The Effects of Chemotherapeutic Agents on the RNA Metabolism of ^{90}Sr-Induced Rat Chloroleukemia (35495)

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The ^{90}Sr-induced rat chloroleukemia, discovered by Dr. Robert K. Jones, Lovelace Foundation, Albuquerque, New Mexico, in March 1966, during studies of ^{90}Sr effects on bone marrow, has been maintained by intraperitoneal transfer into newborn Holtzman rats. All experimental animals develop chloroleukemia with a mean survival time of 27 days (10, 11). In the present study, experiments were conducted to examine the effects of various chemotherapeutic agents on the RNA metabolism of these cells cultured *in vitro*, and to correlate *in vivo* destruction of the chloroleukemia. *In vitro* tests for chemotherapeutic agents have been proposed to predict *in vivo* cytotoxicity (3–5). The present studies have shown that various chemotherapeutic agents appeared to interfere with ^{3}H-uridine incorporation into cytoplasmic 18S and 28S rRNA[1] as well as nuclear 18S and 32S RNA, but correlated poorly with *in vivo* studies.

Materials and Methods. All glassware and autoclavable pieces of equipment were autoclaved to destroy RNAase, and disposable gloves were worn to reduce RNAase contamination. All procedures were conducted at 0° unless stated otherwise. Rat chloroleukemia cells were harvested at 19 ± 3 days from large soft abdominal tumor masses. Total tumor masses weighing from 1 to 3 g were removed aseptically into Eagle's minimum essential medium containing 0.002 *M* L-glutamine (9) and 0.1 part fetal calf serum (MSG), then minced into fragments less than 0.5 mm^3. The fragments were homogenized with a motor driven tissue grinder (Arthur Thomas, size A, No. 4288-B), washed twice with MSG, and reconstituted with MSG to a concentration of 5×10^6 cells/ml. Tumor cells were equilibrated at 37° for 1 hr, then ^{3}H-uridine (New England Nuclear, 26.2 Ci/mmole) was added to a concentration of 10 μCi/ml. Ten-ml samples were removed at intervals and the incorporation was stopped by pouring the sample into 10 ml of ice-cold MSG, centrifuged (800*g*, 5 min), and the cells were resuspended in 2 ml of hypotonic buffer (RSB) (0.001 *M* NaCl, 0.0015 *M* $MgCl_2 \cdot 6H_2O$, 0.01 *M* Tris, pH 7.4) (15). The cells were broken with a precision bore, stainless steel ball homogenizer (0.0015 in clearance) (15). Approximately 10–15 strokes, monitored by phase contrast and Nomarski interference-contrast, produced efficient cell rupture while minimizing nuclear rupture.

The nuclei were removed by centrifugation (800*g*, 5 min) and the supernatant was saved for cytoplasmic RNA extraction. The nuclei were resuspended in 2 ml of RSB, centrifuged as before, and the supernatant was combined with the previous supernatant. The nuclei were again resuspended in 2 ml of RSB and 0.3 ml of a detergent mixture [1 part 10% (w/w) sodium deoxycholate and 2 parts 10% (w/w) Tween-40, polyoxyethylene sorbital monopalmitate] was added and mixed with a Vortex mixer for 5 sec. The nuclei were removed by centrifugation and the supernatant was added to previous ones. At this point the nuclei appeared quite free of cyto-

[1] Abbrev.: ara-C, cytosine arabinoside (1-β-D-arabinofuranocylcytosine); EDTA, ethylenediaminetetraacetic acid, sodium salt; 6-MMPR, 6-methylmercaptopurine ribonucleoside; Poly I:C, polyinosinic–polycytidylic acid; PPO-POPOP, 2,5-diphenyloxazole; *p*-bis[2-(5-phenyloxazoyl)]-benzene; RNAase, ribonuclease; rRNA, ribosomal RNA; SDS, sodium dodecyl sulfate; and VLB, vinblastine sulfate.

plasmic fragments when examined by phase contrast and Nomarski interference-contrast, and there appeared no sign of an outer nuclear membrane indicating that it could be effectively removed by this technique as observed by electron microscopy.

