Biochemical Pharmacology, Vol. 45, No. 3, pp. 569–572, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

CHROMOSOME DAMAGE INDUCED BY NANOMOLAR CONCENTRATIONS OF BLEOMYCIN IN PORATED MAMMALIAN CELLS

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(*Received* 4 *September* 1992; *accepted* 17 *November* 1992)

Abstract—We have examined chromosome damage caused by a wide range of bleomycin (BLM) concentrations in Chinese hamster ovary (CHO-K1) cells reversibly porated by the bacterial cytotoxin streptolysin-O (SLO). Chromosome damage was measured using the micronucleus cytokinesis block technique (employing cytochalasin-B). Treatment of exponentially growing cells with 0.045 IU/mL SLO for 5 min resulted in up to a thousand-fold and a million-fold increase in biological effectiveness, compared to treatment in the absence of SLO for 24 hr and 5 min, respectively. Increases in micronuclei of 4–5 times background level were observed after only 5 min exposure to the drug in the presence of SLO at doses as low as 100 pg/mL (≈70 pmol/L). These results indicate that the use of SLO may facilitate the treatment of cells with BLM for periods of time resembling acute exposure to ionizing radiations.

Permeabilization or poration of cell membranes allows the increased uptake of otherwise excluded agents and several poration methods have been used in the study of agents causing cytogenetic damage [1–6]. Of these, one of the most widely used, and successful, techniques is electroporation. However, this technique can suffer from lysis of a significant fraction of the electroporated cells under physiological conditions [7, 8]. A technique for the poration of mammalian cells to facilitate uptake of clastogenic agents has recently been developed in our laboratories using the bacterial toxin streptolysin-O (SLO†) [8]. SLO produces pores in cell membranes with pore sizes, increasing with dose, to diameters in excess of 12 nm which allows the release of proteins up to a relative mass of 483 kDa [9] permitting the poration into cells of a wide range of agents. The technique developed by Bryant [8] uses low doses of SLO to permit entry of macromolecules into cells while maintaining cell viability. This has permitted the introduction of restriction endonucleases e.g. *Pvu II*, into Chinese hamster ovary-K1 cells (CHO) in order to mimic ionizing radiation in causing cytogenetic damage such as micronuclei or chromosomal and chromatid aberrations. Treatment of CHO-K1 cells with 0.045 IU/mL SLO (an optimal concentration for CHO cells) for 5 min results in high cell recovery compared with electroporation of cells under the same physiological conditions.

As a result of this work, and our interest in the DNA damaging properties of bleomycin (BLM), we investigated the effect of BLM in inducing lesions in the absence of a continuous membrane barrier.

BLM [10] is a radiomimetic glycopeptide with chemotherapeutic antitumour properties. It is widely used clinically, particularly in the treatment of malignant lymphomas, Hodgkins' disease and squamous cell carcinoma [11]. The action of BLM is believed to be mediated by the oxidative production of single and double stranded DNA breaks in the presence of Fe(II) [12]. In addition, BLM causes membrane peroxidation [13] and aggregation of DNA [14] with the latter being preferential in newly replicated DNA. BLM-induced damage results in chromosome aberrations and growth inhibition [15, 16]. Generally, the lesions induced by BLM are similar in nature and repair pathways to damage produced by ionizing radiations [17].

However, entry of BLM into cells is known to be strongly hindered by the cell membrane with only approximately 0.1% of available BLM being taken up by HeLa cells and only 20% of this amount entering the nucleus [18]. As a result of this, in order to induce damage in cells comparable to that produced by X-rays, high concentrations of BLM and long exposure times are required. In addition, BLM action is not uniform across the cell population treated [19]. The permeabilization of the cell membrane by lysophosphatidylcholine (LPC) has been shown to facilitate both increased uptake of BLM (as measured by repair synthesis and DNA cleavage), and an increase in the proportion of cells damaged [1]. A drawback of this method is that it only facilitates entry of relatively small molecules (<2 kDa), limiting the range of agents that can be examined [20]. Electroporation has also been shown to enhance uptake of BLM *in vitro* [21] and *in vivo*, electric pulses applied directly to tumour sites have successfully increased the antitumour activity of

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† Abbreviations: BLM, bleomycin; SLO, streptolysin-O; EMEMS, supplemented Eagles' minimal essential medium; HBSSM, Hanks' balanced salts solution + $MgCl_2$; CHO, Chinese hamster ovary K1 cells; LPC, lysophosphatidylcholine.

