Modulation of the clastogenic activity of bleomycin by reduced-glutathione, glutathione-ester and buthionine sulphoximine

A.Chattopadhyay, S.Choudhury and A.Chatterjee[1]

Genetics Laboratory, Department of Zoology, North-Eastern Hill University, Mawlai, Shillong-793022, India

[1]To whom correspondence should be addressed

In this study an attempt has been made to establish a relationship between bleomycin (BLM)-induced DNA damage and buthionine sulphoximine (BSO)-mediated modified endogenous glutathione (GSH) status in normal human lymphocytes. Present results demonstrate that depletion of endogenous GSH by BSO reduced the clastogenic action of BLM, whereas elevation of endogenous GSH by treating the cells with GSH and GSH-ester, potentiates the cytotoxicity of BLM. A significant reduction in the frequency of deletions and chromatid breaks was observed when BSO-treated cells were treated with BLM. Again the frequency of these two types of aberrations was increased significantly when GSH- and GSH-ester-treated cells were treated with BLM. The observed reduction in the effect of BLM in GSH-depleted cells could be explained on the basis of the failure of reactivation of the oxidized BLM by reducing agent GSH which is present endogenously. Similarly, it appears that radicals which are generated due to reduction of oxidized BLM by the increased level of cellular GSH, after treating the cells with GSH or GSH-ester, could be responsible for the increasing frequency of deletion and chromatid breaks.

Introduction

Bleomycin (BLM) is a glycopeptide antibiotic, anti-tumour drug that has been shown to produce chromosome aberrations (CAs) comparable with those induced by X-irradiation (Promchainant, 1975; Scott and Zampetti-Bosseler, 1985; Chatterjee and Jacob-Raman, 1988) and it induces CAs at all stages of the cell cycle (Povirk and Austin, 1991). As in the case of indirect action of radiation, BLM is also known to induce DNA breaks through the production of free radicals (Sausville et al., 1976; Lown and Sim, 1977; Takeshita et al., 1978).

The role of reduced glutathione (GSH) on cellular metabolism has been well established and therefore it is of interest to see the effects of cellular GSH level on the cytotoxicities of chemicals. Tumour cells cultured in vitro, in particular those of human origin, were shown to contain extremely high levels of GSH (Biaglow et al., 1983; Mitchell et al., 1985). It has been evident that tumour cells made resistant to some anti-cancer drugs, e.g. melphalan, cis-platin and adriamycin, have increased cellular GSH concentrations (Green et al., 1984; Hamilton et al., 1985). For these reasons much current interest has focused on techniques of reducing cellular levels of GSH prior to treatment with cytotoxic agents. The development of buthionine sulphoximine (BSO), a specific inhibitor of GSH synthesis, has removed much of the uncertanties due to

unwanted side-effects that were associated with GSH depleting agents having less specificity. The concentration of GSH in various cells grown in tissue culture decline rapidly in the presence of BSO (Dethmers and Meister, 1981).

The ability of BSO to potentiate the antitumour activity of anticancer drugs has been demonstrated convincingly in vitro in human tumour lines for adriamycin, melphalan and cis-platin (Hamilton et al., 1985) and for in vivo for cyclophosphamide (Ono and Shrieve, 1986), bleomycin and cis-platin (Tsutsui et al., 1986). Russo et al. (1984) reported that depletion of cellular GSH concentration enhanced the cytotoxicity of BLM treatment and protection was observed when the GSH level was elevated.

On the other hand, an attempt has been made to increase the level of endogenous GSH and observe its effect on BLM action. By allowing the simultaneous presence of GSH and BLM during treatment, Chatterjee et al. (1989) demonstrated that the presence of GSH potentiates the clastogenic action of BLM in muntjac lymphocytes in vitro. This potentiation was attributed to GSH acting as a reducing agent in reactivating oxidized BLM. A dose-dependent potentiation by the cationic thiol radioprotector WR-1065 in the induction of micronuclei by BLM in G_0 lymphocytes has been reported (Hoffmann et al., 1993). Results of certain earlier biochemical studies also showed an enhanced reduction in the molecular size of DNA when BLM treatment was coupled with mercaptoethylamine (MEA) pretreatment in the case of synthetic DNA (Nagai et al., 1969) and in DNA isolated from HeLa or Escherichia coli cells (Suzuki et al., 1969).