The nuclear pellet was disrupted by digestion with deoxyribonuclease (Worthington Biochemical Corp., electrophoretically purified from ribonuclease) in high salt buffer (HSB) (0.5 *M* NaCl, 0.5 *M* $MgCl_2$, 0.01 *M* Tris–HCl, pH 7.4) (15). Two ml of HSB were added to the nuclear pellet, warmed to 37°, and approximately 100 μg of deoxyribonuclease was added. The mixture was stirred with a sterile glass rod for approximately 2 min until a less viscous solution resulted.

Phenol extraction of RNA. HSB–Mg buffer (0.24 *M* NaCl, 0.01 *M* $MgCl_2$, 0.01 *M* Tris, pH 7.8), 2.5 ml, was added to the digested nuclear fractions. The following were then added to the cytoplasmic and nuclear fractions: 0.5 ml of 10% SDS, 0.5 ml of 2.5% bentonite in 0.01 *M* sodium acetate, and 5 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline (2). After shaking at 0° for 20 min, the phases were separated by centrifuging at 800*g* for 10 min. The aqueous layer was saved while the phenol layer was re-extracted with 5 ml of HSB–Mg by shaking for 5 min at 50°. The emulsion was chilled and centrifuged at 800*g* for 10 min. The aqueous layer was combined with the previous aqueous portion and re-extracted twice with 5 ml of phenol at 50° for 5 min. After the final phenol extraction, the aqueous fraction was centrifuged at 15,000*g* for 30 min to remove the bentonite. One-tenth vol of 20% sodium acetate and 2 vol of 95% ethanol at —20° were added to the supernatant and the RNA was allowed to precipitate overnight at —20°.

Density gradient separations of RNA. The RNA was centrifuged at 12,000*g* for 30 min; the ethanol was decanted; and the precipitate was dried by inverting the tube at 5° for 30 min. The RNA was dissolved in SDS buffer (0.1 *M* NaCl, 0.001 *M* EDTA, 0.01 *M* Tris–HCl, pH 7.4, 0.2% SDS), layered on a 10–30% linear sucrose gradient, and centrifuged in a SW 40 Ti rotor for 19 hr at 25,000 rpm (77,560*g*). The centrifuged gradients were displaced with a 40% sucrose solution and continuously monitored at 254 mμ through a Model D Density Gradient Fractionator (Instrumentation Specialties Co., Inc., Lincoln, Nebraska); 0.3-ml fractions were collected. Serum bovine albumin (50 μg) was added as carrier and each fraction diluted tenfold with 10% trichloroacetic acid. The precipitate was collected on Whatman glass filter (GF/C) after standing 30 min at 4°, washed with 15 ml of 10% trichloroacetic acid, dried, and counted in a Nuclear-Chicago, Model 725, liquid scintillation counter utilizing 10 ml of toluene and Nuclear-Chicago Spectrofluor (PPO-POPOP).

RNA fractionation on acrylamide gel. The RNA was phenol extracted as described above and purified RNA was electrophoresed in acrylamide and methylene bisacrylamide according to Loening (13).

Incubation with chemotherapeutic agents. When *in vitro* chemotherapeutic agents were used, the agent was added during the 1-hr equilibration. The remainder of the procedure was performed as described. The particular agents were chosen because some have been used clinically (L-asparaginase, Daunomycin, VLB, ara-C, hydrocortisone) and others were experimental (Poly I:C, DL threonine, 6-MMPR). The concentrations chosen represented minimal *in vitro* concentrations which inhibited RNA synthesis in human or murine leukemia cells.

