/ml) was added to the medium for 2 hr and the radioactivity incorporated into the different fractions was determined.

* AS, acid-soluble fraction.
Effect of AMS and Inosine on the ATP and GTP Content of the Cultures. We then determined the ATP and GTP content of the cultures treated with AMS or AMS with inosine. Fig. 2 shows the results calculated according to the ratio of nanomolar nucleotide to mg of protein.

Although at 24 hr there appeared to be no significant differences between the ATP content of the AMS-treated and nontreated cultures (Fig. 2a), differences were observed at the later time points. The cause of the increase over that of the control at 48 hr in the cells treated with AMS with inosine is not clear because it was seen in some experiments as early as 24 hr after seeding the cells. The increase in the ATP/protein ratio observed at 72 hr in nontreated cultures may be due to a decrease in the protein content per cell, which occurs when the cultures reach saturation density. On the other hand, the protein content of AMS-treated cells with or without inosine was not reduced at 72 hr, possibly because the cells had not yet reached the stationary phase.

The GTP content of the cultures in the same experiment is shown in Fig. 2b. In the presence of AMS, the GTP/protein ratio was increased as compared with the ratio in the other cultures. With AMS and inosine, the GTP/protein ratio was decreased as compared with that in cultures treated with AMS alone.

Effect of AMS and Adenosine on the RNA Content of FL Cells. Because adenosine is also an inhibitor of AMS-induced differentiation (2), the effect of this compound on RNA accumulation also was studied. When adenosine instead of inosine was added to the cultures in combination with AMS, the RNA/protein ratio was increased compared with that in cells treated with AMS alone (Fig. 3). After 48 hr, the action of adenosine was similar to that of inosine in overcoming the effect of AMS on the RNA accumulation in the cells.

Effect of Different Inducers on the RNA Content of FL Cells. The observation that AMS caused an inhibition of RNA synthesis that was not accompanied by a parallel inhibition of protein synthesis suggested that this might be an event required for the induction of differentiation. In order to determine whether this phenomenon was common for other inducers, the effects of Me$_2$SO and sodium butyrate, both of which are potent inducers, were examined. The RNA/protein ratios of FL cells treated with either 2% (vol/vol) Me$_2$SO or sodium butyrate (2 mM) are compared to that of cells treated with AMS (20 μM) in Fig. 4. In cells treated with each of the three inducers, the RNA/protein ratio was reduced compared to the nontreated cells. After 24 hr of treatment, the lowest ratio was observed in butyrate-treated cells, although the concentration used was not cytotoxic. After 48 hr of treatment, the cells of all treated cultures showed a decrease of about 44% in the RNA/protein ratio as compared with the ratio in the nontreated cells. In these experiments, the percentage of benzidine-positive cells scored at 96 hr in the cultures treated with AMS, Me$_2$SO, or sodium...
butyrate was 80%, 77%, and 70%, respectively, as compared to 4% in the control cultures.

**DISCUSSION**

The results of this study show that AMS, a potent inducer of differentiation (2), inhibits RNA synthesis in FL cells as it does in other cell lines (3–5). The biosynthesis of purine nucleotides from labeled inosine is depressed in AMS-treated FL cells. However, the inhibition of RNA synthesis cannot be attributed solely to the inhibition of purine biosynthesis as has been suggested (13) because the level of depression is much greater than can be accounted for by the decrease in purine biosynthesis. On the contrary, it is possible that purine biosynthesis is feedback-inhibited as a consequence of the inhibition of RNA synthesis. This hypothesis agrees with the results of Albanese and Studzinski (14), who showed that RNA synthesis in vitro in the nucleoli isolated from AMS-treated HeLa cells was inhibited as compared with that of nontreated cells.

Protein synthesis in AMS-treated FL cells is also inhibited, but to a much lesser extent than is RNA synthesis. The content of protein per cell is not significantly modified, but the content of RNA is decreased by 30–40%. This is reflected in the decrease in the RNA/protein ratio.

When [14C]inosine is added to the medium, most of the incorporated radioactivity is found in ATP, GTP, and RNA as is to be expected from what is known of the metabolism of these compounds. ATP/protein and GTP/protein ratios are slightly modified by AMS. The variation in the GTP/protein ratio seems to be related to the variations in RNA synthesis. When cellular RNA, which contains a large proportion of GMP, is inhibited, an accumulation of GTP occurs; conversely, when RNA synthesis is increased, the GTP/protein ratio is decreased. Thus, when the cells are treated with inosine at a concentration that inhibits AMS differentiation, the RNA/protein ratio of the cells increases as compared with that of cells treated with AMS alone. Such enhancement of RNA synthesis may be a reflection of the ability of inosine to counteract the effect of AMS in inducing differentiation. The mode of action of adenosine appears to be the same as that of inosine.

Thus far, the mechanism of action of the many unrelated compounds that modulate gene expression in FL cells has remained elusive. It has not been clear whether these agents act through the same or different mechanisms because evidence can be found to support each hypothesis (1). It has been postulated that the increase in G1 phase, after a complete S phase traversed in culture with the inducer, is the necessary event for FL cell differentiation (15). However, the lag in cell growth observed here with AMS alone was not eliminated by inosine, which prevents the induction of differentiation. Therefore, our observations tend to support the findings of Friedman and Schildkrut (16) that an arrest in G1 may not be a prerequisite for induction.

We have reported that the inducers can be grouped into two classes based on whether or not they have the ability to stimulate ornithine decarboxylase activity (17), and we now demonstrate that inducers of both classes cause a decrease in the RNA content of the treated FL cells. Me2SO, which falls into the first class of inducers that stimulate ornithine decarboxylase, had been observed by us (8, 18) and others (19) to cause a decrease in RNA and protein synthesis. The latter investigators had noted that the decline in the RNA content of cells treated with Me2SO for 96 hr was of greater magnitude than the decline in protein content. AMS and sodium butyrate, both of which are representatives of the second class of inducers that have little effect on ornithine decarboxylase activity, also reduce the RNA/protein ratio of treated FL cells. Our results point to a correlation between the induction of differentiation of FL cells and their RNA content and suggest that the necessary event to trigger differentiation may be an inhibition of RNA synthesis that is not coordinated with an equivalent inhibition of protein synthesis.

This hypothesis is supported by the reports that actinomycin D, a well-known inhibitor of ribosomal RNA synthesis, is an inducer of FL cell differentiation (20) and that purine analogues, such as 6-thioguanine, which inhibit de novo purine synthesis (21) and most likely RNA synthesis, induce thioguanine-resistant FL cells (22). The decrease of RNA synthesis might be either the first consequence of an inducer such as actinomycin D or might be a secondary effect, a consequence of a cascade of events produced by an inducer such as Me2SO. For example, Me2SO inhibits phosphoprotein synthesis first, then ATP turnover, and RNA synthesis (8).

Noncoordinated inhibition of RNA and protein synthesis does not appear to be restricted to FL cells induced to differentiate. That it may be a general phenomenon associated with differentiation is suggested by the report that the transcription of the 45S precursor rRNA is inhibited during myoblast differ-
entiation, although the rate of synthesis of ribosomal proteins remains the same (23).

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