Specific G_1–S phase cell cycle block by beryllium as demonstrated by cytofluorometric analysis

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Inhibition of cell division by beryllium (Be^{2+}) has been examined in synchronized cultures of a liver-derived cell line (BL9L cells) using cytofluorometric cell cycle analysis. Results show that a selective dose-related block of the G_1–pre-S transition is produced, with other periods of the cell cycle appearing relatively insensitive.

The notional separation of the cell division cycle into the DNA synthetic phase (S), the first gap (G_1) between mitosis (M) and S, and the second gap (G_2) between S and M, in addition to the concept of resting cells (G_0), is well established (Howard & Pelc, 1953; reviewed by Stein & Baserga, 1972). Recent use of selective fluorescent stains to identify both the amount and structural form of DNA present in nuclei in conjugation with flow cytometry measurements has facilitated the routine and rapid analysis, with commercially available instruments, of the cell cycle into $G_1(G_0)$, S and G_2/M phases (Darzynkiewicz *et al.*, 1977; Gray *et al.*, 1979). Accordingly the procedure of cytofluorometric cell cycle analysis has become extremely useful to study the effects of exogenous chemicals on the cell cycle, particularly in synchronized dividing cell culture systems.

A number of genotoxic or carcinogenic metals have been examined by cytofluorometric cell cycle analysis, and, for example, arsenic, nickel and cobalt have been shown to produce an S phase specific cell block (Costa *et al.*, 1982), whereas chromium prolongs the G_2/M phase (Bakke *et al.*, 1982). Our interest has been concerned with the metallocarcinogen beryllium (Be^{2+}), which for some years has been known to inhibit both cell division of cultured cells (Chevremont & Firket, 1951) and the process of DNA synthesis in hepatic cell regeneration (Witschi, 1970). Furthermore, indirect evidence, from time lapse cinematographic analysis, of an increase in cell interdivision time in $BeCl_2$-treated fibroblasts (Absher *et al.*, 1983) and the inhibition by $BeSO_4$ of histone phosphorylation in regenerating liver (Kaser *et al.*, 1980), have suggested that Be^{2+} may act by inhibition of the G_1 to S phase. The purpose of the present investigation was to examine

directly by cytofluorometric cell cycle analysis the effects of Be^{2+} on the synchronized division of a liver-derived cell line (BL9L) *in vitro* in order to determine the Be^{2+}-susceptible cell cycle phase.

Experimental

Culture and synchronization of cells

Rat-liver-derived epithelial cells (BL9L) indistinguishable from the previously reported BL8L cell line (Judah *et al.*, 1977) were used throughout as monolayer cultures on 100 mm plastic petri dishes. Cell synchrony was achieved by seeding 3×10^6 cells in 9 ml of culture medium [Williams E medium (Flow Laboratories) containing 2 mM-glutamine, 0.5% gentamycin and 5% (v/v) foetal calf serum (Sera Lab)], followed by culture for 3 days at 37°C and pH 7.3 in a humidified atmosphere of O_2/CO_2 (1:19) to achieve a confluent monolayer (approx. 9×10^6 cells). At this stage cytofluorometric cell cycle analysis estimated 80–85% of cells to be in the $G_1(G_0)$ phase, 10–15% in D phase and the remainder in G_2/M. Cells were dispersed by exposure to 10% trypsin (Flow Laboratories) in phosphate-buffered saline [Dulbecco A (Oxoid)] for 2 min at room temperature, followed by removal of the medium and incubation of the cells at 37°C for 8 min prior to elution from the dish by suspension in culture medium. Cells were then centrifuged at 350 g for 2 min and washed once with phosphate-buffered saline. Cells (3×10^6) were next subcultured in 9 ml of serum-free culture medium for 4 h [during which time attachment occurs without multiplication (Temin *et al.*, 1972)] followed by replacement in the normal culture medium (with 5% serum) containing any other additions, to initiate the cell division cycle. Under these conditions S phase commenced at about

16h, and was maximal at 20h (50–60% of cells). The greatest proportion of cells in the G_2/M phase (15–20%) was also observed at about 20h and was followed by a gradual return of cells to $G_1(G_0)$ between 24h and 72h.

