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Serum enhances the cycling and survival of HeLa cells treated with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

(time-lapse cinemicrography/frequencies of mitoses and deaths/heterogeneous nuclear RNA synthesis)

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ABSTRACT We have defined the growth kinetics of HeLa cell populations by determining the frequencies of mitoses and deaths and the lengths of intermitotic intervals. This was done by time-lapse cinemicrography. Untreated control cells proliferated at closely similar rates in medium enriched with 5% or 15% fetal calf serum, with an average of 4% dividing and <0.1% dying per hr. The mean intermitotic interval was 16 hr during exponential growth of the control populations. In contrast, in cultures treated with 40 or 60 μM 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a selective inhibitor of heterogeneous nuclear RNA synthesis, the frequency of mitoses was markedly and directly dependent on serum concentration, whereas the frequency of deaths was inversely dependent. DRB prolonged the intermitotic interval in cells cycling in the presence of the drug, but the effect was less in 15% than in 5% serum. After prolonged treatment of HeLa cells with DRB, the inhibition of heterogeneous nuclear RNA synthesis by DRB appeared to be reduced, which was not due to inactivation of DRB in the culture medium.

The β-linked D-riboside of 5,6-dichlorobenzimidazole (DRB) (1-3) has been shown to inhibit the proliferation of animal cells in culture (1, 4, 5). In diploid human fibroblasts, the inhibition of cell proliferation appears to be reversible upon removal of DRB (5). DRB selectively inhibits the transcription of heterogeneous nuclear RNA (hnRNA) by prematurely terminating ≈70-80% of the nascent chains ≈100-500 nucleotides downstream from initiation sites (6-8). The population of hnRNA chains that is susceptible to premature termination by DRB comprises the great majority of message-containing transcripts, as indicated by the fact that 75 μM DRB inhibits the appearance of mRNA in the cytoplasm by >95% (9). In short-term experiments, the inhibition of RNA synthesis by DRB is promptly reversible upon removal of the compound and independent of serum concentration in the medium (10). After treatment of cells with DRB for 1 hr or longer, the rate of protein synthesis becomes depressed, probably as a result of inhibition of mRNA synthesis (10, 11).

In the course of an investigation to define the effects of DRB on the proliferation of aneuploid HeLa cells derived from an epidermoid carcinoma of the cervix, we made the unexpected observation that the extent of inhibition by DRB was inversely related to serum concentration in the medium. By using time-lapse cinemicrography, we characterized the growth kinetics of DRB-treated cell populations in terms of three parameters: the frequency of mitoses, the frequency of deaths, and the length of the intermitotic intervals. We report that serum enhances both the cycling and the survival of cells treated with DRB for several days.

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MATERIALS AND METHODS

Cells and Culture Conditions. HeLa cells were grown to a density of 1.6-2 × 10^7 cells per flask (75 cm^2 growth area) in reinforced Eagle's medium (12) supplemented with 5% fetal calf serum. Cells were trypsinized and passaged twice a week at a split ratio of 1:10 or 1:20. Cultures were incubated at 37°C in an atmosphere of 5% CO_2/95% air.

Time-Lapse Equipment. Two time-lapse units (13) were used to record control or DRB-treated cultures incubated with 5% or 15% serum. Films were analyzed with the aid of a stop-motion projector (13).

Time-Lapse Experiments. HeLa cells were planted at a density of 5 or 10 × 10^3 cells per cm^2 in 25 ml of reinforced Eagle's medium supplemented with 5% or 15% fetal calf serum. Twenty-four hours after planting, the medium was replaced with fresh medium with or without 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB; 40 or 60 μM), and the flasks were gassed with 5% CO_2/95% air. Cultures were examined by time-lapse cinemicrography under phase-contrast optics with a ×6.3 objective and a long-working-distance condenser. The cultures were photographed every 2 or 4 min over 5 days.

A mitosis was readily recognized as a rapid rounding of a cell followed by division, observed over a period of 20-80 min. The beginning of separation of daughter cells during telophase could be resolved in time to within 4-8 min, and this point was used in the determination of intermitotic intervals.

