

Interaction of radiation- and bleomycin-induced lesions and influence of glutathione level on the interaction

A.Dutta, A.Chakraborty¹, A.Saha¹, S.Ray and A.Chatterjee*

Genetics Laboratory, Department of Zoology, North-Eastern Hill University, Shillong-793 022, India and ¹Inter University Consortium, Jadavpur University 2nd Campus, Bidhan Nagar, Salt Lake, Kolkata-700 098, India

Radiation-induced exchange aberrations are thought to arise as a consequence of misrejoining of free ends of DNA double strand breaks (dsbs). In quiescent mammalian cells this process of misrejoining is prevalently taken up by the non-homologous end joining (NHEJ) process. In order to investigate the role of glutathione (GSH) in DNA dsb rejoining, the interaction of the lesions induced by bleomycin (Blem) and by radiation was studied since the lesions caused by both have similar and apparent rapid rates of repair. Endogenous GSH was depleted by buthionine sulfoximine (BSO) and chromosome aberrations (CAs) of human lymphocytes were scored from first cycle metaphases. Gamma radiation was administered 2 h after Blem treatment in combined studies. In the case of BSO, the treatment was given 3 h before Blem treatment. The BSO-treated samples showed higher sensitivity to radiation than BSO-untreated ones. Combined treatment of Blem and radiation induced higher frequency of CAs, in particular the exchange aberrations and interstitial deletions. However, such increased frequency of exchange aberrations was reduced drastically and the frequency of terminal deletions was increased significantly when combined treatment was given to BSO-pretreated cells. The consistent level of Ku70 protein in all the treated samples, with undetectable level of Rad51 in the G₀-lymphocytes indicates the involvement of NHEJ pathway in misrejoining of DNA dsbs. It may be hypothesized that reduction in the frequency of exchange aberrations as induced by Blem + radiation combined treatment in BSO-treated samples could be because of reduced NHEJ pathway.

Introduction

A large number of studies suggests that DNA double strand break (dsb), induced by ionizing radiations, is the critical lesion which, if unrepaired or misrepaired, can cause chromosome aberrations (CAs), cell death as well as mutations and cell transformation (1,2). Radiation induces exchange aberrations, which are thought to arise as a consequence of illegitimate reunion (misrejoining) of free ends from different DNA dsbs (3). Such misrejoining may be expected to depend on the number and proximity of the breaks. Preston (4) demonstrated that if the DNA damage produced by two agents is repaired at very different rates, the probability of producing a synergistic effect on aberration frequency is low. However, if the damage

is repaired rapidly, there is a high probability of a synergistic or interactive effect. The present study considered these possibilities and selected γ -rays and bleomycin (Blem) as two mutagenic agents since both show similar and apparently rapid rates of repair of the DNA damage (5,6).

Endogenous thiols, especially the tripeptide reduced glutathione (GSH), have long been thought to affect the sensitivity of cells to irradiation (7). Buthionine sulfoximine (BSO) specifically depletes the endogenous GSH level by inhibiting the enzyme γ -glutamylcysteine synthetase and increases cellular radiosensitivity (7,8). It was demonstrated in our earlier study that BSO-mediated GSH depletion increased radiation-induced CAs except exchange aberrations and this could be because of the reduction in the DNA shielding effect of GSH and failure in rejoining of DNA dsbs (9). It has been reported that the rejoining of radiation induced single strand breaks (ssbs) in oxic condition differs from that of hypoxically induced ssbs and that it is clearly dependent on GSH (10). It was also shown earlier that under the influence of higher GSH level in irradiated cells, the frequency of deletions was reduced and the frequency of exchange aberrations was increased (9). This observation is important since the role of GSH has been clearly demonstrated in DNA synthesis under certain conditions (11) and as a cofactor in enzymatic repair processes in the cell (12). By allowing the simultaneous presence of GSH and Blem during treatment, it was demonstrated that the presence of GSH potentiates the clastogenic action of Blem in mammalian lymphocytes and was attributed to GSH acting as a reducing agent in reactivating the oxidized Blem (13,14). Therefore, the present study was carried out with an aim to investigate the role of increased or decreased GSH levels (by treating GSH ester exogenously or with BSO, respectively) on DNA dsb rejoining by treating the cells with Blem and γ -radiation.