Cytofluorometric cell cycle analysis

At the indicated times cells were removed from the culture dishes by the trypsinization procedure described above, prior to dispersion in 5 ml of filtered (0.22 μm) fluorochrome stain comprising 0.1% sodium citrate, 0.005% ethidium bromide (Sigma) and 0.1% Triton X-100. To this suspension was added 0.25 ml of 0.01% ribonuclease A (Sigma) and the mixture was placed on ice for at least 10 min prior to cytofluorometric analysis. In principle the procedure involves passage of the fluorescently (ethidium bromide) labelled nuclei, derived from the cells, as a single stream through a suitable activating laser light source such that the fluorescent light emitted (which is proportional to DNA content) can be quantified. In the present studies nuclear DNA content was determined at an analysis rate of 1000 nuclei/s for a total of 20000 nuclei by use of an Ortho Cytofluorograf System 50-H model 2150 (Ortho Diagnostics Systems, Westwood, MA, U.S.A.) fitted with an argon ion laser. The fluorochrome was excited at 480 nm and the emission measured above 630 nm. This instrument computes the data to present $G_1(G_0)$, S and G_2/M cell cycle phases in the form of an integrated histogram from which percentage phase values can be calculated by using the Ortho program 'DNADISC'. This program also permits discrimination of doublets in the nuclear population.

Additional procedures

Beryllium uptake by cells was measured radiochemically in separate experiments using media containing $^7BeSO_4$ (1–500 μM; 0.37 MBq/μmol) according to procedures previously described (Skilleter & Paine, 1979). Cell protein was estimated by the method of Markwell et al. (1978) and DNA synthesis by [^{3}H]thymidine incorporation (Terasima & Tolmach, 1963).

Results and discussion

Continuous exposure of synchronized BL9L cells to 50 μM-$BeSO_4$ caused a marked inhibition of cell division. Fig. 1 illustrates that whereas the proportion of S-phase cells in controls increased from a starting level of 13–15% to about 55% at 20h, in the presence of 50 μM-$BeSO_4$ a corresponding increase to only 20–23% was observed. At 24h control cells showed a decrease of cells in S phase to 30–40%, which continued for the next 48h to 10–15% as the cells recycled to the status of 80–85% estimated to
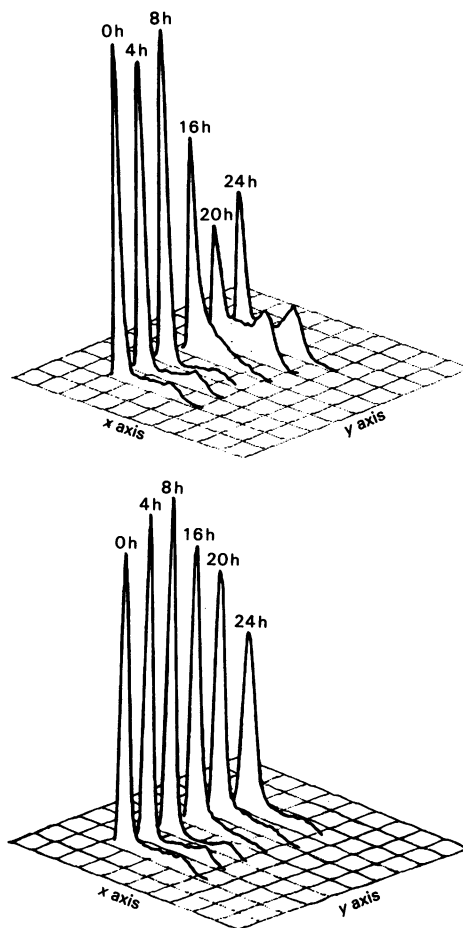

Fig. 1. *Beryllium inhibition of cell division in vitro*
Monolayer cultures of BL9L cells were incubated in the absence (a) or presence (b) of 50 μM-$BeSO_4$, then cytofluorometric cell cycle analysis was performed at the times indicated according to the procedure described in the Experimental section. Results are presented as a series of stacked histograms (y axis) each comprising the Cytofluorograf-computed data of the cell cycle analysis (DNA fluorescence) at each time point [x axis: initial peak, $G_1(G_0)$; intermediate region, S; second peak, G_2/M].

be in $G_1(G_0)$. During this period Be^{2+}-treated cells also showed a decrease in the proportion of S-phase cells to about 9% but this was associated with detachment of 7% of the cells at 24h and 34% at 72h. In the presence of 100 μM- or 500 μM-$BeSO_4$, complete impairment of cell division was observed but this resulted in the detachment of 23% and 40% respectively of the cells within 24h. These initial investigations therefore suggested that the pre-S phase was the most susceptible to Be^{2+} inhibition.