BLM in spontaneous mammary tumours in mice [22]. Other factors which undoubtedly play a part in the clastogenicity of BLM and other radiomimetics are accessibility of the drug to chromatin and the cellular half life of the agent in question since experiments have shown that DNA specifically binds 1 mol of BLM per 10^8 bp for cellular treatments, whereas *in vitro*, DNA binds at an average of 1 mol BLM per 3.1 bp [18]. Cells are known to break down BLM using the BLM-inactivating enzyme, bleomycin hydrolase [23] with varying activities occurring in different cell types [24].

In order to quantify BLM damage to chromosomes in SLO porated mammalian cells we examined the production of micronuclei by BLM, using the cytokinesis block technique [25]. We have examined the frequencies of micronuclei produced by a logarithmic range of BLM concentrations in both untreated cells or cells porated by SLO. CHO-K1 cells were exposed to either chronic (24 hr) treatment in the absence of SLO or acute (5 min) treatment in the presence or absence 0.045 IU/mL SLO.

MATERIALS AND METHODS

Cell culture. CHO cells were grown as monolayers in Eagles' minimal essential medium supplemented with 10% (v/v) calf serum (Gibco BRL, Uxbridge, U.K.), 100 μmol/L $FeCl_3$, 50 U/mL, penicillin and 50 mg/mL streptomycin (EMEMS). All incubations were carried out at 37° in an atmosphere of 5% CO_2, unless otherwise stated. Exponentially growing cells were obtained by seeding 1×10^6 cells per 75 cm^2 tissue culture flasks (Sterilin) and incubating for 24 hr.

Cell poration and bleomycin treatment. Bleomycin sulphate (Lundbeck) and SLO (Wellcome Diagnostics, Beckenham, U.K.) were made up as stock solutions (BLM = 1 mg/mL in H_2O, SLO was made up as per manufacturers instruction to 1.9 IU/mL) and these were stored in aliquots at −20°. Cytochalasin-B (Sigma Chemical Co., Poole, U.K.) was made up as 3 mg/mL stock in dimethyl sulphoxide (Sigma) and stored at −20°. All solutions, except cytochalasin B, were filter sterilized.

Poration was carried out by the method of Bryant [8]. Cells were collected by briefly washing the monolayers twice with 5 mL 0.05% trypsin (Difco Laboratories, Detroit, MI, U.S.A.), 0.02% EDTA followed by 6 min incubation at 37° and resuspension in 10 mL EMEMS. Cells were washed twice by centrifugation with Hanks' balanced salt solution supplemented with 6 mmol/L $MgCl_2$ (HBSSM) at room temperature. For acute BLM treatments (5 min exposure) cells were resuspended at 2×10^6 cells/mL in HBSSM and 100 μL aliquoted into 1.5 mL eppendorf tubes (Treff). HBSSM (100 μL) containing dilutions of BLM ± 0.090 IU/mL SLO was added to give a final cell concentration of 1×10^6 cells/mL and SLO 0.045 IU/mL. The cells were incubated for 5 min at room temperature before addition of 1 mL ice-cold EMEMS. They were then immediately centrifuged (6500 rpm) for 1 min in a microfuge (MSE Micro-centaur) and the supernatant aspirated. The cell pellet was resuspended in 1 mL EMEMS + 3 μg/mL cytochalasin-B before plating out in tissue culture grade multi-well plates (Sterilin). For chronic BLM treatments (24 hr exposure) 2×10^5 cells were plated out in 1 mL EMEMS containing 3 μg/mL cytochalasin-B plus various dilutions of BLM. All cells were incubated for 24 hr in a humidified incubator at 37°.