Rejoining of DNA dsbs induced in the DNA by ionizing radiation and other physical or chemical agents can be achieved either by homologous recombination, which depends on extensive sequence homology or by non-homologous end joining (NHEJ), which depends on limited or no sequence homology (15). In quiescent mammalian cells, this process has been reported to take place prevalently by the NHEJ (16) rather than homologous recombination pathway, which is mainly important during S and G₂ phases (17). Therefore, an attempt was also made to see which DNA dsb-repair pathway is active in the G₀ human lymphocytes, a well established and suitable system for the assessment of chromosomal aberrations (18).

Materials and methods

Chemicals

DL-Buthionine-S,R-sulfoximine (BSO), 5,5'-dithiobis(2-nitro benzoic acid) (DTNB), GSH-ethyl ester, Hoechst 33258, 5-bromodeoxyuridine (BrdU), GSH-reductase, NADPH, Ficoll-hypaque, Nonidet P-40, sodium dodecylsulfate

*To whom correspondence should be addressed. Tel: +91 364 272 2326; Fax: +91 364 255 0076; Email: anupamchatterjee@nehu.ac.in

and aprotinin were obtained from Sigma Chemical Company (St Louis, MO, USA). Bleomycin sulfate (Blem) was obtained from Biochem Pharmaceutical Industries (Mumbai, India). The culture medium RPMI 1640, antibiotic penicillin and streptomycin and mitogen phytohaemagglutinin (PHA) were obtained from Gibco, USA. Foetal calf serum (FCS) was used from Biological Industries, Israel. Giemsa stain was obtained from BDH chemicals, UK. Other chemicals used in this study were of analytical grade from reputed manufacturers.

Determination of GSH-level in human PBLs

The level of total GSH in PBL was estimated by the method of Akerboom and Sies (19). A total of six blood samples was collected from healthy male donors; of these, all were used for control and five were used for a single treatment with BSO. All the rules of 'Ethical Guidelines for Biomedical Research on Human Subject' of Indian Council of Medical Research, India were followed in all the experiments. Lymphocytes were separated out from heparinized whole blood on a Ficoll-hypaque density gradient after 5 h of BSO treatment. Freshly collected lymphocytes were washed in ice-cold 0.1 M phosphate-buffered saline solution (pH 7.4) and the volume was made up to 1 ml. Cells were counted in a haemocytometer and processed for determination of total GSH level as described earlier (9). In brief, after deproteinization by 10% ice-cold 5-sulfosalicylic acid, 50 μ l sample suspension was taken and to it were added 1 ml buffer (0.1 M EDTA phosphate buffer, pH 7.0), 50 μ l NADPH (4 mg/ml), 20 μ l DTNB (1.5 mg/ml) and 20 μ l GSH reductase (6 units/ml). The optical density of the samples was measured at 412 nm using the UV-visible spectrophotometer (Beckman model DU-640). A standard curve was prepared from a stock solution of 1 mM GSH in 5% 5-SSA diluted to 0.1–8 μ mol.

Treatment with gamma radiation

In a separate set of experiments, heparinized peripheral blood from three healthy male donors was used immediately after venipuncture. An aliquot of 1 ml of whole blood was taken in a sterilized flat bottom 25 ml glass beaker. To the blood sample in the beaker, 5 mM BSO (dissolved in phosphate buffer solution; pH 7.4) was added. The samples were kept at 37°C for 5 h before irradiating them to 2 and 4 Gy in a ⁶⁰Co γ -chamber (dose rate 0.6 Gy/min). Blood samples were kept at 37°C for 1 h after irradiation so as to allow normal cellular repair before setting up cultures.

Treatment with BLM and γ -rays, with and without BSO and GSH ester

In another experiment, heparinized peripheral blood from six healthy male donors was used immediately after venipuncture. An aliquot (1 ml) of whole blood was taken in a sterilized flat bottom 25 ml glass beaker. Blem was hydrated with sterilized triple distilled water and a fresh working solution of 100 μ g/ml was prepared by the addition of RPMI 1640 just prior to each use and added to 1 ml aliquot of whole blood to make a final concentration of 20 μ g/ml. Radiation was given to Blem-treated cells 2 h after Blem treatment. In two donors, GSH ester was added 1 h before Blem treatment. The amount of GSH ester required to make a final concentration of 15 mM for the volume of the blood sample to be treated was weighed out and dissolved in 0.2 ml of the medium, mixed well with the blood and the samples were incubated at 37°C.