Chemotherapeutic agents were obtained from the following sources: vinblastine sulfate (Dr. C. J. Jansen, The Lilly Research Laboratories, Indianapolis, Indiana); Poly I:C (Miles Laboratories, Elkhart, Indiana); L-asparaginase (Dr. James A. Page, Merck Sharp and Dohme, West Point, Pennsylvania); cytosine arabinoside (Dr. Paul W. O'Connell, The Upjohn Co., Kalamazoo, Michigan); Daunomycin (Farmitalia Research Laboratory, Milan, Italy); hydrocortisone (The Upjohn Co., Kalamazoo, Michigan); 6-methylmercaptopurine ribonucleoside (Dr. Harry P. Wood, National Institutes of Health, Bethesda, Maryland); DL-threonine (Sigma Chemical Co., St. Louis, Missouri).

In vivo *Experiments. Poly I:C.* The schedule of treatment used was as follows: starting 24

TABLE I. Effect of Chemotherapeutic Agents on Specific Activity[a] at 60 min.

	Cytoplasm				Nucleus			
	18S	% of control	28S	% of control	18S	% of control	32S	% of control
Control (4)[b]	64,160	100	37,687	100	72,236	100	206,931	100
Vinblastine sulfate (2) 2×10^{-6} *M*	26,622	42	13,620	36	23,240	32	72,685	35
Cytosine arabinoside (2) 1×10^{-3} *M*	53,294	83	24,845	66	20,503	28	104,236	50
Daunomycin (2) 1 μg/ml	12,538	20	6011	16	5858	8	21,595	10
L-Asparaginase (2) 5 units/ml	55,040	86	28,944	77	7787	11	83,247	40
Hydrocortisone (2) 5×10^{-4} *M*	15,861	25	10,788	26	5929	8	62,376	30
Total RNA, OD_{254} units[c]	0.39 ± 0.04		0.79 ± 0.12		0.07 ± 0.01		0.10 ± 0.02	

[a] Sp act, cpm/OD_{254} unit; OD_{254} unit = (OD_{254}) (vol).
[b] Chloroleukemia of control and test animals 16–17 days postinoculation; all incubations were performed in duplicate; controls, in quadruplicate. Specific activities are averages.
[c] Av ± 1 SD in each sample.

hr after intraperitoneal transfer of the chloroleukemia 5 μg/g was given intraperitoneally to each of 4 rats on Mondays, Wednesdays, and Fridays for 3 weeks (12, 19) at which time they were sacrificed, examined for the extent of tumor growth as compared to a control group and the RNA extracted and analyzed as described above. Twenty-four hr prior to sacrifice 100 μCi of ^{3}H-uridine (New England Nuclear, 26.2 Ci/mmole) was given intraperitoneally.

VLB. 100 μg/day at days 19 and 20 posttransfer was given intraperitoneally to 4 rats (7); 24 hr prior to sacrifice on day 21, ^{3}H-uridine was given as described above.

L-Asparaginase. 200 units/rat to 4 rats was given intraperitoneally each day for 5 days before sacrifice on day 21, ^{3}H-uridine administered 24 hr prior to sacrifice (1), and the chloroleukemia analyzed as described above.

Appropriate controls of 4 rats with each group received an equal volume of drug diluent.

Results. Sedimentation coefficients in sucrose density gradients were determined by using known *E. coli* 16S and 23S rRNA as markers.

Table I lists the effects of various chemotherapeutic agents on the incorporation of ^{3}H-uridine into nuclear and cytoplasmic RNA. All of the agents tested decreased the incorporation of ^{3}H-uridine into nuclear and cytoplasmic RNA. Increasing the dosage of L-asparaginase to 500 units/ml decreased ^{3}H-uridine incorporation compared to controls as follows: cytoplasm, 3% (18S), 4% (28S); nucleus, 0% (18S), 9% (32S). Also increasing the dosage of ara-C to 4×10^{-3} *M* decreased ^{3}H-uridine incorporation as follows: cytoplasm, 3% (18S), 9% (28S); nucleus, 0.3% (18S), 5% (32S). These preliminary results suggest that at higher concentrations of L-asparaginase and cytosine arabinoside ^{3}H-uridine incorporation is markedly decreased in nucleus and cytoplasm. Single experiments with Poly I:C, 6-MMPR, and DL-threonine have suggested decreased ^{3}H-uridine incorporation into both cytoplasmic and nuclear RNA. The incorporations were decreased as follows: (i) Poly I:C (5.25 mg/ml): cytoplasm, 14% (18S), 38% (28S); nucleus, 25% (18S), 23% (32S). (ii) 6-MMPR (1×10^{-4} *M*): cytoplasm, 7% (18S), 20% (28S); nucleus, 3% (18S) 9% (32S). (iii) DL-threonine (0.4 mg/ml): cytoplasm, 7% (18S), 26% (28S); nucleus 31%

