Effects of bleomycins on synchronized cells by centrifugal elutriation

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ABSTRACT Chinese hamster ovary (CHO) cells and human oat cell lung carcinoma (ME-MAR) cells were synchronized by centrifugal elutriation. The study of bleomycins (BLM) cytotoxicity to CHO cells showed that G2/M phase was sensitive to BLM, S phase intermediate, and G0/G1 phase resistant. In synchronized MEMAR cells the late S phase was sensitive, G2/M phase intermediate, and G0/G1 phase resistant to BLM toxicity. DNA distribution patterns measured by flow cytometry showed that a significant G2 block was obtained in CHO cells exposed to BLM 4-15 μg/ml. BLM also prevented cell passage from S to G2 phase. A similar G2 block was found in MEMAR cells. However, no S phase accumulation was found up to BLM 15 μg/ml.

KEY WORDS bleomycins; cell cycle; cytotoxicity; cell separation; oat cell carcinoma

The oncolytic agent bleomycins (BLM), a mixture of antibiotic glycopeptides, has been used in the treatment of a wide range of cancers, especially squamous cell carcinoma and lymphoma. In contrast to most anticancer drugs, BLM has little or no bone marrow toxicity and immuno suppressive effect.

BLM can cause single strand breaks of DNA in the treated cells as determined by alkaline elution assay. In this report emphasis was placed on the mode of action of BLM at the cellular level. BLM is a cell cycle specific agent but the data were conflicting. Most of the techniques used to synchronize cells at a given stage of the cell cycle may have some perturbation on the cell kinetic and drug sensitivity. In order to clarify this situation and try to give insight to the use of BLM with other agents in combination the cell cycle kinetic effects of BLM treatment were studied.

Centrifugal elutriation has been used to obtain highly synchronized population of yeast, mouse fibroblasts, mouse lymphoid cells and human lymphoid cells. Since this method gives a satisfactory degree of synchrony with the least perturbation to the cell cycle and drug sensitivity and is applicable to cells in suspension culture, it was chosen to synchronize cells in this study.
MATERIALS AND METHODS

Cell lines CHO cells were grown in monolayer culture and MEMAR (Metastasis/ Martin) cells were maintained in suspension culture[1]. Both cell lines were maintained in F10 medium + 10% fetal calf serum incubated at 37°C in humidified 5% CO2 and kept in exponential growth by subculturing every 2-3 d. All experiments on synchron�ed populations were performed on cells in the logarithmic phase of growth.

Drug Bleomycin (Bristol Laboratories) is a mixture of cytotoxic glycopeptide antibiotics isolated from Streptomyces verticillus. It was dissolved in balanced salt solution prior to use. The concentration of BLM is expressed on the μg/ml basis.

Centrifugal elutriation Cells were elutriated on a Beckman J2-21 centrifuge equipped with JE-6 elutriator (Beckman Instruments, USA). The rotor parts, medium, reservoir and accessory hardware were sterilized in autoclave before being assembled, then 75% methanol was pumped through the entire elutriator system 1 d previously.

Before separation 500 ml sterile water followed by ice cold culture medium (McCoys 5A + 10% fetal calf serum) were pumped through the separation chamber to replace the methanol, so the reservoir, rotor and collecting tubes were kept at 4°C during elutriation.

The CHO and MEMAR cells were centrifuged and concentrated in 20 ml medium. About 106 cells were loaded into the separation chamber at a flow rate of 40 ml/min and a rotor speed of 1730 × g. Then the rotor speed was decreased progressively (16-28 × g each adjustment) and the 40-ml samples were collected for each rotor speed.

Each fraction sample was counted and sized using Coulter Counter Channelizer. All together 20-24 duplicate samples were collected for each separation procedure. Then the synchronized G1, S and G2/M phase cells were selected from the fraction samples for the cell survival study. Each fraction separated by centrifugal elutriation was then analyzed for its position in the cell cycle by determination of DNA content by flow cytometry.

Cell survival study After centrifugal elutriation the desired number of synchronized CHO or MEMAR cells were plated onto Petri dishes or double layer soft agar and incubated at 37°C in humidified 5% CO2 for 12 or 21 d, respectively. Colonies were counted and the plating efficiency and surviving fraction for related cells were calculated[1].

Flow cytometric measurement (FCM) After BLM treatment or centrifugal elutriation the CHO and MEMAR cells were spun down. The pellet cells were dispersed and fixed with 70% methanol and kept at 4°C until analysis. For quantitative DNA analysis the fixed cells were centrifuged, and the cell pellet was resuspended and stained with mithramycin dye solution (2.5 μg mithramycin suspended in 18.75 ml 0.85% saline and 6.25 ml MgCl2, 60 mM/L) overnight, using 1 ml dye solution for 106 cells. The relative fluorescence intensity of the cell nuclei, corresponding to the DNA content, was measured with EPICS V flow cytometer (Coulter Electronic, Inc) using argon laser beam 457 nm (200 mW). passing through 515 IF + 495 LP and 530 LP filters combination to a green PMT. The % of cells in each of these 3 phases was calculated using a computer program based on the mathematical model[1].