In case of Blem treatment to BSO-pretreated cells, BSO (5 mM) was added to 1 ml aliquot of whole blood for 3 h and then Blem was added. Radiation (4 Gy) was given to BSO + Blem-treated samples 2 h after Blem treatment. In all these cases, the Blem-, Blem + radiation-, GSH ester + Blem + radiation-, BSO + Blem- and BSO + Blem + radiation-treated samples were washed twice with prewarmed medium at the end of 3 h Blem treatment.

Culture procedure and cell fixation

Cultures were set up in RPMI 1640 medium supplemented with 10% heat inactivated FCS. Lymphocytes were stimulated with PHA. BrdU (6 μ g/ml) was added in each culture during initiation of culture. All cultures were incubated at 37°C and were harvested at 52 h. Colcemid was added at a concentration of 0.01 μ g/ml during the last 3 h in all cultures. Hypotonic treatment was done for 18 min and the cells were fixed in acetic acid and methanol (1:3) and slides were prepared.

Differential staining for sister chromatids

The method of Goto *et al.* (20) was followed. Slides were treated for 10 min with Hoechst 33258 (50 μ g/ml) at room temperature in dark, rinsed in distilled water, mounted in 2 \times SSC (NaCl–sodium-citrate, pH 6.8) and kept in sunlight for 30–40 min, depending upon the intensity of sunlight. After rinsing in distilled water, slides were stained in 2% Giemsa for 4 min.

Western blot analysis

Lymphocytes were separated out from heparinized whole blood on a Ficoll-hypaque density gradient 1 h and 3 h after radiation and/or BLM treatment with

or without BSO or GSH ester. These cells were lysed in radioimmunoprecipitation buffer (0.1% SDS, 2 mM EDTA, 1% NP-40, 1% sodium deoxycholate, 50 mM sodium fluoride and 100 U/ml aprotinin). After 30 min of incubation on ice, the cell lysates were centrifuged for 15 min at 4°C. Equal amount of protein (80 μ g) from each sample was loaded in each well, and equal loading was verified by immunoblotting with actin antibodies. Electrophoresis was performed in 12% SDS–polyacrylamide separating gel. Proteins were transferred to a 0.45 μ m nitrocellulose membrane (Millipore) using Bio-Rad Trans Blot Cell. After overnight incubation at 4°C in blocking solution of 5% non-fat dried milk mixed in TBST buffer, pH 7.6 (1 M Tris–Cl, 5 M NaCl and 0.05% Tween-20), the membrane was probed with a 1:1000 dilution of a mouse monoclonal antibody against anti-Ku Ab-4 (N3H10) or anti-Rad51 Ab-1 (51Rad01) (Neo-Markers, Fremont, USA) in TBST with 5% non-fat dried milk for 1 h. A primary antibody for β -actin (anti-actin ACTN05; Neo-Markers) 1:5000 dilution was also used as control for sample loading.

Blots were washed three times for 10 min each in TBST and incubated with secondary antibody (alkaline phosphatase conjugated anti-mouse IgG, 1:2000 dilution; Bangalore Genei, Bangalore, India) for 1 h at room temperature. After extensive washing the blot was immersed in 4 ml substrate solution of BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Bangalore Genei). Within 20 min bands developed sufficient colour. The whole experiment was repeated twice.

Scoring and statistical analysis

Slides were coded at random. CAs scored in human PBLs were from the first cycle metaphases (M₁) and they were—exchanges including dicentric and rings (with or without fragments), deletions and chromatid breaks. Deletions were scored as terminal deletions (bigger fragments) and interstitial deletions (smaller fragments). For scoring cell-cycle kinetics, metaphases were categorized as in the first, second or subsequent cycles based on their differential staining patterns. Statistical significance of the difference between the control and treated groups for the frequency of aberrant metaphases was evaluated using 2 \times 2 contingency χ^2 -test and for different types of aberrations simple χ^2 -test was used. The difference of GSH level between BSO-treated and untreated groups was evaluated using Student's *t*-test.

