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EFFECT OF tRNA FROM 5-AZACYTIDINE-TREATED HAMSTER FIBROSARCOMA CELLS ON PROTEIN SYNTHESIS *IN VITRO* IN A CELL-FREE SYSTEM*

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Abstract—The effect of transfer RNA (tRNA) isolated from 5-azacytidine (5-aza-C)-treated A(T_1)Cl-3 hamster fibrosarcoma cells on protein synthesis was investigated in a cell-free system derived from rabbit reticulocytes. The cell-free system, which contained polyribosomes, partially purified enzymes and cofactors, was dependent on the addition of tRNA for maximal protein synthesis. tRNA from 5-aza-C-treated fibrosarcoma cells was 2·5 times less active in supporting protein synthesis than normal fibrosarcoma tRNA and 4 times less active than reticulocyte tRNA. The addition of an equal amount of tRNA from 5-aza-C-treated cells to reaction mixture containing tRNA from either reticulocytes or untreated fibrosarcoma cells produced a 50 per cent inhibition of protein synthesis. The extent of inhibition was dependent on the amount of 5-aza-C tRNA added. These data suggest that part of the inhibition of protein synthesis produced by 5-aza-C in mammalian cells may be a result of functional modification of the tRNA molecule by this nucleoside analog.

5-Azacytidine (5-aza-C), a nucleoside analog of cytidine, has been demonstrated to be an active agent against experimental murine leukemia [1, 2]. More recently 5-aza-C has also been shown to be an effective agent for the treatment of acute leukemia in man [3]. The complex biochemical action of 5-aza-C on mammalian cells involves an inhibition of protein, RNA and DNA synthesis [2, 4]. It appears that 5-aza-C must be first converted to a nucleotide in order to be an active inhibitor, since cells resistant to the inhibitory effects of this analog have been shown to have a decrease in uridine cytidine kinase activity [5], the enzyme that catalyzes the phosphorylation of 5-aza-C [6]. The nucleotide derivatives of 5-aza-C inhibit *de novo* pyrimidine biosynthesis by blocking orotic acid decarboxylase [7]. In addition, the incorporation of 5-aza-C into RNA and DNA [2, 8] may be responsible for some of the biological effects produced by this analog. For example, tRNA that contains 5-aza-C shows a reduced amino acid-accepting activity *in vitro* [9]. In this report, in order to further understand the biochemical mode of action of 5-aza-C, we have isolated tRNA from 5-aza-C-treated hamster fibrosarcoma cells and have studied the effect of this tRNA on protein synthesis in a cell-free system derived from rabbit reticulocytes that is dependent on the addition of exogenous tRNA for maximal activity.

MATERIALS AND METHODS

Unlabeled L-amino acids were obtained from ICN Pharmaceuticals, Inc. (Cleveland, Ohio). Uniformly labeled [4, 5-^{3}H]L-leucine was obtained from New England Nuclear Corp. (Boston, Mass.); 5-aza-C was obtained from the National Cancer Institute (Bethesda, Md.); and dithiothreitol, phosphoenolpyruvate and pyruvate kinase were obtained from Calbiochem (La Jolla, Calif.). DEAE-cellulose (Cellex D) was obtained from BioRad Laboratories (Richmond, Calif.). Sephadex G-200 was obtained from Pharmacia Fine Chemicals Inc. (Piscataway, N.J.). The A(T_1)Cl-3 hamster fibrosarcoma cells were kindly donated by Dr. W. F. Benedict [10] and were maintained in suspension culture in McCoy's spinner medium (Flow Laboratories, Rockville, Md.) containing 10% fetal calf serum (Grand Island Biological Co., Grand Island, N.Y.). The polyribosomes and enzymes involved in protein synthesis were prepared from reticulocytes of phenylhydrazine-treated rabbits as described by Gilbert and Anderson [11]. The tRNA from rabbit reticulocytes or hamster fibrosarcoma cells was purified by modification of the method described by Gilbert and Anderson [11]. After phenol extraction, the RNA was fractionated on a column of Sephadex G-200 using a buffer containing 100 mM Tris–HCl, pH 7·5, 100 mM KCl, 1 mM $MgCl_2$, 10 mM EDTA and 1 mM β-mercaptoethanol. The u.v. absorbance peak at 260 nm corresponding to tRNA was precipitated with ethanol, and the precipitate was dissolved in 10 mM Tris–HCl, pH 7·5, containing 0·01 mM EDTA at a concentration of 25–30 $E_{260\,nm}$ (extinction at 260 nm with 1-cm light path).