Table 1. *Sensitivity of G₁-S phase of cell cycle to Be²⁺*

Monolayer cultures of BL9L cells, maintained for 24 h, were exposed to 50 μM-BeSO$_4$ for the periods indicated prior to cell cycle analysis at 24 h as detailed in Fig. 1.

Phase of cell cycle	Proportion of cells present at 24 h (%) after exposure to BeSO$_4$ for times indicated						
	Control (no Be²⁺)	0–4 h	0–24 h	4–24 h	8–24 h	16–24 h	20–24 h
G$_1$(G$_0$)	50	82	80	67	54	56	51
S	36	14	14	19	29	34	33
G$_2$/M	14	4	6	14	17	10	16

Table 2. *Dose–response relationship of Be²⁺ inhibition of G₁–S phase of cell cycle*

Monolayer cultures of BL9L cells maintained for 24 h were exposed to various concentrations of BeSO$_4$ (1–100 μM) for the first 0–4 h followed by subsequent incubation in Be²⁺-free media for 20 h prior to measurements of cell cycle analysis, cell Be²⁺ content and protein, or DNA synthesis ([³H]thymidine added at change of medium) at 24 h as described in the Experimental section. Data for uptake are quoted as means ± S.D. for four determinations. Values for relative cell protein are relative to protein at zero time assumed as 1.0.

	Concentration of BeSO$_4$ (μM) present during 0–4 h						
	0	1	5	10	20	50	100
Be²⁺ uptake (nmol/mg of cell protein)	0	0.48 ± 0.16	1.12 ± 0.41	2.43 ± 0.67	3.72 ± 1.01	6.20 ± 0.92	9.28 ± 0.81
Relative cell protein	2.24	2.25	2.27	2.24	2.02	1.63	1.36
Inhibition of G$_1$–S phase	—	—	—	—	−/+	+	+
Inhibition of DNA synthesis (%)	0	0	0	0	15	64	96

To examine further the period(s) of the cell cycle most sensitive to Be²⁺, continuous exposure of cells to 50 μM-BeSO$_4$ was compared with the addition of 50 μM-BeSO$_4$ to the culture medium only for the periods 0–4 h, 4–24 h, 8–24 h, 16–24 h or 20–24 h; for the remaining times cells were cultured in Be²⁺-free media. Cell cycle analysis at 24 h revealed that Be²⁺ inhibition was only observed if cells had been exposed to BeSO$_4$ prior to 8 h incubation, after which no significant effects were seen (Table 1). More interestingly, it was noted that the critical exposure period was 0–4 h, since incubation of cells in the presence of Be²⁺ at this stage was as effective as continuous exposure; addition of BeSO$_4$ after 4 h being only partially effective (Table 1). These results indicated that Be²⁺ selectively inhibited the G$_1$–pre-S phase of the cell cycle.

Cellular uptake of Be²⁺ in the presence of a range of BeSO$_4$ concentrations (1–100 μM) was measured as ascertain the dose–response relationship for BeSO$_4$ inhibition of cell division. Table 2 indicates that inhibition of cell division was difficult to detect after cells had accumulated less than about 4 nmol of Be/mg of cell protein, but was readily observed when cells had taken up above 6 nmol of Be/mg of cell protein. Assuming a value of 3 × 10⁶ cells equivalent to 1 mg of cell protein for this type of cell preparation (Skilleter & Paine, 1979), the latter estimate corresponds to 2 nmol of Be/10⁶ cells, which has been previously calculated as the cell dose

of Be²⁺ necessary to cause cytotoxicity to liver cells both *in vivo* (Skilleter & Price, 1978) and *in vitro* (Skilleter & Paine, 1979). Table 2 shows that the effects of Be²⁺ on cell division were also consistent with the inhibitory effects seen on the increase in total cell protein and DNA synthesis during the 24 h incubation period.