Results

Level of reduced GSH

The level of reduced GSH in PBLs, with or without BSO was measured. The concentration of GSH in normal PBLs showed a range between 3.67 and 6.55 with an average of 5.61 \pm 0.56 μ mol, in 10⁶ cells. This GSH concentration was depleted by 85% of the control value after 5 h treatment with BSO and the range showed between 0.78 and 0.98 μ mol, with an average of 0.86 \pm 0.1 μ mol, in 10⁶ cells. The statistical difference between the mean GSH concentrations of these two groups was significant.

Effect of BSO on chromosome aberrations in lymphocytes

Gamma-ray-induced CAs in BSO-treated and untreated three human samples are shown in Table I. The induction of CAs and delay in cell cycle kinetics (frequency of M₁) by radiation were found to be clearly dose dependant however, the frequency of chromatid breaks was low at all radiation doses. Significant increment in the induction of deletions was observed in all the BSO-treated samples at all radiation doses and the frequency of aberrant metaphases and chromatid breaks was also increased. This is also clear from the mean data presented as a bar diagram in Figure 1A. Interestingly, the frequency of exchanges did not increase at all in BSO-treated samples rather a tendency of reduction was observed in all cases. A reduction in the frequency of exchanges was observed in the mean data in BSO-treated samples with 2 and 4 Gy (from 17 to 12% and 33 to 28%, respectively; Figure 1A). The effect of BSO (5 mM) itself on the spontaneous CAs was also studied and was observed to be not significant. Presence of BSO alone did not induce any delay and failed to bring in any increase in the radiation-induced delay in cell cycle kinetics.

Table I. Effect of γ -radiation with or without BSO on chromosome aberrations in HPBLs

Donor no.	Experimental condition	Total metaphase	Abt M (%)	Aberrations per cell			
				Exchanges	Chtd Bk	Deletion	M ₁ (%)
1	Untreated	094	03	0	0.01	0.01	69
	BSO	128	04	0	0.01	0.02	70
	2 Gy	214	39	0.19	0.01	0.10	74
	BSO + 2 Gy	219	48	0.12	0.03	0.31	71
	4 Gy	185	80	0.32	0.04	0.84	82 ^a
	BSO + 4 Gy	113	81	0.27	0.18 ^b	1.26 ^c	77
2	Untreated	147	02	0	0.01	0.01	67
	BSO	117	03	0	0.01	0.02	69
	2 Gy	163	34	0.18	0.02	0.10	72
	BSO + 2 Gy	133	46 ^a	0.11	0.03	0.19	70
	4 Gy	152	82	0.35	0.05	0.92	79 ^a
	BSO + 4 Gy	129	90	0.27	0.16 ^b	1.26 ^b	74
3	Untreated	158	02	0	0	0	67
	BSO	109	03	0	0.01	0	68
	2 Gy	123	35	0.15	0.02	0.13	73
	BSO + 2 Gy	120	43	0.12	0.04	0.26	70
	4 Gy	154	80	0.32	0.05	0.79	79 ^a
	BSO + 4 Gy	142	89	0.29	0.20 ^b	1.30 ^c	72

Abt M, Aberrant metaphases; Chtd Bk, chromatid break; M₁, first cycle metaphases.

^aSignificant at $P < 0.05$, 2×2 contingency χ^2 -test (BSO-treated and untreated).

^bSignificant at $P < 0.05$.

^cAt $P < 0.01$ simple χ^2 -test at d.f. = 2 (BSO-treated and untreated).