The composition of the reaction mixture for measurement of protein synthesis *in vitro* in a cell-free system is shown in Table 1 (see Results). The reaction was terminated by addition of 2 ml of 10% TCA. The reaction tubes were then heated at 90–95° for 10 min to hydrolyze any radioactive acylated tRNA, and the mixture was filtered on 2·4-cm diameter Whatman GF/C glass-fiber discs. The discs were washed twice

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Table 1. Effect of tRNA from reticulocytes, fibrosarcoma cells and 5-aza-C-treated fibrosarcoma cells on protein synthesis*

tRNA	[^{3}H]leucine incorporated (pmoles)	Relative activity (%)
None	1·6 ± 0·7	18
Reticulocyte	8·8 ± 0·8	100
Fibrosarcoma	8·3 ± 0·6	94
Fibrosarcoma (5-aza-C)	4·7 ± 0·2	53

* The reaction mixture (50 μl) contained 1·0 μmole Tris-HCl, pH 7·5; 4·4 μmoles KCl; 0·2 μmole $MgCl_2$ 50 nmoles ATP; 10 nmoles GTP; 150 nmoles phosphoenolypyruvate; 4·0 nmoles L-amino acid mixture (except leucine); 200 pmoles of [^{3}H]leucine ($4·8 \times 10^5$ cpm); 0·4 E_{260} unit of polyribosomes; 0·2 unit of pyruvic kinase; 90 μg protein of reticulocyte enzyme fraction that is free of tRNA and 0·086 E_{260} unit of indicated tRNA. The mixture was incubated at 37° for 30 min and the amount of [^{3}H]leucine incorporated into the acid-insoluble fraction was measured as described in Methods. Data presented are mean ± S. D.

with 10% TCA and twice with absolute ethanol, dried and placed in vials containing scintillation fluid for measurement of radioactivity.

The fibrosarcoma cells were incubated with 100 μM freshly prepared 5-aza-C for 3 hr; then an additional 100 μM freshly prepared 5-aza-C was added to the cells for 3 additional hr. The cells were then washed by centrifugation in fresh drug-free medium and the cell pellet was stored at −60° until fractionation of the tRNA was performed.

RESULTS

The data summarized in Table 1 compare the activity in protein synthesis of tRNA from reticulocytes, fibrosarcoma cells and 5-aza-C-treated fibrosarcoma cells. There were only 1·6 pmoles of [^{3}H]leucine incorporated into protein with no addition of tRNA to the reaction mixture. When reticulocyte tRNA was added to the reaction, there was a 5-fold increase in the amount of [^{3}H]leucine incorporated into protein. tRNA from 5-aza-C-treated fibrosarcoma cells was 44 per cent less active in supporting protein synthesis than normal fibrosarcoma tRNA and 47 per cent less active than reticulocyte tRNA.

When an equal amount of tRNA from 5-aza-C-treated fibrosarcoma cells was added to the reaction mixture containing reticulocyte tRNA there was a 45 per cent inhibition of protein synthesis (Table 2). Under identical conditions, the addition of tRNA from untreated fibrosarcoma cells produced only a 15 per cent inhibition of protein synthesis.

Table 3 demonstrates the effect of adding different concentrations of tRNA from 5-aza-C-treated fibrosarcoma cells to a reaction mixture containing a constant amount of reticulocyte tRNA. As the concentration of the tRNA from the 5-aza-C-treated cells increases, the amount of inhibition of protein synthesis increases. As little as 0·14 E_{260} unit of the 5-aza-C tRNA produced almost a complete inhibition of protein synthesis.

The effect of increasing concentrations of reticulocyte tRNA on the inhibition of protein synthesis produced by a constant amount of tRNA from 5-aza-C-treated cells is shown in Table 4. In the presence of 0·04 E_{260} unit of reticulocyte tRNA, 0·03 E_{260} unit of 5-aza-C tRNA produced a 31 per cent inhibition of protein synthesis. When the amount of reticulocyte tRNA was increased to 0·08 E_{260} unit, the inhibition produced by 0·03 E_{260} unit of aza-C tRNA was reduced to 15 per cent. In the presence of low amounts of reticulocyte tRNA, the tRNA from the 5-aza-C-treated cells did not inhibit protein synthesis.

