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ORIGINAL PAPER

# Comparison of the effects of bleomycin and ionizing radiation in two sublines of murine lymphoma L5178Y

**Marcin Kruszewski,**
**Jolanta Zaim,**
**Iwona Grądzka,**
**Irena Szumiel**

**Abstract** We compared the effects of bleomycin (BLM) and ionizing radiation on two sublines of murine lymphoma L5178Y (LY): LY-R, radiation resistant and LY-S, radiation sensitive. This radiosensitivity difference is related to the ability to rejoin DNA double strand breaks. LY-S cells were about two times more sensitive to BLM than LY-R, similarly as in the case of sensitivity to X rays. Since there was no difference in the P-glycoprotein-related drug transport system between the sublines, it could be expected that the enhanced sensitivity of LY-S cells to BLM was caused by the DNA repair defect. Growth disturbances in BLM treated cell populations were proportional to the lethal effect and their duration was observed until elimination of dead cells (3–6 days after 50 μM BLM, 1 h at 37°C). There was no slow growth phase accompanied by normal viability, as previously described for X-irradiated LY-S cells. Initial DNA damage, estimated with the single cell gel electrophoresis method was linearly related to BLM dose in LY-S cells; in LY-R cells – in the low dose range (up to 10 μM) – there was more damage than in LY-S cells, however, at higher doses the dose – effect curves became identical. The dose-effect relationship for γ rays was linear and identical in both cell sublines. DNA damage distribution in BLM treated cells was much less uniform as compared to that in irradiated cells and indicated the presence of cells with severely damaged DNA, a feature typical for BLM action *in vitro*.

**Key words** bleomycin • comet assay • DNA damage • gamma radiation • single cell gel electrophoresis

M. Kruszewski✉, I. Grądzka, I. Szumiel
Department of Radiobiology and Health Protection,
Institute of Nuclear Chemistry and Technology,
16 Dorodna Str., 03-195 Warsaw, Poland,
Tel.: +4822/ 8110736, Fax: +4822/ 8111532,
e-mail: marcinkr@orange.ichtj.waw.pl

J. Zaim
Institute of Biochemistry and Biophysics,
Polish Academy of Sciences,
5a Pawińskiego Str., 02-106 Warsaw, Poland

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## Introduction

Bleomycin (BLM) is an anticancer antibiotic isolated from the supernatant of *Streptomyces verticillus* culture [21]. The cytotoxic activity of BLM depends on generation of single- and double-strand breaks in DNA (for review, see [16]). Indispensable for this BLM activity is the presence of metal ions, specifically $Fe^{2+}$. $Fe^{2+}$ BLM complexed with $O_2$ or $Fe^{3+}$ BLM complexed with $H_2O_2$ leads to formation of an active BLM molecule, $HOO^{-\bullet}$ $Fe^{3+}$ • BLM.

BLM specifically induces DNA double strand breaks and apurinic/apyrimidinic sites in the complementary strands with a higher frequency than would result from randomly induced damage [9, 18]. It is expected that double strand break is generated by a BLM molecule which is reactivated after nicking the first strand [17]. The reactivation consists of reduction of BLM • $Fe^{3+}$ (formed after reaction with DNA) to BLM • $Fe^{2+}$, and a subsequent addition of an oxygen molecule leading to the generation of the active form of BLM. So, the double strand break is a candidate for being the lethal lesion both after exposure to low LET radiation and BLM treatment.

In contrast with the random location of low LET radiation damage, BLM causes DNA damage mainly in the transcriptionally active chromatin domains; the lesions are concentrated at the linker regions (DNA between nucleosomes). Experiments with the use of isolated nuclei from

various cell lines indicated that nucleosomes were dissociated, whereas in isolated nucleosomes there was no further degradation of DNA by BLM. So, the sites of DNA nicking are located only in the linker regions: one or two sites per linker [11]. BLM also is able to nick RNA in a site-specific way (e.g. yeast 5S rRNA in the $tRNA^{Phe}$ locus, in *Bacillus subtilis* in $tRNA^{His}$) [11].

Similarly to low LET radiation, BLM induces both chromatid and chromosome type aberrations (deletions, di- and multicentrics, rings, exchanges, breaks), but does not induce sister chromatid exchanges [16]. Accordingly, interphase cells after BLM treatment show the presence of micronuclei [11].

