PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 173, 238–243 (1983)

Inhibition of HeLa-S3 Cell Proliferation and Biosynthesis by 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (41638)

IGOR TAMM AND TOYOKO KIKUCHI

The Rockefeller University, New York, New York 10021

Abstract. Treatment of HeLa-S3 cells in suspension cultures with 60 μ*M* 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) for 18–30 hr stops the growth of the cell population when treatment is carried out at 37°C in Eagle's spinner culture medium supplemented with 5% fetal bovine serum. The length of the period of no growth after termination of treatment is directly related to the duration of DRB treatment. Upon resumption of growth, the rate becomes exponential and is not distinguishably different from the control rate (doubling time: 19 hr). The growth of the progeny population of the previously DRB-treated cells is as sensitive to inhibition by DRB as the growth of control populations not treated with DRB. After treatment of cells with DRB for 30 hr at 39.5–40°C, the population which grows out has a prolonged doubling time. DRB treatment at 37°C for 5 hr markedly inhibits uridine uptake and cellular RNA synthesis in the presence either of 5 or 15% serum. After treatment for 48 hr in 15% serum, inhibition of RNA synthesis by DRB is significantly decreased. DRB treatment does not inhibit leucine uptake in HeLa cells growing in suspension cultures. Protein synthesis is moderately inhibited in 5% serum and only slightly inhibited in 15% serum after either 5- or 48-hr period of treatment.

Studies of the action of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) in mammalian cells have shown that this compound selectively inhibits the transcription by RNA polymerase II of heterogeneous nuclear RNA (hnRNA) from DNA, and thereby suppresses mRNA synthesis (1–6). Comparative investigation of the effects of DRB on the biosynthesis of hnRNA and the proliferation of HeLa cells in monolayer cultures has revealed the following: (i) Under stringent conditions of treatment of cells (60–80 μ*M* DRB, 24- to 40-hr exposure, 5% serum concentration), there is a net decrease in cell number and the culture enters an apparent "dormant" phase which is ultimately followed by resumption of proliferation at the control rate several days following termination of treatment (unpublished data); (ii) The rate of hnRNA synthesis does not remain severely depressed on prolonged treatment; instead inhibition becomes partially reversed between 5 and 24 hr from the beginning of continuous treatment (unpublished data); (iii) In long-term experiments, the inhibition of cell proliferation by DRB is inversely related to serum concentration in the range from 5 to 30%, as serum enhances both the cycling and survival of DRB-treated cells (7); the inhibition of hnRNA synthesis is similarly affected but to a lesser extent (unpublished data).

In the present study we have investigated the effects of DRB on the proliferation and the RNA- and protein-synthesizing activities of HeLa-S3 cells in suspension cultures, and have demonstrated that the cell population that grows out after termination of severe DRB treatment is as sensitive to the growth-inhibitory action of DRB as the parent population. If treatment is carried out at 39.5–40°C instead of at 37°C, the population that grows out proliferates at a reduced rate. We also report on the inhibition of RNA and protein synthesis after 5 or 48 hr of treatment with DRB in medium supplemented with 5 or 15% fetal bovine serum.

Materials and Methods. *Cells and chemicals.* HeLa-S3 cells were grown in suspension culture at 37°C in Eagle's minimum essential medium modified for spinner culture (8) and supplemented with 5% fetal bovine serum (GIBCO, Grand Island, N.Y.). When cells reached 4×10^5/ml, they were centrifuged and washed once with warm medium prior to treatment with 60 μ*M* 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), which

0037-9727/83 $1.50

was dissolved by shaking a 120 μM stock preparation in medium without serum overnight at 37°C.

Cell counts. Daily counts were done in a Coulter counter and a hemocytometer. Viability of cells was determined by erythrosine B exclusion (9).