The death of a cell was recognized as a sudden change in cell shape, which usually took the form of a contraction followed by cessation of cell movement. Not uncommonly, the cell would subsequently disintegrate. Sometimes a dying cell would undergo apparent osmotic lysis with ballooning of the cytoplasm. Time-lapse cinemicrography made possible a series of consecutive observations that permitted the determination that a cell had died. The death, once established as having occurred, was timed to the frame showing the sudden contraction of the cell. As with mitoses, this was accurate to 4-8 min.

[^{3}H]Uridine-Incorporation Experiments. Cells were processed for the determination of total and acid-precipitable radioactivity as described (10, 11). To determine the effect of DRB on RNA synthesis, the ratio of acid-precipitable to total radioactivity in DRB was expressed as a percentage of that ratio in control cells.

RESULTS

Control Cells. HeLa cells not treated with DRB proliferated at closely similar rates in 5% and 15% serum, with a doubling time of 17-18.5 hr, as determined by enumeration of mitoses

Abbreviation: hnRNA, heterogeneous nuclear RNA; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole.

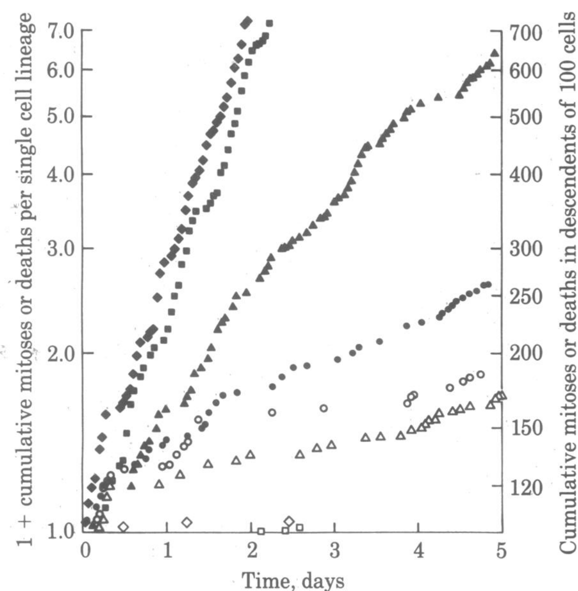

FIG. 1. Cumulative mitoses (solid symbols) and deaths (open symbols) in control HeLa cell populations and in populations treated with 60 μM DRB in the presence of 5% or 15% serum. Controls: ◆ and ◇, 5% serum; ■ and □, 15% serum. With DRB: ● and ○, 5% serum; ▲ and △, 15% serum. The curves are based on mitotic and death frequency data, which are available in numerical form upon request. Mitoses or deaths (if any occurred) were recorded hourly. Frequencies were calculated by using the cell number at the beginning of the period during which the events occurred. A new cell number was calculated at the end of each period based on the number of mitoses and deaths during that period and was used for the calculation of frequencies in the subsequent period, etc. Cumulative values for mitoses and deaths for consecutive intervals after the beginning of the experiment were calculated as follows: cumulative mitoses or deaths at $t_n = (1 + f_1)(1 + f_2)(1 + f_3) \ldots (1 + f_n)$, where t_n = time and f = number of mitoses or deaths during an interval of 1 hr/number of cells at the beginning of the interval. The ordinate on the left is descriptive of the calculated values; for convenience, the ordinate on right gives values applicable to a cohort of 100 cells. Time zero is the beginning of treatment.

and deaths (Figs. 1 and 2). On the average, 3.8–4.1% of the cells in the control populations divided per hr (Table 1). Because very few deaths occurred, the curves for cumulative mitoses (Fig. 1) and for cell number (Fig. 2) are nearly identical. With each mitosis, the cell number in the culture increased by one when no deaths occurred within a given period of observation. As would be expected, the control cells appeared to proliferate at a constant exponential rate from the beginning of the experiment. Minor deviations might be random in nature or might reflect some synchronization of the cells in the culture. For the aneuploid HeLa cells, serum at concentrations as low as 2.5% contains growth factors and other components in sufficient amounts to assure proliferation of the cell population at a maximal exponential rate (data not shown).

