Specific G₁–S phase cell cycle block by beryllium as demonstrated by cytofluorometric analysis

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Inhibition of cell division by beryllium (Be²⁺) 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 G₁-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 (G₁) between mitosis (M) and S, and the second gap (G₂) between S and M, in addition to the concept of resting cells (G₀), 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 conjunction with flow cytometry measurements has facilitated the routine and rapid analysis, with commercially available instruments, of the cell cycle into G₁(G₀), S and G₂/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 carcinogetic 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 G₂/M phase (Bakke et al., 1982). Our interest has been concerned with the metallocarcinogen beryllium (Be²⁺), 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 BeCl₂-treated fibroblasts (Absher et al., 1983) and the inhibition by BeSO₄ of histone phosphorylation in regenerating liver (Kaser et al., 1980), have suggested that Be²⁺ may act by inhibition of the G₁ to S phase. The purpose of the present investigation was to examine directly by cytofluorometric cell cycle analysis the effects of Be²⁺ on the synchronized division of a liver-derived cell line (BL9L) in vitro in order to determine the Be²⁺-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 × 10⁶ 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 O₂/CO₂ (1 : 19) to achieve a confluent monolayer (approx. 9 × 10⁶ cells). At this stage cytofluorometric cell cycle analysis estimated 80–85% of cells to be in the G₁(G₀) phase, 10–15% in D phase and the remainder in G₂/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 × 10⁶) 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
16 h, and was maximal at 20 h (50–60% of cells). The greatest proportion of cells in the $G_2/M$ phase (15–20%) was also observed at about 20 h and was followed by a gradual return of cells to $G_1(G_0)$ between 24 h and 72 h.

**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 $\mu$m) 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 20 000 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 $^7$BeSO$_4$ (1–500 $\mu$m; 0.37 MBq/$\mu$mol) 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 $\mu$m-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 20 h, in the presence of 50 $\mu$m-BeSO$_4$, a corresponding increase to only 20–23% was observed. At 24 h control cells showed a decrease of cells in S phase to 30–40%, which continued for the next 48 h to 10–15% as the cells recycled to the status of 80–85% estimated to 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 24 h and 34% at 72 h. In the presence of 100 $\mu$m- or 500 $\mu$m-BeSO$_4$, complete impairment of cell division was observed but this resulted in the detachment of 23% and 40% respectively of the cells within 24 h. These initial investigations therefore suggested that the pre-S phase was the most susceptible to Be$^{2+}$ inhibition.
Table 1. Sensitivity of G_{1}-S phase of cell cycle to Be^{2+}
Monolayer cultures of BL9L cells, maintained for 24 h, were exposed to 50\mu M-BeSO_{4} for the periods indicated prior to cell cycle analysis at 24 h as detailed in Fig. 1.

<table>
<thead>
<tr>
<th>Phase of cell cycle</th>
<th>Control (no Be^{2+})</th>
<th>0–4 h</th>
<th>0–24 h</th>
<th>4–24 h</th>
<th>8–24 h</th>
<th>16–24 h</th>
<th>20–24 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>G_{1}(G_{0})</td>
<td>50</td>
<td>82</td>
<td>80</td>
<td>67</td>
<td>54</td>
<td>56</td>
<td>51</td>
</tr>
<tr>
<td>S</td>
<td>36</td>
<td>14</td>
<td>14</td>
<td>19</td>
<td>29</td>
<td>34</td>
<td>33</td>
</tr>
<tr>
<td>G_{2}/M</td>
<td>14</td>
<td>4</td>
<td>6</td>
<td>14</td>
<td>17</td>
<td>10</td>
<td>16</td>
</tr>
</tbody>
</table>

Table 2. Dose–response relationship of Be^{2+} inhibition of G_{1}-S phase of cell cycle
Monolayer cultures of BL9L cells maintained for 24 h were exposed to various concentrations of BeSO_{4} (1–100\mu M) for the first 0–4 h followed by subsequent incubation in Be^{2+}-free media for 20 h prior to measurements of cell cycle analysis, cell Be^{2+} content and protein, or DNA synthesis ([^{3}H]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.