Uptake of precursors and RNA and protein synthesis. Cultures of HeLa-S3 cells were set up in spinner medium containing 5 or 15% serum, with or without 60 μM DRB. Five and 48 hr after beginning of treatment, triplicate 2×10^5 cell aliquots of each of four cultures were labeled for 15 min with [5-^{3}H]uridine (25 Ci/mmole, 5 μCi/ml) or L-[4,5-^{3}H(N)]leucine (5 Ci/mmole, 10 μCi/ml) (New England Nuclear, Boston, Mass.). Samples were centrifuged at 4°C, washed twice with ice-cold PBS, and resuspended in 1 ml of phosphate-buffered saline (PBS) and an equal volume of 10% TCA. Aliquots were taken for determination of total cell-associated radioactivity (1, 10). Uridine-labeled samples were then precipitated at 4°C, whereas leucine-labeled samples were first incubated at 37°C for 2 hr and then precipitated at 4°C. The precipitates were collected for determination of radioactivity. Inhibition of RNA or protein synthesis by DRB was estimated by calculating the ratios of acid-precipitable to total counts in DRB-treated and control samples, and expressing the value for DRB as a percentage of that for control.

Results. *Effect of the duration of treatment with DRB (60 μM) on the reversibility of inhibition of cell proliferation.* Figure 1A shows that the proliferation kinetics of populations of HeLa cells that have been treated with 60 μM DRB for 18, 24, or 30 hr are characterized by two features: (i) The population growth ceases and remains in abeyance for a period which is directly related to the length of treatment with DRB; and (ii) after a period during which the cell number does not increase, proliferation resumes at an exponential rate similar to the control rate. The small early increase in total cell number in DRB-treated cultures reflects two circumstances: (i) numerous mitoses as well as cell deaths occur during the initial treatment period (7); and (ii) by Coulter counting, both living and dead cells are enumerated (see below).

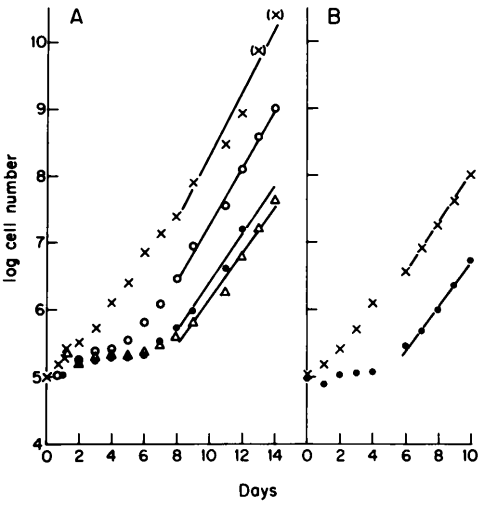

FIG. 1. Reversibility of inhibition of proliferation of HeLa-S3 cells by 60 μM DRB. (A) Effect of the duration of treatment. Cells were suspended at a density of 10^5 cells/ml in spinner medium containing 5% serum. Cells were treated with DRB for 18, 24, or 30 hr at 37°C. Controls received no DRB. After treatment, cells were washed once by centrifugation, resuspended in DRB-free medium, and incubation was continued. Cell counts were done daily, and each time cultures diluted to a concentration of 10^5 cells/ml. Control, × (geometric mean values for 18-, 24-, and 30-hr controls, except the last two points are means for 18- and 30-hr controls only); DRB: 18 hr (○), 24 hr (●), 30 hr (△). The slopes were obtained by linear regression analysis beginning with the eighth day after the start of the experiment. (B) Response of previously DRB-treated cells to second treatment. Eleven days after reversal of the 24-hr DRB-treated cultures (see A), control and DRB-treated cells were washed once by centrifugation and resuspended in control or DRB-containing medium, respectively. The second DRB treatment was carried out for the same period as the first. DRB was then removed and incubation of cells was continued in DRB-free medium. Cell counts were determined daily, and cultures diluted to a concentration of 10^5 cells/ml. Control (×); DRB (●). The slopes were obtained by linear regression analysis beginning with the sixth day after the start of the experiment.