TABLE II. Effect of Chemotherapeutic Agents on Specific Activity[a] at 60 min.

	Cytoplasm				Nucleus			
	18S	% of control	28S	% of control	18S	% of control	32S	% of control
				Vinblastine sulfate				
Control[b]	139,939	100	58,462	100	243,250	100	363,578	100
$2 \times 10^{-8} M$	89,977	64	44,150	76	38,290	16	218,887	60
$2 \times 10^{-7} M$	43,579	31	20,432	35	21,605	9	110,451	30
$2 \times 10^{-6} M$	13,409	10	13,792	2	5649	2	23,279	6
$2 \times 10^{-5} M$	6348	5	7581	1	1665	1	3802	1
Total RNA, OD_{254} units[c]	0.37 ± 0.09		0.78 ± 0.23		0.09 ± 0.02		0.14 ± 0.04	
				Cytosine arabinoside				
Control[b]	159,011	100	57,274	100	156,245	100	252,211	100
$1 \times 10^{-5} M$	141,187	89	58,047	101	117,937	76	166,663	66
$1 \times 10^{-4} M$	109,497	69	46,551	81	86,180	55	148,514	59
$1 \times 10^{-3} M$	54,477	34	24,077	42	43,891	28	70,011	28
$1 \times 10^{-2} M$	21,787	14	8143	14	21,121	14	20,947	8
$1 \times 10^{-1} M$	1365	1	685	1	1120	1	1571	1
Total RNA, OD_{254} units[c]	0.38 ± 0.11		0.87 ± 0.29				0.16 ± 0.04	
				Daunomycin				
Control[b]	20,387	100	12,339	100	13,966	100	37,077	100
1×10^{-1} μg/ml	21,817	107	13,882	112	4881	35	33,264	90
1 μg/ml	21,345	104	6112	50	2694	19	17,072	46
10 μg/ml	631	3	111	1	1120	8	906	2
Total RNA, OD_{254} units[c]	0.33 ± 0.02		0.66 ± 0.04		0.05 ± 0.01		0.16 ± 0.02	

[a] Sp act, cpm/OD_{254} unit; OD_{254} unit = (OD_{254}) (vol).
[b] Chloroleukemia 18–19 days of age.
[c] Av ± SD in each sample.

(18S), 78% (32S).

DL-Threonine was examined because human chronic granulocytic leukemic leukocytes have been shown to reduce incorporation of DNA, RNA, and protein precursors when cultured in a serine-deficient medium (16). Our preliminary evidence suggests that DL-threonine inhibits ^{3}H-uridine incorporation into cytoplasmic and nuclear RNA; Meinke and Holland have shown that its effects are not limited to a single biochemical step and that DL-threonine (0.4 mg/ml) is a competitive antagonist to serine in bacteria.

Table II tabulates the effects of VLB, ara-C, and Daunomycin at various concentrations on the incorporation of ^{3}H-uridine into nuclear and cytoplasmic RNA in cells 18–19 days postintraperitoneal transfer. As shown, higher concentrations of these drugs successively decreased ^{3}H-uridine incorporation into the nucleus and cytoplasm.