DISCUSSION

Treatment of mammalian cells with 5-aza-C results in a profound inhibition of protein synthesis [2]. It is not known whether the inhibition of protein synthesis produced by this analog is due to the direct

Table 2. Effect of tRNA from fibrosarcoma cells or 5-aza-C-treated fibrosarcoma cells on activity of reticulocyte tRNA in protein synthesis*

tRNA	[^{3}H]leucine incorporated (pmoles)	Inhibition (%)
Reticulocyte	7·2 ± 0·1	0
Reticulocyte + fibrosarcoma	6·1 ± 0·1	15
Reticulocyte + fibrosarcoma (5-aza-C)	3·2 ± 0·7	55

* The experimental conditions were the same as those given in Table 1 except that the total E_{260} added for reticulocyte tRNA, when used alone, was 0·16 unit. In experiments with two types of tRNA, 0·08 E_{260} unit each of reticulocyte tRNA and either fibrosarcoma or fibrosarcoma (5-aza-C) tRNA were added to the reaction mixture. The data presented are mean ± S. D.

Table 3. Effect of different concentrations of tRNA from 5-aza-C-treated fibrosarcoma cells on activity of reticulocyte tRNA in protein synthesis*

tRNA fibrosarcoma (5-aza-C) (E_{260} unit)	[^{3}H]leucine incorporated (pmoles)	Inhibition (%)
	9·1 ± 0·3	
0·035	7·0 ± 0·2	23
0·07	3·4 ± 0·2	62
0·14	0·2 ± 0·2	99

* The experimental conditions were same as those given in Table 1 except that 200 pmoles of [^{3}H]leucine (5·1 × 10^5 cpm), 0·086 E_{260} unit of reticulocyte tRNA and the indicated E_{260} of tRNA from 5-aza-C-treated fibrosarcoma cells were used in the reaction mixture. The data presented are mean ± S. D.

interaction of 5-aza-C nucleotides with polyribosome complex, the inhibition of messenger RNA synthesis or the incorporation of 5-aza-C into various RNA species producing nonfunctional molecules.

A preliminary report by Kalousek *et al.* [9] suggests that the incorporation of 5-aza-C into tRNA [2, 8] may, in fact, be responsible for part of the inhibition of protein synthesis observed since tRNA that contains 5-aza-C shows a reduced amino acid-accepting activity *in vitro*. In this report we have extended these studies and investigated the effect of tRNA from 5-aza-C-treated cells on protein synthesis in a cell-free system derived from rabbit reticulocytes. There are two advantages of using this system *in vitro*. First, the system is dependent on the addition of exogenous tRNA in order to obtain maximal synthesis of protein [11]. Second, each of the components of the system has been partially purified, permitting a more profound analysis of drug effects.

The tRNA from untreated hamster fibrosarcoma cells was about 6 per cent less active than reticulocyte tRNA in supporting protein synthesis (Table 1). Perhaps this difference in activity is related to the species specificity of the system *in vitro* [11]. However, tRNA from 5-aza-C-treated cells was 47 per cent less active than reticulocyte tRNA, suggesting that the defect in the 5-aza-C tRNA is not due to a species difference, but possibly due to the incorporation of the 5-aza-C into this tRNA or a direct effect of 5-aza-C nucleotides on synthesis of tRNA.

When tRNA from 5-aza-C-treated cells was added to a cell-free system containing an equal amount of reticulocyte tRNA, there was marked inhibition of protein synthesis (Table 2). It appears that this inhibition may be due to the reversible binding of 5-aza-C tRNA to the polyribosome complex since increasing concentrations of reticulocyte tRNA partially reversed the inhibition produced by the 5-aza-C tRNA (Table 4). When the concentration of the 5-aza-C tRNA was increased, the inhibition of protein synthesis eventually approached 100 per cent (Table 3).