Cell fixation and scoring. Cells were collected by trypsinization and approximately 5×10^4 cells cytospun (Shandon Cytospin II) onto glass slides (BDH, Poole, U.K.) and air dried. The cells were fixed for 10 min in methanol and again air dried before staining in 10% filtered aqueous Giemsa solution for 20 min. Slides (without coverslips) were examined under high magnification (oil immersion × 1800) and the number of micronuclei per 100 binucleate cells were scored. These frequencies were normalized by subtracting the background level of micronuclei per 100 binucleate cells in the untreated control for each experimental group.

RESULTS AND DISCUSSION

The number of micronuclei induced per 100 binucleate cells by BLM in the presence or absence of 0.045 IU/mL SLO are shown in Fig. 1. It can be seen that SLO markedly increases the frequency of micronuclei from chronic treatments in the absence of SLO especially in the low dose range where concentrations to give the same effect are approximately 10^{-4} times that required in the absence of SLO. Acute treatment (5 min) without SLO gave very low levels of micronuclei even at 100 μg/mL. The dose–response relationship for both acute plus SLO and chronic treatments minus SLO gave maximal micronucleus induction at 10 ng/mL and 10 μg/mL, respectively. At higher concentrations a reduction in frequency occurred especially in the case of BLM plus SLO. The absolute frequencies of micronuclei achieved in the two cases were also different with maximum frequencies of 70.2 (±16.3) and 108.7 (±15.4) micronuclei per 100 binucleate cells scored for chronic and acute treatments, respectively. Background levels of micronuclei for the three experimental groups were: 24 hr, 3.33 (±1.09); 5 min −SLO, 5.0 (±2.04); 5 min +SLO, 4.14 (±0.63). This would indicate that SLO treatment does not increase the numbers of micronuclei by itself, however the possibility of synergism between BLM and the effects of poration cannot be ruled out. The reason for a downturn in numbers of micronuclei at high doses of BLM in porated and non-porated (24 hr) cells is at present uncertain but it may be the result of different forms of damage occurring which cause cell death prior to mitosis.

In order to examine whether SLO treatment increases either the number of binucleates containing damage or the amount of damage within individual damaged binucleate cell, Fig. 2 shows the mean number of micronuclei per damaged binucleate cell versus the percentage of binucleate cells containing micronuclei. This shows the total number of micronuclei is a linear function of both the number of binucleates containing damage and the number of micronuclei per damaged binucleate. Poration would appear to increase the total number of

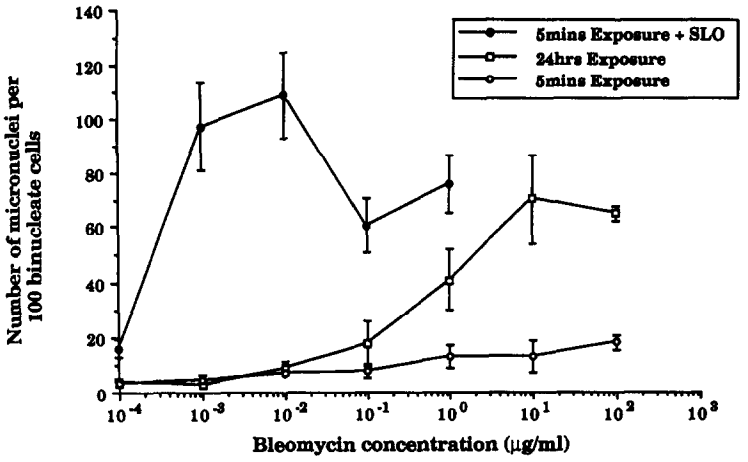

Fig. 1. Number of micronuclei per 100 binucleate cells as a function of BLM concentration, in the presence (filled circles) or absence (squares and open circles) of 0.045 IU/mL SLO. Treatment with BLM was for 5 min (circles) or 24 hr (squares). SLO treatment was concurrent with BLM. Vertical bars represent values of the SEM micronucleus frequency per 100 binucleate cells (normalized with respect to the control). Results are from 2–5 independent experiments.