The curve for the culture treated with DRB for 18 hr indicates that rapid proliferation resumed by the fifth day after termination of DRB treatment; that for the 30-hr treated culture indicates resumption of rapid proliferation by the eighth day. The actual recovery periods may be somewhat shorter as the recording by the Coulter counter of dead cells

that are present obscures the behaviour of living cells.

After establishment of close to exponential growth following recovery from the effects of DRB treatment, the doubling times for populations which had been treated for 18, 24, or 30 hr were 17, 20, and 21 hr, respectively. These values were calculated by linear regression analysis, beginning with the eighth day after the start of the experiment; the respective correlation coefficients were 0.9965, 0.9898, and 0.9844. The doubling times for the corresponding control cultures were 14, 28, and 16 hr, and the correlation coefficients were 0.9584, 0.9840, and 0.9909. It is evident that the doubling times for the control cultures and also those for cultures which had been treated with DRB did not show any systematic variation. The mean doubling time for the three control cultures was 19 hr as was also that for the three experimental cultures.

Effect of a second DRB (60 μM) treatment on proliferation kinetics of cells. Figure 1B shows that when the progeny population of the previously treated HeLa cells was treated with DRB for 24 hr, population increase ceased, but subsequently resumed at a rate similar to the rate of proliferation of the control population. The apparent recovery period after termination of the second DRB treatment (approximately 5 days) was similar to that observed after the first treatment. The doubling time during exponential growth of the cells which survived and had recovered from the second treatment was 22 hr as compared to the doubling time of 19 hr for the corresponding controls, with linear correlation coefficients of 0.9874 and 0.9982, respectively, for curves starting with the 6-day time point.

Effect of elevated temperature during DRB (60 μM) treatment on the reversibility of inhibition of cell proliferation. Figure 2 shows that incubation of HeLa cells during 30-hr DRB (60 μM) treatment at 39.5–40°C, instead of at 37°C, prolonged the doubling time during postrecovery exponential growth of the surviving population. The Coulter counts again showed a small early increase, whereas hemocytometer counts for viable cells indicated a 40% decrease in cell number 2–3 days from the beginning of the 30-hr treatment period (i.e., 1–2 days after termination of treatment). The two curves join on the 10th day of the experiment and follow a closely similar course to the 24th and last day of the experiment. During this later exponential period of growth, the doubling time in the control was 32 hr whereas in the DRB-treated culture it was 58 hr. This is equivalent to a 45% decrease in the population growth rate of the DRB-treated cells relative to control cells. The culture which had been treated with DRB for 24 hr showed a smaller increase in doubling time during the exponential postrecovery period, i.e., 47 hr (data not shown). The control curve for the entire 24-day culture period shows some fluctuation: over the first 10 days the doubling time was 23.5 hr; then the growth slowed somewhat, but accelerated toward the