Effects of 60 μM DRB. Cells treated with 60 μM DRB in medium containing 5% serum continued to undergo mitoses at a markedly reduced frequency throughout a 5-day period of observation (Fig. 1). During the first 1.5 days of treatment, the frequency of mitoses was similar to that of deaths, but the mitotic frequency was considerably higher than the death frequency during the last 3.5 days. Increasing the serum concentration from 5% to 15% greatly increased the mitotic frequency and lowered somewhat the death frequency in the culture treated with 60 μM DRB.

It is evident for cultures incubated at either serum concentration that, after an initial period, the curves for cumulative mitoses assume essentially constant slopes which are main-

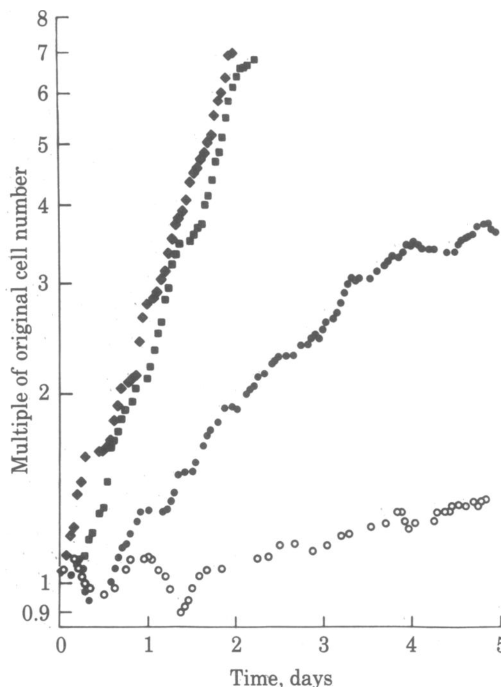

FIG. 2. Effects of 60 μM DRB on the overall rate of proliferation of HeLa cells in the presence of 5% (○) or 15% (●) serum. Controls: ◆, 5% serum; ■, 15% serum. Consecutive cell numbers were calculated based on cell number at the beginning of a period and the numbers of mitoses and deaths occurring during the period. Time zero is the beginning of treatment.

tained for several days. The curves for cumulative deaths display similar characteristics. Thus, it appears that, after an initial period, steady-state conditions are established under which DRB-treated cells incubated with 15% serum are capable of more frequent mitoses and liable to fewer deaths than those incubated in 5% serum.

For a quantitative overview, Table 1 lists mean frequencies, expressed as the percentage of cells undergoing mitoses or dying per hr, for days 1–5 of the experiment. The considerable decrease in the mitotic frequency and increase in death fre-

Table 1. Effects of serum concentration on the frequencies of mitoses and deaths in control and 60 μM DRB-treated HeLa cell populations

	Mean frequency, % cells per hr			
	5% serum		15% serum	
Day	Mitoses	Deaths	Mitoses	Deaths
	Control*			
1	4.5	0.10	3.2	0
2	4.0	0.050	4.6	0
3	3.7†	0.021†	3.5	0.054
Mean	4.1	0.057	3.8	0.018
	DRB‡			
1	1.5	1.1	2.1	0.85
2	0.78	0.80	1.9	0.43
3	0.46	0.22	1.4	0.15
4	0.63	0.25	1.5	0.25
5	0.71	0.25	0.78	0.59
Mean	0.82	0.52	1.54	0.45

* Zero-hour cell number per frame in 5% serum was 26 and in 15% serum was 28.

† Based on 18 hr.

‡ Zero-hour cell number per frame in 5% serum was 50 and in 15% was 32.

quency during day 5 of incubation of DRB-treated cells in 15% serum may have been due to the development of unfavorable conditions in the culture. Of the cells in the original population, 67% divided at least once in 5% serum while 22% died; the corresponding figures for 15% serum are 79% and 21%, respectively. Any small difference between the total number of cells originally present and the sum of those cells that either divided or died is due to cells that did neither or moved out of the field.