Differential sensitivity to BLM was observed in apparently uniform cell populations; the reason for this remains unknown [5, 15, 21]. Explanation of this phenomenon may have practical implications for BLM use in chemotherapy of cancer. One possible approach is by studying the biological effects of BLM treatment in well characterized cell model systems, usually consisting of parental wild type and mutant sublines, the latter with a modified sensitivity to the drug. Povirk [16] describes as hypersensitive to BLM the rodent mutants from 3 complementation groups with defects in DNA double strand break repair as well as *ataxia telangiectasia* cells. Also murine lymphoma L5178Y-S (LY-S) cells show a DNA repair defect [22–24]. These cells, as well as the parental LY-R cells are a convenient model system for studying BLM effects and for comparing such effects to those of low LET radiation; LY-S cells were obtained by a prolonged in vitro culture of LY-R cells and this conversion was accompanied by an altered susceptibility to various DNA damaging agents. We used this model system to study survival, growth and DNA damage after BLM and X-ray treatment.

## Materials and methods

### Cell treatment

Murine lymphoma cells L5178Y-R (LY-R) and L5178Y-S (LY-S) were grown in suspension culture in Fischer's medium, as described in [19].

In BLM experiments, BLM sulphate for injection (Lundbeck, Copenhagen) was used. The preparation was sterile, desiccated and kept at 4°C. The stock solution (1 mM) prepared in sterile saline was used for further dilutions. Incubation of LY cells with BLM was carried out in Fischer's medium. Then, the cells were centrifuged at room temperature, washed with PBS, resuspended in fresh, warm medium, counted and diluted for cloning or growth tests. Aurintricarboxylic acid (ATA, a nuclease inhibitor) was used to determine the effect of apoptosis inhibitors on growth of BLM-treated cells. Treatment schedule is presented in Fig. 1.

In radiation experiments, cell cultures were exposed to γ rays from a $^{136}Cs$ source at a dose rate of 0.44 Gy/min. For this, the cells were placed in agarose layer (see below) on ice and immediately after irradiation transferred to lysing solution for determination of the initial DNA damage.

### Survival determination by cloning

Soft-agar cloning was carried out as previously described [19]. After incubation with BLM, the cells were centrifuged and resuspended in drug free-medium supplemented with sodium pyruvate (0.05%, Sigma), heat-inactivated foetal calf serum (4%) and "Special Agar Noble" (0.19%, Difco). LY-R cell cultures were also supplemented with β-mercaptoethanol (0.08%, Sigma). The soft-agar cultures were incubated in 5% $CO_2$ atmosphere, 100% humidity at 37°C. Clones were scored after 8–10 days (LY-S) or 14 days (LY-R). Mean results from 3–5 experiments are presented.

### DNA damage determination by single cell gel electrophoresis (comet assay)

After incubation with BLM, cells were suspended in 1% agarose (low melting point, type IV, Sigma) and placed on microscopic slides, covered with cover glasses and cooled on a metal plate on ice. Lysis and electrophoresis were carried out as previously described [7]. The Fencomet software from Fenestra Vision v. 1.4 software package (Confocal Technologies, Liverpool, UK) was used to evaluate DNA damage in γ-irradiated cells. For BLM treated cells, the Comet v.3.1 software (Kinetic Imaging, Liverpool, UK) was used. One hundred cells per slide were analyzed from 3 independent experiments with BLM for each LY subline or 50 cells per slide from 2 experiments with γ radiation. The tail moment was the measure of DNA damage.

### Rhodamine 123 efflux

Activity of the drug transport system was determined from the rate of rhodamine 123 efflux [12]. Cell cultures ($4\times10^5$

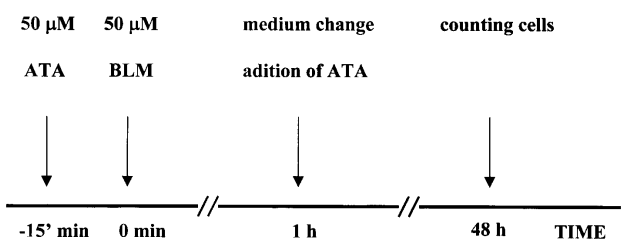


**Fig. 1.** Treatment schedules used in experiments with apoptosis inhibitors.

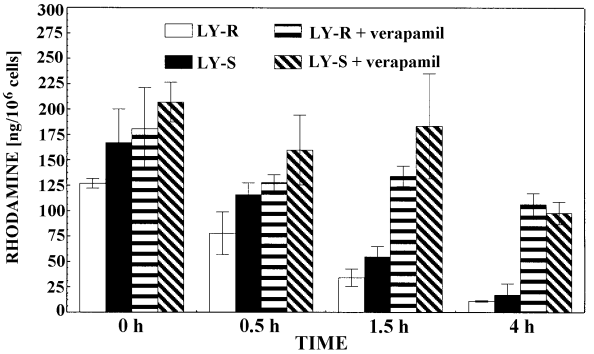


**Fig. 2.** The content of rhodamine 123 in LY cells after incubation in the presence or absence of verapamil. Mean results from 3–5 experiments ± SD.