<table>
<thead>
<tr>
<th>Concentration of BeSO_{4} (\mu M) present during 0–4 h</th>
<th>0</th>
<th>1</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>50</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Be^{2+} uptake (nmol/mg of cell protein)</td>
<td>0.48 ± 0.16</td>
<td>1.12 ± 0.41</td>
<td>2.43 ± 0.67</td>
<td>3.72 ± 1.01</td>
<td>6.20 ± 0.92</td>
<td>9.28 ± 0.81</td>
<td></td>
</tr>
<tr>
<td>Relative cell protein</td>
<td>2.24</td>
<td>2.25</td>
<td>2.27</td>
<td>2.24</td>
<td>2.02</td>
<td>1.63</td>
<td>1.36</td>
</tr>
<tr>
<td>Inhibition of G_{1}-S phase</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—/+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Inhibition of DNA synthesis (%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>15</td>
<td>64</td>
<td>96</td>
</tr>
</tbody>
</table>

To examine further the period(s) of the cell cycle most sensitive to Be^{2+}, continuous exposure of cells to 50\mu M-BeSO_{4} was compared with the addition of 50\mu M-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 Be^{2+}-free media. Cell cycle analysis at 24 h revealed that Be^{2+} 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 Be^{2+} 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 Be^{2+} selectively inhibited the G_{1}–pre-S phase of the cell cycle.

Cellular uptake of Be^{2+} in the presence of a range of BeSO_{4} concentrations (1–100 \mu M) 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 \times 10^{6} 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/10^{6} cells, which has been previously calculated as the cell dose of Be^{2+} 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 Be^{2+} 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 Be^{2+} 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 Be^{2+} effect on the S–G_{2} phases might be due to a markedly decreased rate of Be^{2+} accumulation during this period. This suggestion had some support from the observation that in the presence of 50 \mu M-BeSO_{4}, monolayer Be^{2+} 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 \mu M-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 \mu M-BeSO_{4} to the cells at 16 h (Fig. 2). It must therefore be concluded that,
Table 3. Comparison of Be\textsuperscript{2+} uptake by cells at different times during cell cycle

Monolayer cultures of BL9L cells were incubated in the presence of either 50 or 500\mu M Be\textsubscript{SO\textsubscript{4}} for the periods indicated prior to measurement of cellular Be\textsuperscript{2+} content as described in the Experimental section. Data are quoted as means \pm S.D. for four determinations. Values in parentheses are nmol of Be/mg of cell protein.

<table>
<thead>
<tr>
<th>Concentration of Be\textsubscript{SO\textsubscript{4}} present (\mu M)</th>
<th>Uptake of Be\textsuperscript{2+} (nmol) by monolayer at the times indicated</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>0–4 h   16–20 h  20–24 h</td>
</tr>
<tr>
<td></td>
<td>2.34 \pm 0.2 1.22 \pm 0.3 1.26 \pm 0.2</td>
</tr>
<tr>
<td></td>
<td>(6.20)  (1.41)  (1.27)</td>
</tr>
<tr>
<td>500</td>
<td>0–4 h   16–20 h  20–24 h</td>
</tr>
<tr>
<td></td>
<td>3.10 \pm 0.3 4.13 \pm 0.4 5.88 \pm 0.4</td>
</tr>
<tr>
<td></td>
<td>(5.89)  (3.86)  (4.67)</td>
</tr>
</tbody>
</table>

Fig. 2. Insensitivity of G\textsubscript{1}/M phase of cell cycle to Be\textsuperscript{2+}

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 Be\textsuperscript{2+}-free media for 16, 24 h and 48 h respectively. Samples 3 and 5: incubation in the presence of 500\mu M Be\textsubscript{SO\textsubscript{4}}, 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 G\textsubscript{1}–pre-S phase to Be\textsuperscript{2+} cannot be provided from data presented in the present paper. However, previous studies have shown that Be\textsubscript{SO\textsubscript{4}} can inhibit nuclear protein phosphorylation processes (Kaser et al., 1980; Williams & Skilleter, 1983) and Be\textsuperscript{2+} 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 G\textsubscript{1}–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 Be\textsuperscript{2+} inhibition of cell division.

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References


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