The cumulative cell numbers in DRB-treated cultures, expressed as multiples of the original cell numbers, are plotted against time in Fig. 2. The doubling time of the DRB-treated population between 42 and 90 hr after the beginning of treatment was 52 hr in 15% serum, but it was 180 hr in 5% serum. Thus, the population growth rates in 15% and 5% serum were reduced to 36% and 9.4%, respectively, of the control rates.

Effects of 40 μM DRB. Comparison of the effect of serum in 40 μM DRB-treated cultures also shows that the mitotic frequency was higher and the death frequency was lower in 15% serum as compared to 5% serum (Fig. 3). At either serum concentration, the frequency of mitoses was higher than that of deaths, but the difference was much greater in 15% than in 5% serum.

The daily mean mitotic and death frequencies (Table 2) confirm that these frequencies were most stable during the middle portion of the 5-day period of observation.

In the 40 μM DRB-treated culture incubated in 5% serum, 89% of the cells originally present divided at least once, while 6% died. Between 42 and 90 hr after the beginning of treatment with 40 μM DRB, the population doubling time was 33 hr in 15% serum and 61 hr in 5% serum. Thus, the growth rates in 15% and 5% serum were reduced to 55% and 31%, respectively, of the exponential control rates of 18 and 19 hr (data not shown).

Intermitotic Intervals. Treatment of cells with 60 μM DRB increased the mean intermitotic interval (of the cells able to divide) 3.5-fold in 5% serum but only 1.8-fold in 15% serum (Table 3). Treatment with 40 μM DRB in 5% serum increased the mean intermitotic interval 2.5-fold; strictly comparable data for 15% serum are not available. Comparisons are based on measurements of the lengths of the first and second intermitotic intervals because the subsequent intervals are increasingly biased towards artificially low values; the longer intervals will fail, with increasing frequency, to be recorded because of termination of the experiment (14–16). The data for intermitotic intervals are consistent with those for mitotic frequencies in Figs. 1 and 2 and Tables 1 and 2, in which mitotic events are recorded as a function of time.

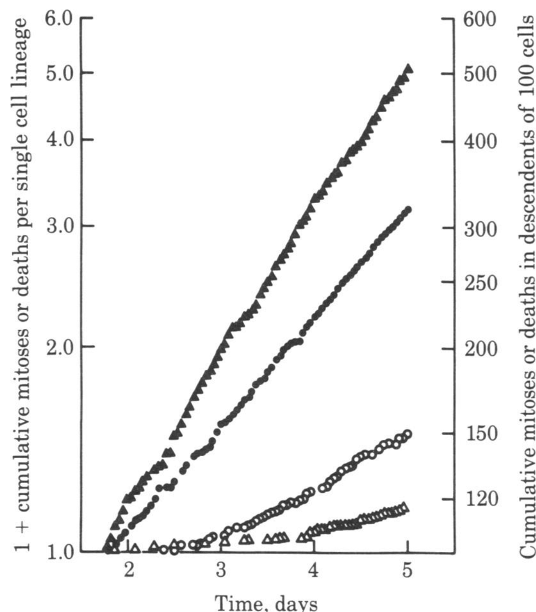

FIG. 3. Cumulative mitoses (solid symbols) and deaths (open symbols) in HeLa cell populations treated with 40 μM DRB in the presence of 5% (●, ○) or 15% (▲, △) serum. Cumulative values for mitoses and deaths were calculated as described in the legend for Fig. 1. Time zero is the beginning of treatment.

Table 2. Effects of serum concentration on the frequencies of mitoses and deaths in HeLa cell populations treated with 40 μM DRB

	Mean frequency, % cells per hr*			
	5% serum		15% serum	
Day	Mitoses	Deaths	Mitoses	Deaths
1	2.55	0.67	—	—
2	1.69	0.10	2.88	0.10
3	1.53	0.23	2.22	0.08
4	1.50	0.68	2.17	0.18
5	1.54	0.70	1.78	0.35
Mean, days 1–5	1.77	0.48	—	—
Mean, days 3–5	1.54	0.54	2.10	0.20

* Mean over a 24-hr period, except that day-2 values for 15% serum are based on the last 6 hr of day 2. Zero-hour cell number per frame in 5% serum was 84 and in 15% was 57. The observations in 15% serum began at 43 hr with a count of 177 cells per frame.