The 5-aza-C tRNA fraction contained some functional tRNA molecules, since addition of this tRNA alone to the reaction mixture produced a limited stimulation of protein synthesis (Table 1). The addition of increasing amounts of reticulocyte tRNA to a fixed amount of 5-aza-C tRNA resulted in a curve of protein synthetic activity with a maximum inhibition at almost equal amounts of the two tRNA mixtures (Table 4). In the presence of low concentrations of reticulocyte tRNA, the addition of 5-aza-C tRNA produced some stimulation of protein synthesis. At high concentrations of reticulocyte tRNA, the inhibitory effect of the 5-aza-C tRNA was partially overcome. Presumably, when most of the available binding sites on the polyribosome complex were saturated with reticulocyte tRNA, the inhibition produced by the 5-aza-C tRNA was more evident.

The inhibition of protein synthesis in the cell-free system by 5-aza-C tRNA could be explained by the incorporation of this analog into a resulting fraudulent tRNA [9]. Since the hamster fibrosarcoma cells were treated with 5-aza-C for only 6 hr and the half-life

Table 4. Effect of different concentrations of reticulocyte tRNA on inhibition of protein synthesis produced by tRNA from 5-aza-C-treated fibrosarcoma cells*

tRNA reticulocyte (E_{260} unit)	[^{3}H]leucine incorporated		Inhibition (%)
	Minus 5-aza-C tRNA (pmoles)	Plus 5-aza-C tRNA (pmoles)	
None	0·7 ± 0·2		
0·01	3·0 ± 0·3	4·0 ± 0·4	
0·02	5·0 ± 0·1	4·4 ± 0·5	12
0·04	7·2 ± 0·1	5·0 ± 0·3	31
0·08	7·2 ± 0·1	6·1 ± 0·1	15

* The experimental conditions were the same as those described in Table 1 except that 200 pmoles of [^{3}H]leucine (5·1 × 10^5 cpm), 0·03 E_{260} unit of tRNA from 5-aza-C-treated fibrosarcoma cells and the indicated E_{260} unit of reticulocyte tRNA were used in the reaction mixture. The data presented are mean ± S. D.

of cellular tRNA appears to be greater than this time interval [12], 5-aza-C could be incorporated into the internal part of only a minor fraction of newly synthesized tRNA molecules. However, it is possible that a large fraction of the cellular tRNA contained 5-aza-C at the pCpCpA terminal end because of the rapid turnover of these terminal nucleotides [13, 14]. At present we are attempting to synthesize enzymatically tRNA containing 5-aza-C in the pCpCpA terminus in order to study further the effect of these fraudulent species of tRNA on cell-free protein synthesis.

REFERENCES

1. F. Sorm and J. Vesely, *Neoplasma* **11**, 123 (1964).
2. L. H. Li, E. J. Olin, H. H. Buskirk and L. M. Reineke, *Cancer Res.* **30**, 2760 (1970).
3. M. Karon, L. Sieger, S. Leimbrock, J. Z. Finkelstein, M. E. Nesbit and J. Swaney, *Blood* **42**, 359 (1973).
4. B. SayeedaZain, R. L. P. Adams and R. C. Imrie, *Cancer Res.* **33**, 40 (1973).
5. J. Vesely, A. Cihak and F. Sorm, *Cancer Res.* **30**, 2180 (1970).
6. T. Lee, M. Karon and R. L. Momparler, *Cancer Res.* **34**, 2482 (1974).
7. J. Vesely, A. Cihak and F. Sorm, *Biochem. Pharmac.* **17**, 519 (1968).
8. M. Jurovick, K. Raska, Z. Sormova and F. Sorm, *Colln Czech. Chem. Commun. Engl. Edn* **30**, 3370 (1965).
9. F. Kalousek, K. Raska, M. Jurovcik and F. Sorm, *Colln Czech. Chem. Commun. Engl. Edn* **31**, 1421 (1966).
10. W. F. Benedict, N. Rucker and M. Karon, *Proc. Am. Ass. Cancer Res.* **15**, 138 (1974).
11. J. M. Gilbert and W. F. Anderson, *J. biol. Chem.* **245**, 2342 (1970).
12. W. Blobel and V. R. Potter, *Biochim. biophys. Acta* **166**, 48 (1968).
13. C. E. Holt, P. B. Joel and E. Herbert, *J. biol. Chem.* **241**, 1819 (1966).
14. R. M. Landin and Y. Moule, *Biochim. biophys. Acta* **129**, 249 (1966).