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Effects of bleomycins on synchronized cells by centrifugal elutriation

WU De-Zheng[1], Peter KENG (*Cancer Center, University of Rochester, Rochester NY 14642, USA*)

ABSTRACT Chinese hamster ovary (CHO) cells and human oat cell lung carcinoma (MEMAR) cells were synchronized by centrifugal elutriation. The study of bleomycins (BLM) cytotoxicity to CHO cells showed that G_2M phase was sensitive to BLM, S phase intermediate, and G_1 phase resistant. In synchronized MEMAR cells the late S phase was sensitive, G_2M phase intermediate, and G_1 phase resistant to BLM toxicity. DNA distribution patterns measured by flow cytometry showed that a significant G_2 block was obtained in CHO cells exposed to BLM 4-15 μg/ml. BLM also prevented cell passage from S to G_2 phase. A similar G_2 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[1]. In contrast to most anticancer drugs, BLM has little or no bone marrow toxicity and immunosuppressive effect[1,2].

BLM can cause single strand breaks of DNA in the treated cells as determined by alkaline elution assay[3]. In this report emphasis has been placed on the mode of action of BLM at the cellular level. BLM is a cell cycle specific agent but the data were conflicting[4]. Most of the techniques used to synchronize cells at a given stage of the cell cycle may have some perturbance 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[5], mouse fibroblasts[6], mouse lymphoma cells[7] and human lymphoid cells[8] Since this method gives a satisfactory degree of synchrony with the least perturbance to the cell cycle and drug sensitivity and is applicable to cells in suspension culture, it was chosen to synchronize cells in this study.

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[1]Now in *Division of Clinical Pharmacology, North Tai-Ping Road Hospital, Beijing 100850, China*

MATERIALS AND METHODS

Cell lines CHO cells were grown in monolayer culture and MEMAR (Metastasis/Martin) cells were maintained in suspension culture[3]. Both cell lines were maintained in F 10 medium + 10% fetal calf serum incubated at 37℃ in humidified 5% CO_2 and kept in exponential growth by subculturing every 2–3 d. All experiments on asynchronized populations were performed on cells in the logrithmic phase of growth.

Drug Bleomycin (Bristol Laboratories) is a mixture of cytotoxic glycopeptide antibiotics isolated from a strain of *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 J 2–21 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 70% methanol was pumped through the entire elutriator system 1 d previously. Before separation 500 ml sterile water followed by ice cold culture medium (McCoys 5 A + 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℃ during elutriation.

The CHO and MEMAR cells were centrifuged and concentrated in 20 ml medium. About 10^8 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 Channelyzer. All together 20–24 duplicate samples were collected for each separation procedure. Then the synchronized G_1, S and G_2M 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℃ in humidified 5% CO_2 for 12 or 21 d, respectively. Colonies were counted and the plating efficiency and surviving fraction for treated cells were calculated[3].

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℃ 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 mg mithramycin suspended in 18.75 ml 0.85% saline and 6.25 ml $MgCl_2$ 60 nmol/L) overnight, using 1 ml dye solution for 10^6 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 models[9].

RESULTS

Degree of synchrony The data in Tab 1 summarize the cell cycle–phase distribution of each fraction. The purity of G_1 fraction was 97% for CHO cells, and 77% for MEMAR cells. In S and G_2M fractions the maximal enrichment for S and G_2M phase cells were 66 and 72% respectively in CHO cells, and 75 and 70% respectively in MEMAR cells. Contamination of the G_2M population by G_1 phase cells was consistently $<10\%$ in both CHO and MEMAR cells.

Tab 1. Cell cycle distribution of CHO cells and MEMAR cells separated by centrifugal elutriation, relative %, $\bar{x} \pm SD$

Fraction	G_1	S	G_2M
Chinese hamster ovary cells			
Unseparated	67±9	18±7	15±2
G_1	97±3	2.4±3.8	0.6±1.4
S	19±7	66±17	15±9
G_2M	7±3	21±9	72±10
Human oat cell lung carcinoma cells			
Unseparated	51±9	27±55	22±5
G_1	77±3	12±5	11±9
S	13±5	75±5	12±3
G_2M	3±0.7	27±15	70±17

Cell phase dependent cytotoxicity of BLM The sensitivity to BLM in G_1, S, and G_2M CHO cells is shown in Fig 1. With 1 h exposure to BLM 15 μg/ml for cells beginning at late S phase the sensitivity to BLM increased progressively, the most sensitive cells in the cell cycle being G_2M cells. At 75 μg/ml (1-h exposure) there was a more increase of drug sensitivity starting from the mid- and late-S phase, and the most sensitive cells were still in late-G_2M 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 G_1, S, and G_2M 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 G_1, S, and G_2M 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>G_2M>G_1 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 cells treated by BLM. After 4-h exposure a clear G_2M phase accumulation (G_2 block) was seen. G_2M accumulation was increased with increasing exposure time of BLM. After 24-h exposure G_2M accumulation was very significant. The G_2 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 G_2M cells occurred at 0.5 μg/ml and increased rapidly up to 15-