Several studies have indicated that combining BSO with cytotoxic agents does not adversely affect normal tissue toxicity. For example, Russo et al. (1986) showed no effect of BSO on CFU-s survival and peripheral white blood cell counts following melphalan treatment. No detectable effect on the lung toxicity of cyclophosphamide as measured by breathing rate (Lee et al., 1987). However, more in-depth studies using normal tissue toxicity models best suited for each cytotoxic drug should be carried out before combination therapy with BSO can safely be used in patients. Therefore, in the present study we made an attempt to establish a relationship between BLM-induced DNA damage in normal and BSO-treated human lymphocytes. In order to increase the endogenous GSH level we used a GSH-ester as well as GSH. GSH is generally not considered to be transported across the cell membrane (Clark, 1986), whereas GSH-ester is readily transported into cells and converted to GSH increasing the level of GSH within 3–4 h (Puri and Meister, 1983; Wellner et al., 1984). By using GSH, GSH-ester and BSO, this study showed that depletion of cellular GSH by BSO reduces the effect of BLM, whereas elevation of cellular GSH potentiates BLM action.
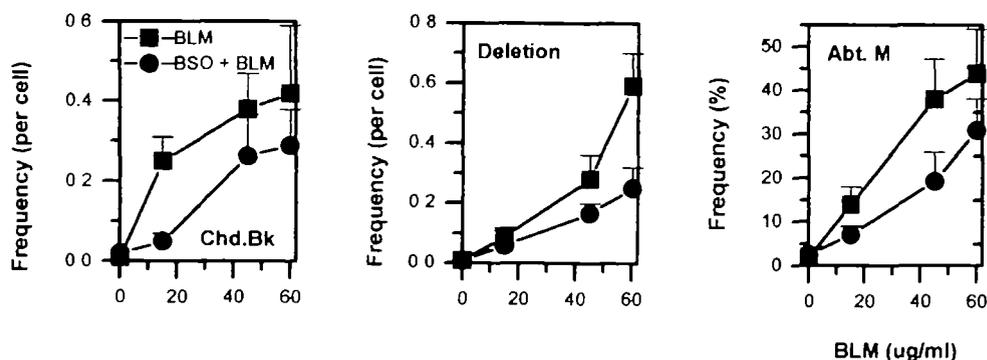
Materials and methods

Fig. 1. The frequency of chromatid breaks, deletions and aberrant metaphases in BLM and BSO + BLM treated sample. Mean ± SEM are indicated in all cases.

10% heat-inactivated fetal calf serum (Biological Industries Ltd, Israel) following phytohaemagglutinin (PHA; Gibco) stimulation and incubated at 37°C To obtain differential sister chromatid staining, 5 μg/ml 5-bromodeoxy-undine (BrdU, Sigma, St Louis, MO, USA) was added to the cultures at the time of initiation. Cells were harvested at 48 h and colcemid (0.1 μg/ml) was added 2 h prior to that

Bleomycin, GSH, GSH-ester and BSO-treatment

Commercially-available bleomycin (Bleocin; Nippon Kayaku, Japan) was hydrated with sterilized triple-distilled water, from which a fresh working solution of 100 μg/ml was prepared by addition of RPMI 1640 (pH 6.8) just prior to each use, and given to cells to make a final concentration of 15, 45 and 60 μg/ml. Buthionine sulphoximine (BSO, Sigma, USA) was dissolved in phosphate buffer solution (pH 7.4) and 1 and 5 mM BSO were added into the 1 ml aliquot of whole blood for 3 h and then BLM was added

In a separate set of experiments GSH and GSH-ester were used. Three hours before BLM treatment, the amount of reduced-glutathione (GSH; Sigma) and glutathione-ester (Sigma) required to make a final concentration of 10 mM for the volume of the blood sample to be treated were weighed out, dissolved in 0.2 ml of medium and mixed well with blood and incubated at 37°C. There were three experiments in this set where the concentration of BLM was 40 and 60 μg/ml In another set of experiments with a different blood donor, 15 mM GSH was added 45 min after 40 μg/ml BLM treatment In all these cases, BLM, GSH + BLM and GSH-ester + BLM, the treated samples were washed twice with pre-warmed medium at the end of 2 h BLM treatment.