The studies described illustrate a Be²⁺ block in the G$_1$–S phase of the cell cycle and imply there is no inhibition of the S–G$_2$/M or G$_2$/M–G$_1$(G$_0$) phases. It is known that both pinocytosis and phagocytosis of a variety of materials by cells is less during the G$_2$/M than G$_1$ phase (Riley & Dean, 1978; Quintart *et al.*, 1979; Berlin & Oliver, 1980) and we have previously shown that Be is taken up by cells primarily by endocytosis (Skilleter & Paine, 1979). Therefore, it was possible that the apparent lack of Be²⁺ effect on the S–G$_1$ phases might be due to a markedly decreased rate of Be²⁺ accumulation during this period. This suggestion had some support from the observation that in the presence of 50 μM-BeSO$_4$, monolayer Be²⁺ uptake between either 16–20 h or 20–24 h was approx. 50% of that measured during 0–4 h (Table 3). However, during the same exposure periods, but in the presence of media containing 500 μM-BeSO$_4$, more comparable levels of Be uptake were observed (Table 3), yet, no inhibition of the S through G$_2$/M to G$_1$ phases was apparent after addition of 500 μM-BeSO$_4$ to the cells at 16 h (Fig. 2). It must therefore be concluded that,

Table 3. *Comparison of Be²⁺ uptake by cells at different times during cell cycle*

Monolayer cultures of BL9L cells were incubated in the presence of either 50 or 500 μM-BeSO₄ for the periods indicated prior to measurement of cellular Be²⁺ content as described in the Experimental section. Data are quoted as means ± S.D. for four determinations. Values in parentheses are nmol of Be/mg of cell protein.

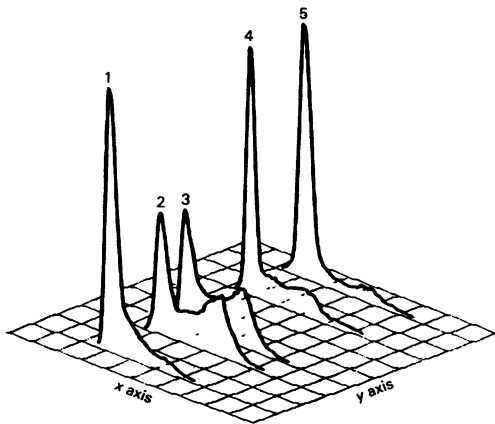| Concentration of BeSO₄ present (μM)	Uptake of Be²⁺ (nmol) by monolayer at the times indicated		
	0–4 h	16–20 h	20–24 h
50	2.34 ± 0.2 (6.20)	1.22 ± 0.3 (1.41)	1.26 ± 0.2 (1.27)
500	3.10 ± 0.3 (5.89)	4·13 ± 0.4 (3.86)	5.88 ± 0.4 (4.67)

Fig. 2. *Insensitivity of G₂/M phase of cell cycle to Be²⁺* Cell cycle analyses were performed on monolayer cultures of BL9L cells as detailed in Fig. 1 after incubation under the following conditions. Samples 1, 2 and 4: incubation in Be²⁺-free media for 16 h, 24 h and 48 h respectively. Samples 3 and 5: incubation in the presence of 500 μM-BeSO₄, added at 16 h and analysed at 24 h and 48 h respectively.

during the periods of DNA synthesis and mitosis, although Be is accumulated by BL9L cells it is either not released intracellularly to exert its effects, or that these phases of the cell cycle are relatively insensitive to the inhibitory action of the metal ion.

Reasons for the sensitivity of the G₁–pre-S phase to Be²⁺ cannot be provided from data presented in the present paper. However, previous studies have shown that BeSO₄ can inhibit nuclear protein phosphorylation processes (Kaser *et al.*, 1980; Williams & Skilleter, 1983) and Be²⁺ has a strong affinity for binding to non-histone nuclear proteins (Parker & Stevens, 1979). It is suggested, therefore,

that since non-histone proteins are actively and selectively phosphorylated during the first few hours of the G₁–S phase of the cell cycle (reviewed by Stein & Baserga, 1972; Ord & Stocken, 1980) it could be the inhibition of these phosphorylation processes that is responsible for the observed Be²⁺ inhibition of cell division.

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