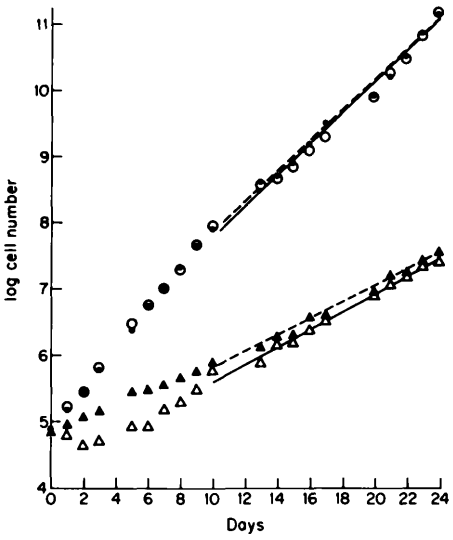

FIG. 2. Reversibility of inhibition of proliferation of HeLa-S3 cells by 60 μM DRB: effect of an elevated temperature during treatment. Cells were suspended at a density of 10^5 cells/ml in spinner medium containing 5% FCS, with or without DRB. Cultures were incubated at 39.5–40°C in a circulating water bath on a Bellco Bellstir Multi-stir (Vineland, N.J.). Thirty hours after addition of DRB, aliquots were washed once by centrifugation, resuspended in DRB-free medium, and incubation continued at 39.5–40°C. Total and viable cell counts were determined daily, and cultures diluted to 10^5 viable cells/ml. Control: Coulter (●), hemocytometer (○); DRB: Coulter (▲), hemocytometer (△). The slopes were obtained by linear regression analysis beginning with the 10th day after the start of the experiment. Coulter (- - -); hemocytometer (——).

end. The overall doubling time for the entire period was 28.8 hr.

Effects of duration of treatment and serum concentration on the inhibition of precursor uptake and RNA and protein synthesis by DRB (60 μM). Table I shows that 5-hr treatment of HeLa-S_3 cells with 60 μM DRB in suspension cultures in medium containing either 5 or 15% serum caused marked inhibition of uridine uptake. The degree of inhibition is closely similar to that observed in monolayer cultures (unpublished data). After 48-hr treatment in 5% serum, but not after treatment in 15% serum, there is even greater inhibition, which also agrees with findings in monolayer cultures (unpublished data). In suspension cultures, no significant effects on leucine uptake were observed, except that after 48-hr treatment in 5% serum the uptake appears to have become slightly reduced (Table II). In monolayer cultures, there was some inhibition of uptake after 5-hr treatment either in 5 or 15% serum and the inhibition increased considerably after 48-hr treatment in 5% but not in 15% serum.

The results in the last columns of Tables I and II provide estimates of RNA and protein synthesis in DRB-treated cultures as percentage of control values. Treatment for 5 hr caused marked inhibition of RNA synthesis either in 5 or 15% serum (Table I), which is in complete agreement with results obtained in monolayer cultures (unpublished data). After 48-hr treatment the value obtained in 15% serum indicates a significantly decreased ($P < 0.01$) inhibitory effect of DRB or RNA synthesis. The result in Table I for RNA synthesis after 48-hr treatment in 5% serum is in parentheses because there was an unusual degree of variation in the precipitable counts for controls and also for DRB-treated samples in the 5 experiments. Experiments in monolayer cultures have shown a substantial reduction in the inhibitory effect of DRB on RNA synthesis between 5 and 48 hr, with the reduction being somewhat greater in 15 than in 5% serum (unpublished data).

The results on protein synthesis (Table II) indicate moderate inhibition by DRB in 5% serum and slight or no inhibition, in 15% serum after either 5- or 48-hr treatment. In HeLa cell monolayer cultures, too, no significant change in the level of inhibition of protein synthesis was observed when treatment was prolonged from 5 to 48 hr at either serum concentration (unpublished data). After either 5- or 48-hr treatment with DRB, there appeared to be somewhat less inhibition of protein synthesis in 15% than in 5% serum (unpublished data), which is in agreement with the present results obtained in suspension cultures (see Table II).

Discussion. The results of the cell proliferation experiments demonstrate that HeLa-

TABLE I. INHIBITION OF [^{3}H]URIDINE UPTAKE AND TOTAL RNA SYNTHESIS IN HeLa-S3 CELLS AFTER TREATMENT WITH 60 μM DRB IN 5 OR 15% FETAL CALF SERUM

	Medium		[^{3}H]Uridine incorporation[a]					
						DRB, % of control		
Time (hr)	Serum (%)	DRB (μM)	Total cpm	Acid-prec. (cpm)	Prec./total (%)	Total	Acid-prec.	Prec./total
5	5	0	438,352 ± 27,560[b]	16,183 ± 2764	3.69 ± 0.83			
5	5	60	125,814 ± 7,254	1,187 ± 256	0.94 ± 0.24	29	7.3	26
5	15	0	480,219 ± 32,807	25,664 ± 4523	5.34 ± 1.15			
5	15	60	122,478 ± 23,033	2,158 ± 583	1.76 ± 0.45	26	8.4	33
48	5	0	456,860 ± 35,043	14,303 ± 5162	3.13 ± 0.90			
48	5	60	92,111 ± 16,158	672 ± 204	0.73 ± 0.11	20	4.7	(23)
48	15	0	502,963 ± 43,808	9,909 ± 1695	2.00 ± 0.37			
48	15	60	160,934 ± 34,374	1,498 ± 403	0.93 ± 0.14	32	15	47