Chromosome preparations were made from hypotonically (KCl, 0.075 M)-shocked cells fixed in 1:3 aceto-methanol. Differential sister chromatid staining was carried out by the method of Goto *et al.* (1975). Slides were coded and CAs were scored as dicentric, ring, deletion (both terminal and interstitial) and chromatid break. Though it is hard to distinguish precisely between terminal and interstitial deletions, however, we considered the deletions which are double minute-like (smaller than the diameter of the chromatids) as interstitial and the rest of the fragments as terminal. Translocation were not scored since reciprocal translocation cannot be distinguished without G-banding. Metaphases were categorized as in first, second and subsequent division cycles based on their differential staining patterns and aberrations were scored only from first cycle metaphases (M₁) only

Statistical analysis

Data were analysed using the 2×2 contingency test for the frequency of aberrant metaphases. In order to see the statistical significance of the differences between the BLM and BSO + BLM groups for the frequency of deletions or chromatid breaks, a simple χ^2 test (3×2 tables) was used. The actual observed data were used during statistical analysis. The mean aberration data along with SEM were plotted by using Microccal Origin implemented on a Toshiba T2100 computer

Results

BLM-induced CAs in normal lymphocytes were studied in human peripheral blood lymphocytes as positive control to BSO-treated lymphocytes in each experiment and their results are presented individually in Table I. Negative control data were shown in two experiments. Contrary to the anticipated sensitizing effect of GSH-depletion by BSO, a significant reduction in the frequency of deletions and chromatid breaks was observed in most of the experiments except experiment

no. 3 where BSO was unable to reduce the frequency of CAs induced by 45 and 60 μg/ml BLM.

Though the frequency of aberrations was observed to vary from sample to sample, the frequency of CAs induced by BLM in BSO-treated samples clearly showed that the frequency of deletions and chromatid breaks was considerably reduced. The category of deletions and chromatid breaks showed the most prominent reduction. Both 1 and 5 mM BSO showed an appreciable reduction in the frequency of BLM-induced CAs. However, 5 mM showed a slightly better degree of reduction than 1 mM BSO-treatment. The frequency of aberrant metaphases was also reduced considerably. From the individual sample data it was clear that the frequency of exchanges did not show any significant reduction. In fact there was insufficient induction of exchanges by BLM in this experiment. The mean frequency of deletions, chromatid breaks and aberrant metaphases induced by BLM in BSO-treated and untreated samples were plotted and are shown in Figure 1.

Table II represents data of individual experiment where GSH was added 45 min after BLM addition (section A) and in section B, GSH or GSH-ester was added 3 h before BLM treatment. In both experiments of section A a significant enhancement in the frequency of deletions and chromatid breaks has been observed. In section B, GSH-ester showed a better degree of potentiation than GSH with respect to the frequency of BLM-induced CAs. Considerable enhancement in the frequency of deletions and chromatid breaks was observed when GSH was added 45 min after or when GSH-ester was added 3 h before BLM treatment. In both GSH and GSH-ester treated samples a good number of interstitial deletions was observed which was extremely low in number only in the BLM-treated sample. From the data it is clear that GSH or GSH-ester mediated potentiation has not occurred for the frequency of exchanges.

It may be noted that BSO (5 mM; Table I) and GSH or GSH-ester treatment alone (Table II) did not induce any type of CAs since no change was observed from the frequency of spontaneous aberrations.

Discussion

It has been shown previously that, like ionizing radiation, BLM can induce CAs (Ohara and Kadotani, 1970; Vig and Lewis, 1978) and the cellular target for CAs induction by BLM is DNA (Evans, 1977). At equal levels of deletions and rearrangements induced by 40 μg/ml BLM and 2 Gy X-rays in muntjac lymphocytes the frequency of aberrant metaphases and the delay in cell cycle kinetics are less in BLM-treated

Table I. Effect of BSO-treatment on chromosome aberrations induced by bleomycin (BLM) in human peripheral blood lymphocytes *in vitro*

BLM (µg/ml)	BSO (mM)	Abt. M. (%)	TM	Aberrations/cell			Expt no.
				Exchange[a]	Deletion	Chd.bk	
0	0	02	101	0	0.01	0.00	1
0	5	03	113	0	0.01	0.02	
15	0	13	145	0.01	0.04	0.12	
	1	17	070	0.00	0.14	0.14	
	5	03[b]	113	0.00	0.02	0.02[c]	
45	0	30	147	0	0.01	0.00	2
	5	06[d]	098	0.07	0.18	0.26[c]	
0	0	02	102	0	0.01	0.02	
0	5	02	110	0.00	0.16	0.02	
15	0	22	124	0.04	0.08	0.32	
	1	14	205	0.01	0.10	0.08[c]	
	5	10[b]	227	0.01	0.04	0.09[c]	
45	0	37	128	0.04	0.33	0.44	
	1	09[b]	066	0.00	0.06[c]	0.26[c]	
	5	11[b]	184	0 01	0.08[c]	0.16[c]	
15	0	08	072	0.01	0.15	0.32	3
	5	07	070	0.06	0.11	0.03[c]	
45	0	22	128	0.01	0.11	0.20	
	1	30	150	0.03	0.29	0.23	
	5	24	053	0.04	0.19	0.38	
60	0	Not responded					
	1	28	178	0.04	0.12	0.30	
	5	25	197	0.04	0.15	0.45	
45	0	54	084	0.25	0.39	0.50	4
	5	36[b]	099	0.03	0.24[c]	0.42	
60	0	63	123	0.13	0.52	0.76	
	5	48[b]	118	0.15	0.38[c]	0.44[c]	
60	0	39	179	0.12	0.82	0.29	5
	1	39	142	0.05	0.46[c]	0.41	
	5	35	106	0.09	0.37[c]	0.20	
60	0	29	150	0.03	0.43	0.20	6
	5	14[e]	055	0.04	0.09[c]	0.07	