In vivo poly I:C decreased ^{3}H-uridine incorporation in the cytoplasm to an average of 50% (18S) and 67% (28S), and in the nucleus to 16% (18S) and 18% (32S) of controls. *In vivo* L-asparaginase decreased ^{3}H-uridine incorporation in the cytoplasm to an average of 20% (18S) and 30% (28S), and in the nucleus to 0% (18S) and 2% (32S) of controls. Vinblastine sulfate intraperitoneally caused chloroleukemic regression to small whitish remnants; no chloroleukemic cells could be seen under the light microscope.

Discussion. Various chemotherapeutic agents had an inhibitory effect on ^{3}H-uridine incorporation into both cytoplasmic 18S and 28S rRNA and nuclear 18S and 32S RNA of the rat chloroleukemic cell. Generally, there appeared to be equal inhibition of ^{3}H-uridine incorporation into cytoplasmic 18S and 28S rRNA, but nuclear 18S RNA inhibition as compared to 32S RNA was

somewhat variable. Recent evidence indicates that rRNA in mammalian cells is first synthesized as a 45S precursor which is subsequently cleaved to a 41S and 36S molecule. The 36S molecule gives rise to the 18S rRNA and 32S precursor, which subsequently is degraded to the 28S rRNA (8). This being the case, 18S and 28S rRNA should be produced in equimolar quantities. Recently, Cooper (6) presented evidence that rRNA production regulates lymphocyte growth by limiting the accumulation of ribosomes, which it does by regulating the quantity of 18S rRNA synthesized and utilized for ribosome production. In our case, nuclear 18S was present in smaller quantities than the 32S, supporting Cooper's proposal. Our nuclear 18S RNA is probably not rRNA contamination from the outer nuclear membrane or cytoplasm since 28S rRNA which would also derive from cytoplasmic contamination is never found. Polyacrylamide gel electrophoresis of the 32S nuclear RNA appeared as a single species. In addition, electron microscopy indicated that the outer nuclear membrane, to which ribosomes (containing 18S and 28S rRNA) are known to be attached, had been removed. The significance of nuclear 18S RNA remains speculative, but after 45S RNA cleaves to 18S and 28S rRNA, the 18S rRNA may transiently remain in the nucleus prior to being transported to the cytoplasm as the ribosomal subunit; whereas the 28S rRNA is transported promptly to the cytoplasm possibly to interact with 7S rRNA (8).

Stevens *et al.* (17) have found that L-asparaginase decreased ^{3}H-uridine incorporation into 28S rRNA greater than 18S rRNA in the P1798 lymphosarcoma, while Wagner and Roizman (18) using VLB with human (HEp-2) cells found 28S rRNA synthesis was preferentially inhibited over 18S rRNA. In our case, cytoplasmic 18S and 28S rRNA were generally inhibited to the same extent, which would suggest inhibition of nucleolar 45S, 41S, or 36S synthesis, or equal inhibition of nucleolar 36S and 32S, direct precursors of 18S and 28S rRNA. The sp act of 18S nuclear RNA was consistently lower than that of 32S nuclear RNA; the various chemotherapeutic agents did not alter this proportion, although the sp act decreased. Neither Stevens *et al.* nor Wagner and Roizman studied nuclear RNA, therefore, we cannot compare all our results with theirs.

Preliminary evidence suggests poor correlation between *in vivo* destruction of tumor and *in vitro* ^{3}H-uridine incorporation into nuclear or cytoplasmic RNA as tested with VLB, L-asparaginase, and Poly I:C. L-Asparaginase given intraperitoneally decreased ^{3}H-uridine in the cytoplasm to an average of 20% (18S) and 30% (28S) and in the nucleus to 0% (18S) and 2% (32S), yet the accumulated mass of chloroleukemia appeared equal to the controls. Similar results were obtained with Poly I:C, although the decrease in ^{3}H-uridine incorporated was not as great. Vinblastine sulfate appeared to destroy most of the chloroleukemic cells within 48 hr and only remnants could be seen. Therefore, *in vivo* ^{3}H-uridine uptake studies into RNA could not be performed, although there appeared to be moderate inhibition *in vitro*.