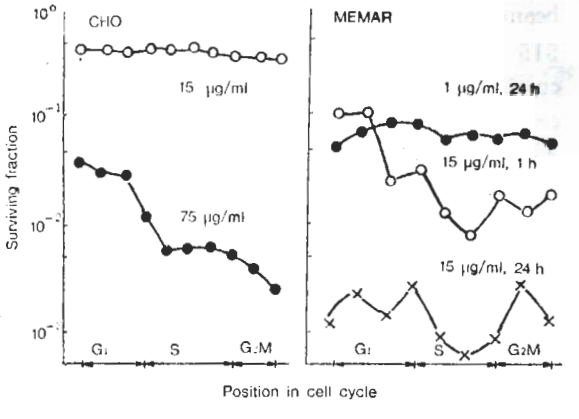

Fig 1. Sensitivity to bleomycin in G_1, S and G_2M phase of Chinese hamster ovary (CHO) cells and human oat cell lung carcinoma (MEMAR) cells. A consistant increase in sensitivity was found from S through G_2M phase. The most sensitive CHO cells were in G_2M phase. However, the most sensitive MEMAR cells were in late S phase.

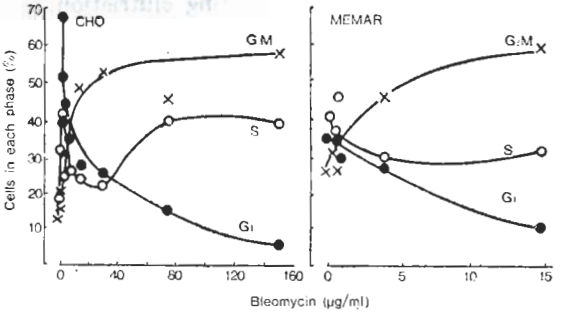

Fig 2. Quantitative cell cycle effect analysis of bleomycin-treated Chinese hamster ovary (CHO) cells and human oat cell lung carcinoma (MEMAR) cells. There is a significant concentration-dependent cell cycle redistribution after 24-h exposure to bleomycin.

30 μg/ml. After 30 μg/ml the relative % of G_2M 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 G_1 population was decreased when the G_2M accumulation increased.

A similar G_2 block pattern was noted with MEMAR cells treated by BLM. The G_2 block took place after 12-h exposure and became more significant after 24-h exposure. The G_2M accumulation started at 0.5 μg/ml BLM. The G_1/G_2 ratio was reversed to 0.6 at 4 μg/ml (before treatment the control value was 1.4), when the BLM concentration reached 15 μg/ml the G_2M population predominated ($G_1/G_2 = 0.18$). There was also a concurrent relative decrease of G_1 population with increased G_2M 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 literature[10-13], CE and FCM provide the least perturbance 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 BLM, 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 BLM might be related to the mode of action of BLM cytotoxicity and also the additivity or supraadditivity of lethality with radiation[3]. The data indicate that BLM pretreatment of CHO cells blocked the cell cycle in G_2 or S phase, the most sensitive phase to both BLM and radiation, since the sensitive cell population was increased and the cytotoxicity of BLM and radiation was enhanced. The cell cycle effect may also be related to the biphasic nature of the survival curve for BLM-treated CHO cells. The % of the resistant cells estimated from the biphasic curve was 54% of the whole population[3] falls in the range of the percentage of G_1 cells in the cell cycle. Therefore the data suggest that the G_1 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 BLM 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 BLM in MEMAR cells. Low activity of the BLM-inactivating enzyme in lung tissue has been described[14,15].

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用离心淘洗法同步化细胞研究博来霉素的效应

吴德政[1]、耿中平　(*Cancer Center, University of Rochester, Rochester NY 14642, USA*)

提要　用中国仓鼠卵巢细胞(CHO)和人小细胞肺癌(MEMAR)细胞，以离心淘洗法使同步化于细胞周期各时相。博来霉素(BLM)细胞毒作用时相特异性研究结果表明，CHO 细胞中以 G_2M 细胞最敏感，S 细胞居中，而 G_1 细胞不敏感；MEMAR 细胞以晚 S 细胞最敏感，G_2M 细胞居中，G_1 细胞不敏感。以流式细胞光度计测定细胞内 DNA 分布图型表明，CHO 细胞经 4-15 μg/ml BLM 处理后发现显著的 G_2 细胞积聚，同时还可见有 S 细胞增加；MEMAR 细胞经 BLM 处理后，仅出现 G_2 细胞积聚。

关键词　博来霉素；　细胞周期；　细胞毒作用；　细胞分离；　燕麦细胞癌

[1]现在北京市北太平路医院临床药理室，北京 100850