[a] cpm/2 × 10^5 cells.
[b] Mean ± SEM, based on five experiments.

TABLE II. INHIBITION OF [^{3}H]LEUCINE UPTAKE AND TOTAL PROTEIN SYNTHESIS IN S3 CELLS AFTER TREATMENT WITH 60 μM DRB IN 5 OR 15% FETAL CALF SERUM

Medium			[^{3}H]Leucine incorporation[a]					
						DRB, % of control		
Time (hr)	Serum (%)	DRB (μM)	Total (cpm)	Acid-prec. (cpm)	Prec./total (%)	Total	Acid-prec.	Prec./total
5	5	0	23,321 ± 2239[b]	1190 ± 91.7	5.10 ± 0.23			
5	5	60	25,170 ± 3806	817 ± 40.6	3.25 ± 0.63	108	69	65
5	15	0	24,452 ± 1640	1261 ± 64.8	5.16 ± 0.52			
5	15	60	23,819 ± 2504	1018 ± 129	4.27 ± 0.71	97	84	84
48	5	0	29,053 ± 5755	1179 ± 227	4.06 ± 0.37			
48	5	60	25,351 ± 3033	660 ± 194	2.60 ± 0.73	87	56	64
48	15	0	21,027 ± 1303	995 ± 141	4.73 ± 0.90			
48	15	60	24,972 ± 2563	1163 ± 343	4.66 ± 0.97	119	117	99

[a] cpm/10^5 cells.
[b] Mean ± SEM.

S3 cells treated with 60 μM DRB for 18–30 hr in suspension cultures in medium supplemented with 5% fetal bovine serum require several days for recovery, but then proliferate at the control rate. Such treatment is sufficiently severe to stop population growth and to cause some cell loss. The population that survives DRB treatment and grows out is fully sensitive to DRB.

After treatment of cells with DRB at 39.5–40°C instead of at 37°C, the surviving population proliferated at a reduced rate following the recovery period. This experiment was done to determine whether the ability of cells to withstand DRB treatment was reduced at an elevated temperature. The result suggests that DRB treatment for a limited period (24–30 hr) at an elevated temperature results in a persistent reduction in the population growth rate. The mechanism underlying this persistent effect is not known.

The outstanding result of the studies of the inhibition of cellular RNA and protein synthesis 5 and 48 hr after the beginning of continuous DRB treatment is the demonstration that inhibition of protein synthesis does not become more marked with prolonged treatment (Table II). If mRNA synthesis in cells treated with 60 μM DRB for a prolonged period remained depressed at the level (i.e., approximately 10%) to which it is reduced after a short period of treatment, a progressive decrease in protein synthesis might be expected to occur with time as preexisting mRNAs decayed. Several possibilities exist to explain the fact that a progressive decrease in protein synthesis was not observed: (i) DRB treatment may stabilize abundant mRNAs. Although it is well known that inhibition of RNA synthesis by DRB does result in the stabilization of certain mRNAs, such as β fibroblast interferon mRNA in poly(I · C)-induced fibroblasts, there is no evidence that such stabilization is a general phenomenon (11–14); (ii) the translation of the still active mRNAs in DRB-treated cells may increase as the total population of mRNAs decays during prolonged treatment with DRB. There is no evidence for or against this possibility; and (iii) the inhibition of mRNA synthesis by DRB may not be maintained at the initial level on prolonged incubation. Although mRNA levels have not been measured after prolonged treatment with DRB, the present results indicate that at least in 15% serum, the inhibitory effect of DRB on total RNA synthesis does decline with time (cf. Table I). Previous results obtained in monolayer cultures of HeLa cells showed that the inhibition of hnRNA as well as of total RNA synthesis becomes partially reversed when treatment is prolonged (unpublished data). The findings of partial reversal of RNA synthesis and of stable levels of protein synthesis when DRB treatment is prolonged from 5 to 24 or 48 hr support the hypothesis that HeLa cells undergo a serum-dependent