Abt. M., aberrant metaphase; TM, total metaphase.
[a]Dicentric + ring.
[b]$P < 0.01$ (2×2 contingency χ^2 test).
[c]$P < 0.001$ (χ^2 test, $df = 2$).
[d]$P < 0.001$ (2×2 contingency χ^2 test).
[e]$P < 0.05$ (2×2 contingency χ^2 test).

cultures than in the irradiated one (Chatterjee and Jacob-Raman, 1988). As outlined in the Introduction, this study was carried out to check the effect of endogenous GSH depletion by BSO on the DNA-damaging ability of BLM in normal cells. Using cell survival as a parameter, Russo *et al.* (1984) reported that depletion of cellular GSH enhanced the cytotoxicity of BLM treatment and protection was observed when the level of GSH was elevated in tumour cell lines. However, there have been no studies on the effect of BSO-mediated GSH-depletion on the clastogenic action of BLM with respect to CAs in normal cells.

The present results showed that BSO depletion of cellular GSH by reduces the effect of BLM with respect to CA induction. Therefore, endogenous GSH whose depletion could sensitize the cells against radiation-induced damage (Bump *et al.*, 1982; Clark *et al.*, 1984), may not be effective in sensitizing the cells against damage induced by radiomimetic drugs such as BLM. The reducing effect of BLM-induced CAs with regard to GSH depletion by BSO observed in the present study was associated with a significant reduction in the frequency of chromatid breaks, deletions and aberrant metaphases. Thus it appears that BSO-mediated GSH depletion reduces the number of induced strand breaks and thereby reduces the frequency of both types of aberrations.

In contrast to GSH-depletion by BSO, when an attempt was made to increase the endogenous level of GSH by treating the lymphocytes either with GSH or GSH-ester, potentiation of BLM action was observed. This is in agreement with the reported effect of GSH and cysteine in muntjac lymphocytes where the frequency of CAs are enhanced significantly (Chatterjee *et al.*, 1989; Chatterjee and Jacob-Raman, 1993).

However, in the present study, potentiation of BLM action was more pronounced in cultures with GSH-ester than with GSH when both were added 3 h before BLM treatment. It is worth noting that GSH added 45 min after BLM addition potentiated BLM action more significantly than GSH added 3 h before BLM treatment. This is in support of the earlier observation in muntjac lymphocytes, where a higher degree of potentiation of BLM action was observed when GSH was added 30 min after rather than 30 min before BLM treatment (Chatterjee *et al.*, 1989). Since GSH is not generally considered to be transported across the cell membrane (Clark, 1986), GSH-ester was used in this study which is readily transported into cells and converted to GSH thereby increasing the level of GSH within 3–4 h (Wellner *et al.*, 1984). Thus, for a true comparison we treated the cells with 10 mM GSH and GSH-ester for 3 h before BLM addition. It may be that very little

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Table II. Effect of GSH and GSH-ester on chromosome aberrations induced by bleomycin (μg/ml) in human peripheral blood lymphocytes

Experimental conditions	Abt. M (%)	TM	Aberrations/cell		
			Exchange[a]	Deletion	Chd.bk.
Section A					
Untreated	02	96	0	0.02	0.01
GSH (15 mM)	03	112	0	0.02	0.02
BLM (40)	52	177	0 03	0.28	0.78
BLM + GSH	71[b]	114	0 03	0.50[c]	1.00[d]
Untreated	02	114	0	0.02	0
GSH (15 mM)	03	135	0	0.01	0 03
BLM (40)	41	205	0.02	0 13	0.55
BLM + GSH	39	141	0.02	0 23[c]	0.65[c]
Section B					
GSH-ester (10 mM)	02	140	0	0.01	0.02
BLM (40)	29	150	0.03	0.43	0.20
GSH (10 mM) + BLM	29	076	0.06	0.10	0.46[d]
GSH-ester + BLM	36	072	0.06	0.38	0 47[d]
GSH-ester (10 mM)	03	105	0	0 01	0.03
BLM (40)	21	107	0.02	0.14	0 08
GSH (10 mM) + BLM	29	161	0.09	0.16	0.47[d]
GSH-ester + BLM	45[b]	244	0.02	0 52[d]	0 75[d]
BLM (60)	63	123	0 13	0.52	0.76
GSH (10 mM) + BLM	62	087	0 15	0.66[c]	0.66
GSH-ester + BLM	58	081	0.17	0.68[c]	0 83