All the chemotherapeutic agents tested appeared to affect nuclear and cytoplasmic RNA synthesis during the formation of rRNA. Whether RNA metabolism in the chloroleukemic cell is deranged or unregulated is unknown, but all the effective chemotherapeutic agents tested were inhibitory to one or more aspects of RNA synthesis and processing. Preliminary evidence indicates poor correlation between *in vivo* destruction of tumor and *in vitro* ^{3}H-uridine incorporation into nuclear or cytoplasmic TNA as tested with L-asparaginase and Poly I:C.

Summary. The metabolism of nuclear and cytoplasmic ribonucleic acid (RNA) of a ^{90}Sr-induced rat chloroleukemia was characterized and the effects of chemotherapeutic agents on this RNA metabolism studied.

The *in vitro* ^{3}H-uridine incorporation into nuclear and cytoplasmic RNA of this tumor is inhibited by certain chemotherapeutic agents. The following chemotherapeutic agents were studied: vinblastine sulfate; polyinosinic–polycytidylic acid; L-asparaginase; cytosine arabinoside; Daunomycin; hydrocortisone; 6-methylmercaptopurine ribonucleoside; DL-threonine (utilized as a serine antagonist). In general, the chemotherapeutic agents tested appeared to interfere with ^{3}H-

uridine incorporation into cytoplasmic and nuclear RNA. Preliminary evidence suggests poor correlation between *in vivo* destruction of tumor and *in vitro* ^{3}H-uridine incorporation into nuclear or cytoplasmic RNA with vinblastine sulfate, L-asparaginase, and polyinosinic–polycytidylic acid.

1. Adamson, R. H., and Fabro, S., Cancer Chemother. Rep. (Part I) **52**, 617 (1968).
2. Cline, M. J., J. Lab. Clin. Med. **68**, 33 (1966).
3. Cline, M. J., Blood **30**, 176 (1967).
4. Cline, M. J., N. Engl. J. Med. **280**, 955 (1969).
5. Cline, M. J., and Rosenbaum, E., Cancer Res. **28**, 2516 (1968).
6. Cooper, H. L., J. Biol. Chem. **244**, 1946 (1969).
7. Creasey, W. A., and Markiw, M. D., Biochem. Pharmacol. **13**, 135 (1964).
8. Darnell, J. E., Jr., Bacteriol. Rev. **32**, 262 (1968).
9. Eagle, H., Science **130**, 432 (1959).
10. Jones, R. K., U.S. At. Energy Comm. Lovelace Found. Annu. Rep. **39**, 217 (1968).
11. Jones, R. K., Brooks, A. L., Ferris, A. C., and Shaffer, D. K., U.S. At. Energy Comm., Myeloproliferative Disorders of Animals and Man, pp. 517-532, USAEC Division of Technical Information Extension, Oak Ridge, Tennessee, 1970 (paperback).
12. Levy, H. B., Law, L. W., and Rabson, A. S., Proc. Nat. Acad. Sci. U.S.A. **62**, 357 (1969).
13. Loening, U. E., Biochem. J. **102**, 251 (1967).
14. Meinke, W. W., and Holland, B. R., J. Biol. Chem. **193**, 535 (1948).
15. Penman, S., Smith, I., Holtzman, E., and Greenberg, H., Nat. Can. Inst. Monogr. **23**, 489 (1966).
16. Regan, J. D., Vodopick, H., Takeda, S., Lee, W. H., and Faulcon, F. M., Science **163**, 1452 (1969).
17. Stevens, J., Mashburn, L. T., and Hollander, V. P., Biochim. Biophys. Acta **186**, 332 (1969).
18. Wagner, E. K., and Roizman, B., Science **162**, 569 (1968).
19. Zeleznick, L. D., and Bhuyan, B. K., Proc. Soc. Exp. Biol. Med. **130**, 126 (1969).

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