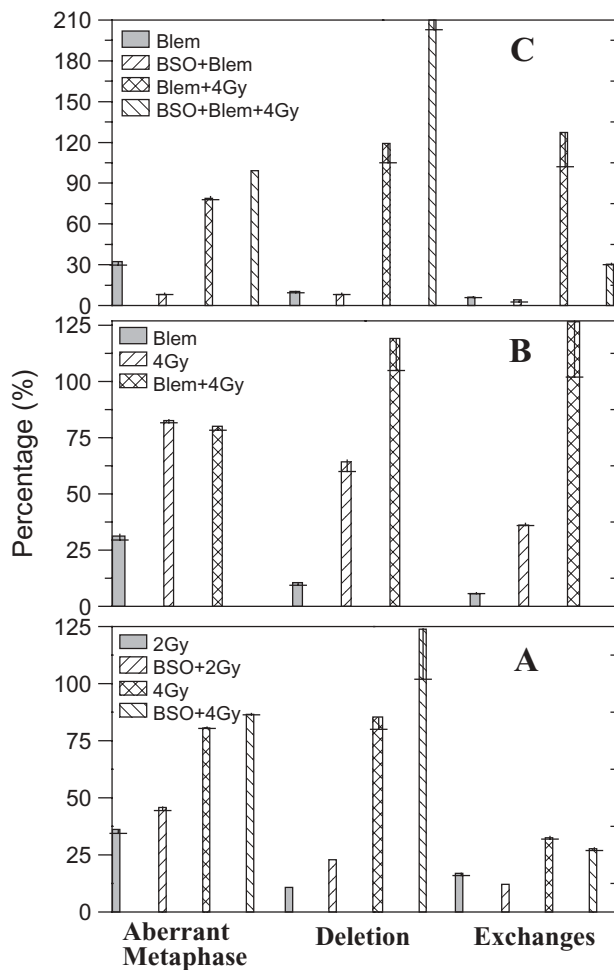


Fig. 1. Comparison of induction of aberrations in (A) radiation and BSO + radiation, (B) Blem and Blem + 4 Gy and in (C) Blem + 4 Gy, BSO + Blem and BSO + Blem + 4 Gy. Data are from Tables I and II. The values shown are the means \pm SEM.

Effect of Blem and radiation alone or in combination with or without GSH ester or BSO on CAs

Blem- and γ -ray-induced CAs in human PBL were studied as positive control to Blem + γ -ray treated samples, and the data are presented in Table II. Deletions and exchanges were the most frequent types of aberrations induced by radiation and the frequency of chromatid breaks was more with Blem. From the frequency of M₁ (Table II), it is clear that Blem alone does not induce delay in cell proliferation. Blem and 2 Gy combined treatment increased the frequency of aberrant metaphases and deletions significantly in each sample. The frequency of other aberrations and M₁ was increased marginally in Blem + 2 Gy treated sample than the 2 Gy and Blem alone treatment. When Blem + 2 Gy combined treatment was given to GSH ester pretreated cells, the frequency of aberrant metaphases, exchanges and chromatid breaks was increased significantly with respect to Blem + 2 Gy treated cells.

The degree of increase in the frequency of deletions and exchanges was significantly more in the sample exposed to Blem + 4 Gy combined treatment than the 4 Gy exposure alone (Tables II and III). The induction of exchange aberrations by the combined treatment was higher than the additive effects of the Blem and 4 Gy given (the exchanges 1.78 per cell were in donor 2 whereas the additive effect of 4 Gy and Blem was 0.42 per cell). This is also clear from the mean data presented as a bar diagram in Figure 1C. The data on the frequency of both terminal and interstitial deletions induced by 4 Gy alone and its combination with Blem are presented in Table III. The data show that radiation alone induced terminal deletion more than interstitial deletion, whereas the frequency of the latter was increased significantly when cells were exposed to Blem + 4 Gy.

With respect to cell-cycle kinetics, the frequency of M₁ was a little higher in the combined treatment than the treatment with 4 Gy alone (Table II).

Interestingly, the Blem + 4 Gy combined treatment to BSO-treated cells showed drastic reduction in the frequency

Table II. Induction of CAs by Blem or γ -rays alone or in combination with or without GSH ester or BSO in HPBLs