Abt. M., aberrant metaphase; TM, total metaphase.
Section A GSH was added 45 min after BLM treatment; Section B GSH or GSH-ester was added 3 h before BLM treatment
[a]Dicentrics + rings
[b]$P < 0.01$ (2×2 contingency χ^2 test).
[c]$P < 0.05$ (χ^2 test, $df = 2$)
[d]$P < 0.001$ (χ^2 test, $df = 2$)

GSH is left in the reduced form in the cell after 3 h pretreatment with GSH and therefore the potentiating effect is much less.

In order to explain how GSH depletion reduces BLM action, one has to consider the mechanism of BLM action itself. It is known that BLM requires a metal ion cofactor for its activity and is capable of binding with Fe(II) to yield an oxygen-sensitive complex Fe(II).BLM (Sausville et al., 1976). The oxidation of this complex to Fe(III).BLM yields a radical which is responsible for DNA damage. The presence of reducing agents such as MEA and dithiothreitol enhance the breakage of DNA by BLM (Suzuki et al., 1969; Umezawa et al., 1973) by reducing the Fe(III).BLM to Fe(II).BLM; the mechanism allows multiple radical production from a single iron BLM. Therefore, the reducing effect of BLM on CAs in GSH-depleted cells observed in the present study could be explained on the basis of the failure of reactivation of the oxidized BLM by the reducing agent GSH which is present endogenously. Similarly it appears that the radicals generated due to reduction of Fe(III).BLM to Fe(II).BLM by increased levels of endogenous GSH, after treating the cells by GSH and GSH-ester, might be responsible for the increased frequency of deletions and chromatid breaks.

GSH-ester- and GSH-mediated potentiation was more pronounced in the production of deletions and chromatid breaks and similarly BSO-mediated reduction was also effective in these two types of aberrations. It is also of interest to note that there was a significant increase in interstitial-type deletions in GSH-ester- and GSH-treated samples in the present study which is also in support of the earlier observation in muntjac lymphocytes (Chatterjee et al., 1989). If it is truly interstitial in nature, this indicates that the BLM molecule bound to the DNA helix causes strand scissions at physically close points. This could be a reflection of the biphasic action of BLM in the presence of a reducing agent which has been discussed earlier. The frequency of exchanges induced by BLM was not influenced either by GSH or GSH-ester or by BSO treatment. We consider this to be due to the intrinsically lower production of exchanges by BLM in human peripheral lymphocytes. There are reports regarding the absence of dicentrics, rings and triradials (Promchainant, 1975) or very few dicentrics (Bornstein et al., 1971) in human lymphocytes after treatment with BLM for 24 h before harvesting. However, it is interesting to note that in spite of considerable increase in BLM-induced DNA strand breaks in GSH-ester and GSH-treated sample, the frequency of exchanges did not increase at all. This is not the case for muntjac lymphocytes where the frequency of exchanges was increased significantly (Chatterjee et al., 1989). Therefore, what appears apparently as a case of differential pattern of sensitization in human or in muntjac may be the only an expression of the intrinsic features of the test systems themselves.

There is interest in using thiol compounds such as WR-1065 or WR-2721 to minimize damage to non-target tissues in cancer chemotherapy and radiotherapy (Glover et al., 1988; Yuhas et al. 1980). Although GSH and WR-1065 may provide protection against some cytotoxic drugs (Berrigan et al., 1982; Wolf et al., 1987; Glover et al. 1988), they enhance the clastogenicity of BLM in G_0 lymphocytes (Chatterjee et al. 1989; Littlefield and Hoffmann, 1993). Enhancement of damage in non-target tissues may pose a risk if thiol compounds

are used in combined therapy with BLM. Therefore, the relative concentration of GSH in normal and tumour cells will be an important factor in determining therapeutic index while BLM is used as an anticancer drug.

Acknowledgement

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