Table 3. Length of intermitotic intervals and growth rate in control and DRB-treated (60 or 40 μM) cultures*: effect of serum concentration

Intermitotic intervals	Mitosis number in cell lineages in			
	5% serum		15% serum	
	1st	2nd	1st	2nd
	Control			
Duration, hr				
Mean	15.5	15.0	16.5	16.5
Range	12–20	11–22	11–21	13–22
Coefficient of variation, %	16	16	11	11
Observations, no.†	34	62	44	85
	DRB, 60 μM			
Duration, hr				
Mean	52	53	30	32
Range	29–86	33–88	21–54	18–60
Coefficient of variation, %	31	35	27	30
Observations, no.†	13	16	32	41
Cell growth rate, % of control	30	29	55	52
	DRB, 40 μM			
Duration, hr				
Mean	40	37	29‡	29‡
Range	17–109	11–85	17–73	16–63
Coefficient of variation, %	48	39	34	26
Observations, no.†	105	137	212	243
Cell growth rate, % of control	39	41	57	57

* Observed for 5 days by time-lapse cinemicrography.

† Zero-time cell numbers per time-lapse frame were as follows: 5% serum control, 18; 15% serum control, 28; 60 μM DRB/5% serum, 50; 60 μM DRB/15% serum, 32; 40 μM DRB/5% serum, 84. The cell number per frame at 43 hr in 40 μM DRB/15% serum was 146.

‡ These mitotic intervals were measured starting at 43 hr from the beginning of treatment with 40 μM DRB in 15% serum.

Table 4. Sensitivity of previously DRB-treated cells to inhibition of hnRNA synthesis by DRB*

Incubation medium			[^{3}H]Uridine incorporation,					
			cpm			% of control		
Serum, %	3 days	45 min	Total	Acid prec.	Ratio,† %	Total	Acid prec.	Ratio,† %
5	Control	Control	438,312	11,981	2.7	—	—	—
	Control	DRB	181,034	1,094	0.6	41	9	22
	DRB	DRB	154,261	1,542	1.0	35	13	37
	DRB	Control	421,480	9,237	2.2	96	77	81
15	Control	Control	419,619	11,832	2.8	—	—	—
	Control	DRB	193,960	1,536	0.8	46	13	29
	DRB	DRB	191,704	2,384	1.2	46	20	43
	DRB	Control	424,456	10,874	2.6	101	92	93
	5 days	45 min						
5	Control	Control	270,644	7,238	2.7	—	—	—
	Control	DRB	103,570	566	0.6	38	8	22
	DRB	DRB	104,838	995	1.0	39	14	37
	DRB	Control	275,182	6,725	2.4	102	93	89
15	Control	Control	390,169	10,797	2.8	—	—	—
	Control	DRB	219,562	1,492	0.7	56	14	25
	DRB	DRB	138,490	1,874	1.4	35	17	50
	DRB	Control	373,956	11,130	3.0	96	103	107

* Cells were planted in reinforced Eagle's medium with 5% serum in flasks (75 cm^2) at initial densities that would yield semiconfluent cultures after the 2- or 4-day treatment period (i.e., 2 days: control, 1×10^6 cells; with DRB, 2×10^6 cells or 4 days: control, 0.5×10^6 cells; with DRB, 1×10^6 cells per flask). The medium was changed 24 hr later, and the cells were continuously treated with 60 μM DRB or mock-treated in reinforced Eagle's medium containing serum at a concentration of 5% or 15%. They were then trypsinized and planted in 35-mm plates in 60 μM DRB-containing or control medium, respectively, at 2×10^5 viable cells per plate [as determined by erythrosine B dye exclusion (17)]. Twenty-four hours later, the medium was changed as indicated in Table 4, and the cells were treated with actinomycin D (0.04 μg/ml, which selectively suppresses preribosomal RNA synthesis) for 30 min and pulse-labeled with 5 μCi (1 Ci = 3.7×10^{10} becquerels) of [^{3}H]uridine (25 Ci/mmol; New England Nuclear) per plate for 15 min in the continued presence of actinomycin D. All radioactivity determinations are expressed per 2×10^5 cells, based on cell counts done on companion cultures. Mean results of three experiments.