Donor no.	Experimental condition	Total metaphase	Abt M (%)	Aberrations per cell			M ₁ (%)
				Exchanges	Chtd bk	Deletion	
1	Untreated	112	2	0	0.02	0	76
	Blem	087	33	0.06	0.12	0.14	77
	2 Gy	079	32	0.10	0.03	0.12	82
	Blem + 2 Gy	088	53 ^{a,b}	0.22 ^{b,c}	0.13	0.82 ^{b,c}	82
2	Untreated	123	3	0	0.02	0.01	78
	Blem	113	37	0.06	0.13	0.09	80
	2 Gy	133	33	0.14	0.04	0.14	83
	Blem + 2 Gy	133	60 ^{a,b}	0.20	0.16	1.46 ^{b,c}	88
	4 Gy	126	87	0.36	0.07	0.59	87
	Blem + 4 Gy	109	82	1.78 ^{b,c}	0.18	1.70 ^{b,c}	100
3	Untreated	199	2	0	0.02	0.01	84
	Blem	183	33	0.06	0.10	0.12	84
	2 Gy	147	35	0.12	0.04	0.12	87
	Blem + 2 Gy	124	63 ^{a,b}	0.17	0.10	1.40 ^{b,c}	92
	Ester + Blem + 2 Gy	121	89 ^{a,b}	0.62 ^{b,c}	0.25 ^c	0.60 ^{b,c}	94
	4 Gy	201	84	0.35	0.05	0.57	94
	Blem + 4 Gy	129	81	1.24 ^{b,c}	0.07	1.20 ^{b,c}	98
4	Untreated	185	3	0	0.01	0.02	77
	Blem	130	21	0.07	0.14	0.07	78
	2 Gy	127	37	0.12	0.03	0.10	81
	Blem + 2 Gy	108	59 ^{a,b}	0.14	0.07	0.92 ^c	86
	Ester + Blem + 2 Gy	118	87 ^a	0.72	0.32	0.94	88
	4 Gy	143	81	0.39	0.05	0.77	87
	Blem + 4 Gy	121	75	0.68 ^{b,c}	0.08	1.12 ^c	92
5	Untreated	109	2	0	0.02	0.01	74
	Blem	183	36	0.05	0.15	0.13	74
	BSO + Blem	147	10 ^a	0.03	0.04	0.10	76
	4 Gy	222	84	0.39	0.06	0.65	98
	Blem + 4 Gy	139	79	1.34 ^{b,c}	0.07	1.06 ^{b,c}	95
	BSO + Blem + 4 Gy	090	100 ^{d,e}	0.29 ^{c,d}	0.26 ^{c,d}	2.20 ^{c,d}	100
6	Untreated	119	3	0	0.02	0.01	74
	Blem	152	34	0.06	0.13	0.08	72
	BSO + Blem	128	8 ^a	0.04	0.03	0.08	74
	4 Gy	117	81	0.37	0.06	0.67	96
	Blem + 4 Gy	132	82	1.31 ^{b,c}	0.05	0.85 ^{b,c}	93
	BSO + Blem + 4 Gy	103	100 ^{d,e}	0.32 ^{c,d}	0.30 ^{c,d}	2.03 ^{c,d}	100

2 × 2 contingency χ^2 -test.

^aAt $P < 0.01$.

^bBetween radiation and Blem + radiation.

^cSignificant at $P < 0.05$, χ^2 -test at d.f. = 2 compared with respect to positive control.

^dBetween Blem + 4 Gy and BSO + Blem + 4 Gy.

^eSignificant at $P < 0.05$.

Table III. Induction of interstitial and terminal deletions by γ -rays alone or in combination with Blem with and without BSO pretreated HPBLs

Donor no.	Experimental condition	Total metaphase	Abt M (%)	Aberrations per cell		
				Exchanges	Deletion	
					Terminal	Interstitial
2	4 Gy	126	87	0.36	0.40	0.19
	Blem + 4 Gy	109	82	1.78 ^a	0.78	0.92 ^a
3	4 Gy	201	84	0.35	0.37	0.20
	Blem + 4 Gy	129	81	1.24 ^a	0.39	0.50 ^a
4	4 Gy	143	81	0.39	0.45	0.20
	Blem + 4 Gy	121	79	1.34 ^a	0.34	0.72 ^a
5	4 Gy	222	84	0.39	0.45	0.20
	Blem + 4 Gy	139	79	1.34 ^a	0.34	0.72 ^a
6	BSO + Blem + 4 Gy	090	100 ^{b,c}	0.29 ^{a,c}	1.70 ^{ac}	0.50 ^{ac}
	4 Gy	117	81	0.37	0.43	0.24
6	Blem + 4 Gy	132	82	1.31 ^a	0.27	0.66 ^a
	BSO + Blem + 4 Gy	103	100 ^{b,c}	0.32 ^{a,c}	1.60 ^{ac}	0.44 ^{ac}

^aSignificant at $P < 0.05$ χ^2 -test at d.f. = 2 compared with respect to positive control.