"adaptive" change to DRB, which may restrict the access of the inhibitor to its target sites or may result in an amplification of the biosynthetic capacity of the cells [(7), unpublished data].

The authors thank Ms. Andrea Gifford and Mrs. Kathleen Pickering for typing the manuscript. The investigation was supported by Research Grant CA-18608 and by Program Project Grant CA-18213 awarded by the National Cancer Institute.

1. Tamm I, Hand R, Caliguiri LA. Action of dichlorobenzimidazole riboside on RNA synthesis in L-929 and HeLa cells. J Cell Biol **69**:229–240, 1976.
2. Sehgal PB, Darnell JE Jr, Tamm I. The inhibition by DRB (5,6-dichloro-1-β-D-ribofuranosylbenzimidazole) of hnRNA and mRNA production in HeLa cells. Cell **9**:473–480, 1976.
3. Tamm I. Definition of subclasses of nucleoplasmic RNA. Proc Nat Acad Sci USA **74**:5011–5015, 1977.
4. Tamm I, Kikuchi T. Early termination of heterogeneous nuclear RNA transcripts in mammalian cells. Accentuation by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Proc Nat Acad Sci USA **76**:5750–5754, 1979.
5. Tamm I, Kikuchi T, Darnell JE Jr, Salditt-Georgieff M. Short capped hnRNA percursor chains in HeLa cells: Continued synthesis in the presence of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Biochemistry **19**:2743–2748, 1980.
6. Tweeten KA, Molloy GR. Inhibition of messenger RNA synthesis by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole without its detectable accumulation in mouse T-lymphoma cells. Arch Biochem. Biophys **217**:332–340, 1982.
7. Tamm I, Kikuchi T, Murphy JS. Serum enhances the cycling and survival of HeLa cells treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Proc Nat Acad Sci USA **79**:2569–2573, 1982.
8. Eagle H. Amino acid metabolism in mammalian cell cultures. Science **130**:432–437, 1959.
9. Phillips HJ. Dye exclusion tests for cell viability. In: Kruse PF Jr, Patterson MK Jr, eds. Tissue Culture. Methods and Applications. New York, Academic Press, pp 406–408, 1973.
10. Sehgal, PB, Tamm I, Vilcek J. Human interferon production: Superinduction by 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole. Science **190**:282–284, 1975.
11. Sehgal PB, Lyles DS, Tamm I. Superinduction of human fibroblast interferon production: Further evidence for increased stability of interferon mRNA. Virology **89**:186–198, 1978.
12. Tamm I, Sehgal PB. New evidence for regulated transcription of hnRNA and for regulated translation of interferon mRNA in human cells. In: Engberg J, Klenow H, Leick V, eds. Alfred Benzon Symposium 13: Specific Eukaryotic Genes. Copenhagen, Munksgaard, pp 424–439, 1979.
13. Sehgal PB, Tamm I. Benzimidazoles and their nucleosides. Antibiotics Chemother **27**:93–138, 1980.
14. Sehgal PB, Gupta SL. Regulation of the stability of poly(I)·poly(C)-induced human fibroblast interferon mRNA: selective inactivation of interferon mRNA and lack of involvement of 2′,5′-oligo (A) synthetase activation during the shutoff of interferon production. Proc Nat Acad Sci USA **77**:3489–3493, 1980.

Received November 29, 1982. P.S.E.B.M. 1983, Vol. 173.