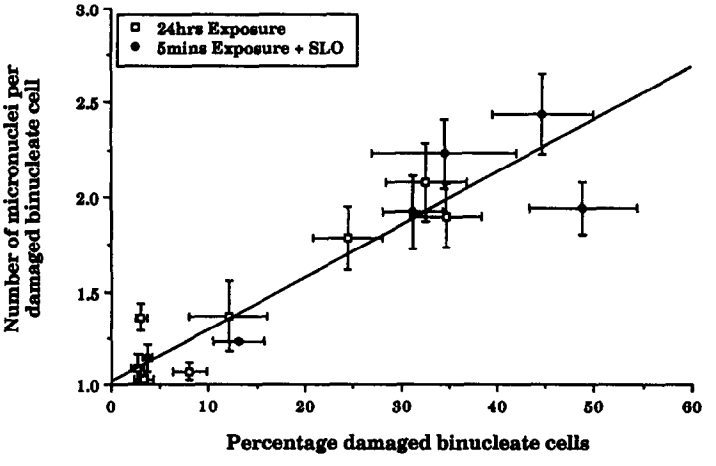

Fig. 2. Correlation between the number of BLM-induced micronuclei per damaged binucleate cell and the percentage of damaged binucleate cells for CHO cells treated in the presence (filled circles) or absence (squares) of 0.045 IU/mL SLO. Treatment with BLM was for 5 min (circles) or 24 hr (squares). SLO treatment was concurrent with BLM. Vertical and horizontal bars represent the SEM for 2–5 independent experiments.

micronuclei by increasing both of these parameters to the same extent, indicating that damage in porated cells is a function of increased amounts of BLM entering all cells of the population rather than either an increased proportion of cells damaged or an increase in numbers of micronuclei within the same fraction of cells susceptible to damage.

From the results presented above it is clear that poration with a low concentration of SLO produces a very large increase in chromosome damage caused by BLM as measured by the production of micronuclei. On a concentration basis, this increase is approximately a thousand-fold and a million-fold compared to treatment in the absence of SLO for 24 hr and 5 min, respectively. The damage caused by BLM in the presence of SLO would appear to be similar since there is no significant variation in the number of micronuclei induced per damaged cell. The cell membrane is thus a very effective barrier to uptake of BLM as also shown by Sidik and Smerdon [1] who have found that the early end-points of DNA breakage and repair are enhanced in LPC-permeabilized human fibroblast cells and also by Poddevin *et al.* [21] who introduced BLM by electropermeabilization and who measured uptake of BLM and relative cloning efficiencies of

transformed Chinese hamster lung fibroblast cells. LPC (80 μg/mL) and electropermeabilization (1500 V/cm) were found to increase the uptake by 50- and 100-fold as measured by repair synthesis levels and relative cloning efficiencies, respectively.

From these results, with BLM, we conclude that SLO can be effectively used to introduce cytotoxic molecules into cells at concentrations much lower than would otherwise be required for non-porated cells. SLO may also permit treatment of cells with other chemical agents on a short timescale that more closely mimics acute treatment with ionizing radiations; although the actual exposure of the cellular target to the introduced drug will still be dependent on the half life of the agent introduced.

Acknowledgements—We thank John Macintyre for valuable technical assistance. This work was supported by funds from the Commission of European Communities and the Cancer Research Campaign.