^bSignificant at $P < 0.05$, 2 × 2 contingency χ^2 -test.

^cBetween Blem + 4 Gy and BSO + Blem + 4 Gy.

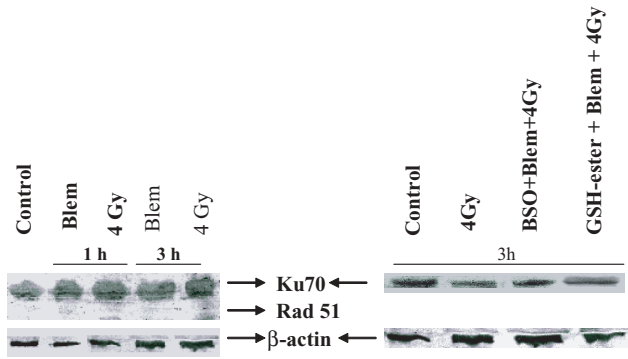


Fig. 2. The levels of Ku70 and Rad51 proteins in HPBL after 1 and 3 h of treatment with radiation and/or bleomycin with or without BSO and GSH ester.

of exchanges (from 1.34 to 0.29 per cell in donor 5 and from 1.31 to 0.32 per cell in donor 6) as compared with BSO-untreated cells. However, the frequency of aberrant metaphases, deletions (particularly terminal deletions, Table III) and chromatid breaks induced by Blem + 4 Gy were increased significantly in BSO-treated cells (Figure 1C). The frequency of M_1 indicates that all the metaphases were in the first cycle stage in BSO + Blem + 4 Gy treated sample and it did not show any significant difference from Blem + 4 Gy or 4 Gy treatment to BSO-untreated cells. It is worth mentioning that Blem treatment to BSO-treated cells showed significant reduction in the frequency of aberrant metaphases and chromatid breaks (Table II and Figure 1B).

Expression of Ku70 and Rad51 in HPBLs

Figure 2 shows the levels of Ku70 and Rad51 proteins in G_0 human lymphocytes after 1 and 3 h of Blem or 4 Gy treatment. The presence of Ku70 was clear in all the samples and the level of this protein was almost similar even after addition of BSO or GSH ester before treating the cells with radiation and Blem. However, the level of Rad51 protein was undetectable in both control and treated samples.

Discussion

In this study, BSO was used to evaluate the effect of GSH depletion on DNA dsb ends joining in human PBLs after exposing the cells to Blem and radiation. The incidence of dicentric and ring types of aberrations in human PBLs has been proven to be a sensitive indicator of radiation damage because of its high frequency relative to other types of radiation-induced aberrations and its low natural incidence (21). Our results indicate that the presence of BSO increased the cellular radiosensitivity and it could be owing to the depletion of endogenous GSH as shown earlier (9). The reduced GSH estimation in this study indicates that 5 h incubation with BSO (5 mM) could deplete the GSH level significantly with respect to control. In cultured cells 75% depletion was achieved within 4–5 h duration by 500 μ M to 10 mM BSO (22,23). It has been shown that such depletion of endogenous GSH by BSO increases the frequency of CAs induced by arecoline (24) and mitomycin C (25).

In order to ascertain the role of endogenous GSH on joining of DNA dsbs we allowed the DNA lesions induced by Blem and γ -ray to interact, since the chemistry of the end of

DNA strand breaks is similar (26). It has also been shown that Blem-induced DNA dsbs are rejoined *in vitro* with an efficiency similar to that measured for radiation-induced DNA dsbs (27). In the present study, CAs induced by Blem (20 μ g/ml) and γ -ray (2 Gy) are qualitatively and quantitatively similar, although Blem induced higher frequency of chromatid breaks and radiation induced more exchange aberrations. In this study, the frequency of exchanges and deletions was increased substantially when Blem combined with radiation, 4 Gy in particular. Similar significant increase in the frequency of exchanges and deletions was observed by Preston (4) in human PBLs when Blem 15 μ g/ml was combined with 1.5 Gy X-ray. It was interpreted that since the DNA damage induced by both Blem and X-rays is repaired rapidly, the probability of obtaining a synergistic effect on aberration frequency with a combined treatment is high. It seems that in this synergistic process misrejoining of DNA dsbs induced by both Blem and radiation may take place with a high frequency and thus, increase the frequency of exchange aberrations and interstitial deletions.