† Ratio of acid-precipitable to total incorporation.

Table 3 also shows that the coefficients of variation of intermitotic intervals were 2–3 times larger for DRB-treated cells than for control cells.

Inhibitory Activity of DRB-Containing Cell Culture Supernatant on RNA Synthesis. DRB-containing supernatant from cultures of human diploid fibroblasts incubated for 3 days at 37°C is fully active in inhibiting RNA synthesis upon addition to a new set of cultures (5). We report now that the supernatant from DRB-treated cultures of HeLa cells, which had been incubated for 3–5 days with 15% serum in time-lapse experiments, had undiminished inhibitory activity on hnRNA synthesis in new cultures of HeLa cells (data not shown). These cultures were treated with 60 μM DRB-containing medium for 60 min and then labeled for 15 min with [^{3}H]uridine in the continued presence of DRB.

Sensitivity of Previously DRB-Treated Cells to Inhibition of RNA Synthesis by DRB. We investigated the level of hnRNA synthesis in the surviving DRB-treated cell populations to determine whether these populations were still sensitive to DRB and capable of recovery from inhibition of hnRNA synthesis upon removal of DRB from the medium. Cells from cultures that had been treated for 3 or 5 days with 60 μM DRB in medium containing 5% or 15% serum remained sensitive to inhibition of hnRNA synthesis by DRB, although the inhibition was less than in cells treated for a short period (Table 4). When DRB was removed from cultures treated for 3 or 5 days, hnRNA synthesis returned to the control level.

DISCUSSION

We dissociated, by time-lapse cinemicrography, the conventional population growth curve for cells in culture into two components—i.e., the mitotic and death frequencies. This revealed that in a drug-treated population in which some cells are dying, others can continue to cycle and undergo mitoses, although with reduced frequency and variably prolonged intermitotic intervals. The outstanding result of the present study is the finding that when HeLa cells are incubated under the restrictive condition of selective inhibition of hnRNA synthesis with DRB, there becomes manifest a serum effect that is supportive of cell cycling and survival. Fig. 4 brings out the fact that the cumulative-events curves obtained with 60 μM DRB/15% serum and with 40 μM DRB/5% serum are similar. Thus, 60 μM DRB in 15% serum is similar in its effects on cell cycling to 40 μM DRB in 5% serum, although when both concentrations of DRB are in 5% serum, 60 μM DRB inhibits cell cycling much more than does 40 μM DRB (compare Fig. 1 with Fig. 4 and Table 1 with Table 2).

Any model to account for the ability of serum to counteract the deleterious effects of DRB on the cycling and survival capabilities of cells should be consistent with the following facts. (*i*) The lower serum concentration (5%) is entirely adequate to support maximal cycling activity and survival of control cells. (*ii*) After several days of treatment of cells with DRB, the culture supernatant shows undiminished inhibitory activity on RNA synthesis in cells not previously treated with DRB. (*iii*) Surviving cells from cultures that are treated with DRB for several days retain sensitivity to the inhibitory effect of DRB on hnRNA synthesis, but the inhibitory effect appears reduced in such cells. (*iv*) The serum concentration-dependent effect is demonstrable with dialyzed serum, although dialyzed serum is less effective in supporting the proliferation of control and DRB-treated cells than is undialyzed serum (unpublished data). (*v*) In short-term experiments (30–60 min), the inhibitory effect of DRB on RNA and protein synthesis is independent of the presence or concentration of serum in the medium (11).