REFERENCES

1. Sidik K and Smerdon MJ, Bleomycin induced DNA damage and repair in human cells permeabilised with lysophospatidyl-choline. *Cancer Res* **50**: 1613–1619, 1990.
2. Bryant PE, Enzymatic restriction of mammalian cell DNA using *Pvu II* and *Bam H1*: evidence for the double strand break origin of chromosomal aberrations. *Int J Radiat Biol* **46**: 57–65, 1984.
3. Natarjan AT and Obe G, Molecular mechanisms involved in the production of chromosomal aberration, III. Restriction endonucleases. *Chromosoma* **90**: 120–127, 1984.
4. Obe G and Winkel E, The chromosome breaking activity of the restriction endonuclease *Alu I* in CHO cells is independent of the S-phase of the cell cycle. *Mutat Res* **152**: 25–29, 1985.
5. Winegar RA and Preston RJ, The induction of chromosomal aberrations by restriction endonucleases that produce blunt-ended or cohesive ended double strand breaks. *Mutat Res* **197**: 141–149, 1988.
6. Winegar RA, Phillips JW, Youngblom JH and Morgan WF, Cell electroporation is highly efficient method for introducing restriction endonucleases into cells. *Mutat Res* **225**: 49–53, 1989.
7. Costa ND and Bryant PE, The induction of DNA double strand breaks in CHO cells by *Pvu II*: kinetics using neutral filter elution (pH 9.6). *Int J Radiat Biol* **57**: 933–938, 1990.
8. Bryant PE, Induction of chromosomal damage by restriction endonuclease in CHO cells porated with streptolysin-O. *Mutat Res* **268**: 27–34, 1992.
9. Buckingham L and Duncan JL, Approximate dimension of membrane lesions produced by streptolysin S and streptolysin O. *Biochim Biophys Acta* **729**: 115–122, 1983.
10. Umezawa H, Maeda K, Takeuchi T and Okami Y, New antibiotics, bleomycin A and B. *J Antibiot (Tokyo)* **19**: 200–209, 1966.
11. Hecht SM, DNA strand scission by activated bleomycin group antibiotics. *Fed Proc* **45**: 2784–2791, 1986.
12. Byrnes RW, Templin J, Sem D, Lyman S and Petering DH, Intracellular DNA strand scission and growth inhibition of Ehrlich ascites tumour cells by bleomycins. *Cancer Res* **50**: 5275–5286, 1990.
13. Ciriolo MR, Peisach J and Magliozzo RS, A comparative study of the interactions of bleomycin with nuclei and purified DNA. *J Biol Chem* **264**: 1443–1449, 1989.
14. Woynarowski JM and Beerman TA, Preferential effect of bleomycin on newly replicated chromatin in nuclei from L1210 cells. *Biochim Biophys Acta* **1007**: 116–119, 1989.
15. Vig BK and Lewin R, Genetic toxicology of bleomycin. *Mutat Res* **55**: 121–145, 1978.
16. Larramendy ML, Lopez-Larraza D, Vidal-Rioja L and Bianchi NO, Effect of the metal chelating agent *o*-phenanthroline on the DNA and chromosome damage caused by bleomycin in Chinese hamster ovary cells. *Cancer Res* **49**: 6583–6586, 1989.
17. Byfield JE, Lee YC, Tu L and Kulhanian F, Molecular interaction of the combined effects of bleomycin and X-rays on mammalian cell survival. *Cancer Res* **36**: 1138–1143, 1976.
18. Roy SM and Horwitz SB, Characterisation of the association of radiolabelled bleomycin A_2 with HeLa cells. *Cancer Res* **44**: 1541–1546, 1984.
19. Östling O and Johanson KJ, Bleomycin, in contrast to gamma irradiation, induces extreme variation of DNA strand breakage from cell to cell. *Int J Radiat Biol* **52**: 683–691, 1987.
20. Miller MR, Castellot JJ Jr and Pardee AB, A permeable animal cell preparation of studying macromolecular synthesis. DNA synthesis and the role of deoxyribonucleotides in S-phase initiation. *Biochemistry* **17**: 1073–1080, 1978.
21. Poddevin B, Orlowski S, Belehradek J Jr and Mir LM, Very high cytotoxicity of bleomycin introduced into the cytosol of cells in culture. *Biochem Pharmacol* **42**: S67–S75, 1991.
22. Belehradek J Jr, Orlowski S, Poddevin B, Paoletti C and Mir LM, Electrochemotherapy of spontaneous mammary tumours in mice. *Eur J Cancer* **27**: 73–76, 1991.
23. Umezawa H, Hori S, Sawa T, Yoshioka T and Tacheuchi T, A bleomycin inactivating enzyme in mouse liver. *J Antibiot (Tokyo)* **27**: 419–424, 1974.
24. Müller WEG and Zahn RK, Bleomycin, an antibiotic that removes thymine from double-stranded DNA. *Prog Nucl Acid Res* **20**: 21–57, 1977.
25. Fennech M and Morley AA, Measurement of micronuclei in lymphocytes. *Mutat Res* **147**: 29–36, 1985.