NHEJ is regarded as the dominant mechanism for dsb repair in vertebrates, especially in G_0 and G_1 phases of the cell cycle (28). Several lines of evidence suggest that cells with an intact NHEJ pathway can give rise to chromosomal rearrangements in response to induction of high frequencies of dsbs (29,30). It also emerges from the present study that NHEJ pathway might be involved in such misrejoining of DNA dsbs and thus increased the frequency of exchanges and interstitial deletions. NHEJ involves DNA end-binding heterodimer Ku70/Ku80, the catalytic subunit of the DNA-PK, the XRCC4 gene product and DNA ligase IV (31). The present study showed higher level of Ku70 protein in untreated, Blem or radiation treated samples with undetectable amount of Rad-51 protein, that involved in homologous recombination, in G_0 lymphocytes, thus justifying the involvement of NHEJ pathway in DNA dsbs joining process. The western analysis also showed that the level of Ku70 protein did not get altered even when treatment was given to BSO-pretreated cells.

The interesting observation in this study is the drastic reduction in the frequency of exchange aberrations and huge increase in the frequency of terminal deletions after exposing the BSO-pretreated cells to the combined treatment of Blem and radiation. It is worth mentioning that Blem treatment to BSO-treated cells reduced the frequency of aberrant metaphases and chromatid breaks as shown earlier (15). This reduction in the effect of Blem in GSH-depleted cells could be explained on the basis of failure of reactivation of the oxidized Blem by the reducing agent GSH that is usually present endogenously. Nevertheless, Blem + 4 Gy combined treatment to BSO-treated cells showed an increased frequency of aberrations perhaps because the frequency of DNA lesions induced by Blem in BSO-treated cells was at normal level initially and failed to increase further owing to failure of reactivation of the oxidized Blem. It has been demonstrated previously that the presence of GSH or thiol radioprotector potentiates the clastogenic action of Blem (14,32). Therefore, in this study we used GSH ester in two samples. The frequency of exchanges was increased and the frequency of deletions was reduced significantly after the cells were treated with Blem + 2 Gy. GSH-ester is readily transported into cells and converted to GSH increasing the level of endogenous GSH within 2–3 h (33). It seems that DNA lesions induced by Blem are subjected to interact or illegitimately unite with radiation-induced

DNA-dsbs and such interaction depends on the level of endogenous GSH.

The question may arise whether the response, as shown by the analysis of CAs in different treated samples in this study, is really the effect of treatment or is simply because of the scoring of different cell populations as a result of cell-cycle shift? In fact, the cells were fixed at 52 h in this study and they are mostly early-arising metaphases. We scored the first, second and subsequent cycles simultaneously with the aberration scoring and observed that Blem did not induce any delay in cell proliferation, whereas radiation did. Similar observation was also made earlier in muntjac lymphocytes (34). However, the differences in the frequency of M1 cells in 2 Gy, Blem + 2 Gy and GSH ester + Blem + 2 Gy and 4 Gy, Blem + 4 Gy and BSO + Blem + 4 Gy were not significant. Therefore, the present data indicate that BSO-mediated GSH depletion did not allow the interaction of DNA lesions induced by Blem and radiation and thus, increased the frequency of terminal deletions and chromatid breaks. That GSH affects DNA dsb rejoining and exchanges has been shown in this study and also previously (9,10,35). Therefore, if the present induced DNA dsbs are the candidates for the NHEJ-repair pathway, the failure in restitution and misrejoining in the absence of endogenous GSH indicates the involvement of GSH either directly or indirectly in the joining of such DNA dsbs.

It may, thus, be concluded that combined treatment of Blem and radiation induces higher frequency of CAs, particularly, exchange aberrations and interstitial deletions, whose frequency was reduced if the cells were pretreated with BSO. A mode of combination therapy with Blem and irradiation has been evaluated in cancer patients (36,37) and increased levels of GSH are commonly found in the drug-resistant human cancer cells (38). Therefore, combined treatment of Blem and radiation to such cancer cells may induce higher DNA damage including exchange aberrations, which, in turn, could lead to apoptotic cell death.

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