It has been estimated that 60 μM DRB inhibits mRNA syn-

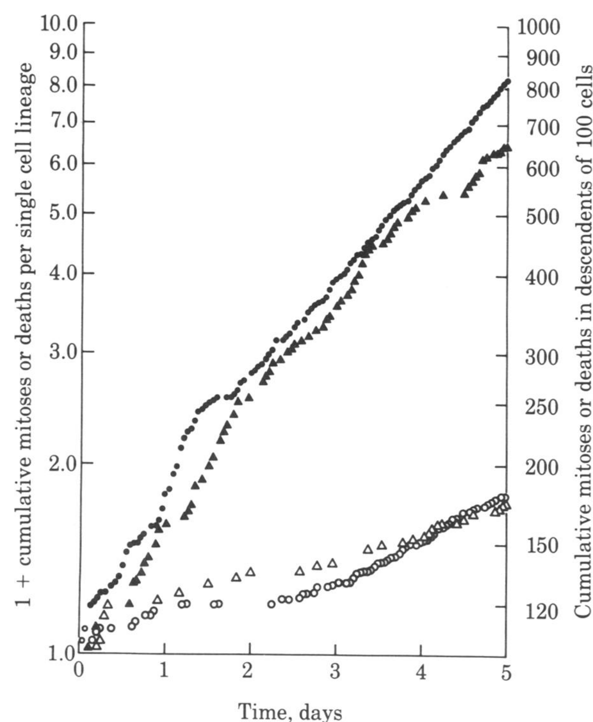

FIG. 4. Comparison of curves for cumulative mitoses (solid symbols) and deaths (open symbols) in HeLa cell populations treated with 60 μM DRB in 15% serum (▲, △) or with 40 μM DRB in 5% serum (●, ○). Time zero is the beginning of treatment.

thesis by ≈90% (unpublished data), and 40 μM DRB, by ≈80% (18). We propose that serum supplies growth factors that are capable of counteracting the effects of DRB at one or more levels of regulation of cellular biosynthesis. It is apparent that serum could cause the effects by limiting access of DRB to its putative target sites. Based on the mechanism of action of DRB, two mechanisms suggest themselves in particular. First, serum factors may counteract the inhibition of certain species of hnRNA and, consequently, of mRNA synthesis by DRB. Second, serum factors may increase the translation of the reduced amounts of mRNA available in DRB-treated cells.

Available evidence indicates that the primary action of DRB is to increase the frequency of premature termination of nascent hnRNA chains (3, 7, 8)— chains that comprise mRNA precursor molecules. DRB may act on some mechanism that controls and possibly regulates the abundance of mRNA precursor molecules in cells and, consequently, also the level of protein synthesis. However, it also has been shown that depression of RNA synthesis by DRB can paradoxically increase ("superinduce") the synthesis of certain inducible proteins such as interferons (11). Superinduction of interferon production by DRB operates through two mechanisms (18, 19): (*i*) increased rate of appearance of interferon mRNA (this represents a quantitatively minor component in superinduction) and (*ii*) stabilization of interferon mRNA (this is responsible for the bulk of additional interferon synthesis under superinducing conditions). Because the effects of DRB on cellular biosynthesis are complex, nonconcordance may be observed when effects on bulk mRNA synthesis are compared to effects on the formation and translation of individual mRNA species (20).

The evidence that we obtained suggests that, after prolonged treatment of HeLa cells with DRB in either 5% or 15% serum, the level of bulk hnRNA synthesis is higher than in cells treated for a short period only. It is possible that in the drug-treated cells, the synthesis of different species of hnRNA (such as polyadenylated vs. nonpolyadenylated hnRNA) may be differentially affected by serum concentration upon prolonged incubation.

K. A. Tweeten and G. R. Molloy (21) have recently found that in the mutant AE_1 mouse lymphoma cells, which are deficient in the transport of purine and pyrimidine nucleosides, 40 μM DRB inhibits the accumulation of cytoplasmic poly(A)-containing RNA by 86%, and 60 μM DRB, by 93%. Neither the AE_1 cells nor the wild-type S49 cells accumulate DRB to a significant extent. Thus, inhibition of mRNA synthesis by DRB may be triggered by interaction of DRB with a surface membrane component.

It has been reported that the effect of a cytostatic or cytocidal drug on colony formation by cells under investigation may be significantly affected by culture conditions, such as the use of dialyzed vs. undialyzed serum, of small-molecular medium supplements, and of feeder layers (22–24). The findings that we obtained by time-lapse cinemicrography provide a striking example of the importance of serum concentration in evaluating the effects of a cytostatic drug on cell proliferation. We have shown that both cycling and survival may be dependent on the concentration of serum within a range in which untreated control cells proliferate at a maximal rate.

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