RESULTS

Degree of synchrony The data in Tab 1 summarize the cell cycle-phase distribution of each fraction. The purity of G1 fraction was 97% for CHO cells, and 77% for MEMAR cells. In S and G2/M fractions the maximal enrichment for S and G2/M phase cells were 66 and 72% respectively in CHO cells, and 75 and 70% respectively in MEMAR cells. Contamination of the G2/M population by G1 phase cells was consistently <10% in both CHO and MEMAR cells.
Cell phase dependent cytotoxicity of BLM. The sensitivity to BLM in G₁, S, and G₂M CHO cells is shown in Fig. 1. With 1 h exposure to BLM 10 μg/ml for cells beginning at late S phase the sensitivity to BLM increased progressively, the most sensitive cells in the cell cycle being G₂M cells. At 75 μg/ml (1-h exposure) there was a more increase of drug sensitivity quarting from the mid- and late-S phase, and the most sensitive cells were still in late-G₂M phase. With 24-h exposure to BLM 15 μg/ml a similar increase in sensitivity was obtained (data not shown in Fig. 1).

The sensitivity to BLM in G₁, S, and G₂M MEMAR cells are shown in Fig. 1. The phase-dependent cytotoxicity was more significant in MEMAR cells than in CHO cells. When MEMAR cells were exposed to BLM 1 μg/ml for 24 h there was no difference in drug sensitivity among G₁, S, and G₂M cells. At 15 μg/ml (for 1 or 24-h exposure) a significant difference of sensitivity to BLM was found. The order of BLM sensitivity in synchronized MEMAR cells was late-S > G₂M > G₁ phase.

Effect on cell cycle progression. Asynchronous CHO cells were treated with BLM 15 μg/ml for 0.5 to 24-h exposure. By the use of FCM, cell accumulation in phases of the cell cycle can be readily observed in the CHO treated by BLM. After 4-h exposure a clear G₂M phase accumulation (G₂ block) was seen. G₂M accumulation was increased with increasing exposure time of BLM. After 24-h exposure G₂M accumulation was very significant. The G₂ block was also concentration-dependent. The quantitative analysis of the cell cycle effect of BLM (24-h exposure) on CHO cells is shown in Fig. 2. The accumulation of G₂M cells occurred at 0.5 μg/ml and increased rapidly up to 10-
30 µg/ml. After 30 µg/ml the relative % of G2-M cells reached a plateau. There was also a concurrent increase of S phase cells starting from 0.5 µg/ml to 150 µg/ml. The relative G2-M population decreased when the G1-M accumulation increased.

For a similar G1 block protocol was noted with MEM-MEM cells treated by BLN. The G1 block took place after 12-h exposure and became more significant after 24-h exposure. The G1-M accumulation started at 0.5 µg/ml BLN. The G1/M ratio was reversed to 0.5 at 4 µg/ml (before treatment the control value was 1.4). When the BLN concentration reached 15 µg/ml the G1/M population predominated (G1/M = 0.12). There was also a consequent relative decrease of G2/M population with increased G1 cell accumulation. But no S phase accumulation was obtained up to 15 µg/ml (Fig 2).

DISCUSSION

In this report the synchronized cells were obtained using the centrifugal elutriation (CE) technique and the cell cycle distribution was measured by FCM. In comparison with the cell synchronization technique used in the literature11-15, CE and FCM provide the least perturbation to the cell cycle so that the most reliable results should be obtained, and the results reported here may be of more clinical significance. For BLN, 4-15 µg/ml should be the appropriate concentrations to be used in the clinical treatment of cancer in order to get the best cell cycle effect.

The above cell cycle kinetic effect of BLN might be related to the mode of action of BLN cytotoxicity and also the additivity or supraadditivity of lethality with radiation.11 The data indicate that BLN pretreatment of CHO cells blocked the cell cycle in G1 or S phase, the most sensitive phase to both BLN and radiation, since the sensitive cell population was increased and the cytotoxicity of BLN and radiation was enhanced. The cell cycle effect may also be related to the biphasic nature of the survival curve for BLN-treated CHO cells. The % of the resistant cells estimated from the biphasic curve was 54% of the whole population12 falls in the range of the percentage of G2 cells at the late cycle. Therefore, the data suggest that the G2 cells might be responsible for the resistant population of CHO cells. So the most effective use of chemotherapeutic agent could be obtained if the knowledge of the mechanism of action and the phase of cell cycle during which an agent exerts its greatest effect were known. Also such knowledge will be helpful in the design of optimal dose schedules or combination use of several agents.

For CHO and MEMAR cells, the cell cycle effects of BLN were different in character. Although the factors which contribute to the differences in phase specific susceptibility are still not clear, this difference possibly arises from the reduced inactivating system for BLN in MEMAR cells. Low activity of the iLiMA-inactivating enzyme in lung tissue has been described16,17.

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用离心淘洗法同步化细胞研究博来霉素的效应

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摘要 用中国仓鼠卵巢细胞(CCHO)和小鼠肉瘤180细胞(MEMAR)细胞，用离心淘洗法使同步化于有丝分裂
各时相，博来霉素(BLM)细胞周期作用的特性研究。实验表明，CCHO细胞经G(2)M期细胞后，G2期细胞数增加，M
MEMAR细胞经BLM处理后，MAP细胞数不增加。

关键词 博来霉素 细胞周期 细胞分裂 细胞分裂中的

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