cells/ml) were incubated for 1 h at 37°C with rhodamine 123 (Sigma, 5 μg/ml) with or without verapamil hydrochloride (Sigma, 10 μg/ml). After incubation, 3 ml aliquots of cell suspension were taken and cooled at 0°C to determine the quantity of rhodamine in the cells at time zero. The remaining cell suspensions were centrifuged and cell pellets resuspended in fresh medium with or without verapamil, as before. The cells were incubated at 37°C and, at appropriate time intervals, 3 ml aliquots were taken and cooled, centrifuged and washed twice with equal volume of cold phosphate-buffered saline (PBS), centrifuged and from cell pellets the stain was extracted. The extraction was carried out by 10 min shaking with n-butanol (3 ml, Merck, RFN). Fluoroscence of the extracted stain was measured with spectrofluorimeter Shimadzu RF-5000 (Japan) (excitation $\lambda_{ex}$ 480 nm, emission $\lambda_{em}$ 532 nm). The results were expressed in ng of rhodamine 123 per 1 million cells.

## Results

### Drug Transport System

The measure of drug transport system (responsible for multidrug resistance, MDR) is the rate of efflux of rhodamine 123 from cells previously loaded with this stain and then placed in fresh culture medium with or without calcium antagonist type α1, verapamil. Fig. 2 shows that the drug transport system acts in both sublines in the same way; therefore, the difference in BLM sensitivity between LY sublines is not dependent on BLM transport by the MDR system.

### Survival and growth

Dose-survival curves obtained for BLM (1 h, 37°C) treated cells are presented in Fig. 3. LY-S cells are more BLM-sensitive than LY-R cells; $D_{10}$ (dose reducing survival to 10%) is equal to 26 and 46 μM, respectively. Fig. 4 shows growth (expressed in relative cell numbers, i.e. ratios of cell numbers in treated cell cultures and those in the control), and viability (percentage of dead cells, as stained with nigrosine) of cell populations after treatment with 10 or 50 μM BLM. Low viability on days 3–6 after 50 μM BLM points out to a delayed lethal effect; after elimination of dead cells, from day 8 on the growth rate returns to the control level, as indicated by the stabilization of relative cell numbers.

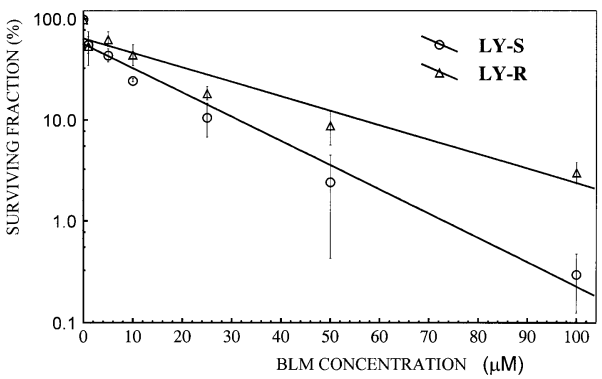


**Fig. 3.** Survival curves of LY cells treated with BLM (concentrations indicated) for 1 h at 37°C and cloned in fresh medium (soft agar). Mean results from 3–5 experiments ± SD.

### Apoptosis inhibitors

ATA, an inhibitor of nucleases was used to prevent apoptosis. As shown in Fig. 5, ATA exerted a statistically significant ($p>0.05$, Student's *t* test) sparing effect in BLM-treated LY cells, especially in LY-S cells, thus, indirectly indicating that this mode of death is present in BLM-treated LY-cell populations.

### Initial DNA damage

BLM-induced initial DNA damage measured by the comet assay is expressed as excess cumulative tail moment (ETM) [20], i.e., the sum of tail moments [14] of BLM treated cells minus the sum of control tail moments. We observed statistically significant differences between the mean ETM values for LY sublines at 1 μM BLM ($p<0.01$) and 10 μM BLM ($p<0.05$, Student's *t* test); the values for LY-R cells are higher than those for LY-S cells (Fig. 6). For concentrations above 25 μM BLM, the dose-effect relationship is linear and the differences between LY sublines disappear (for LY-S cells the relationship is approximately linear in the entire dose range examined). Distributions of the initial DNA damage in LY cells are shown in Fig. 8. When the practically undamaged (tail moment values as in the control) and less heavily damaged (tail moments up to 30) cells are left out, the difference between sublines in the extent of damage inflicted by 1, 10 and 100 μM BLM becomes clearly visible (Fig. 8E-G, 8L-N).

Figure 7 presents the initial DNA damage (expressed as ETM) in γ-irradiated LY cell populations. The level of damage is identical in both sublines; the relation to dose is lin-

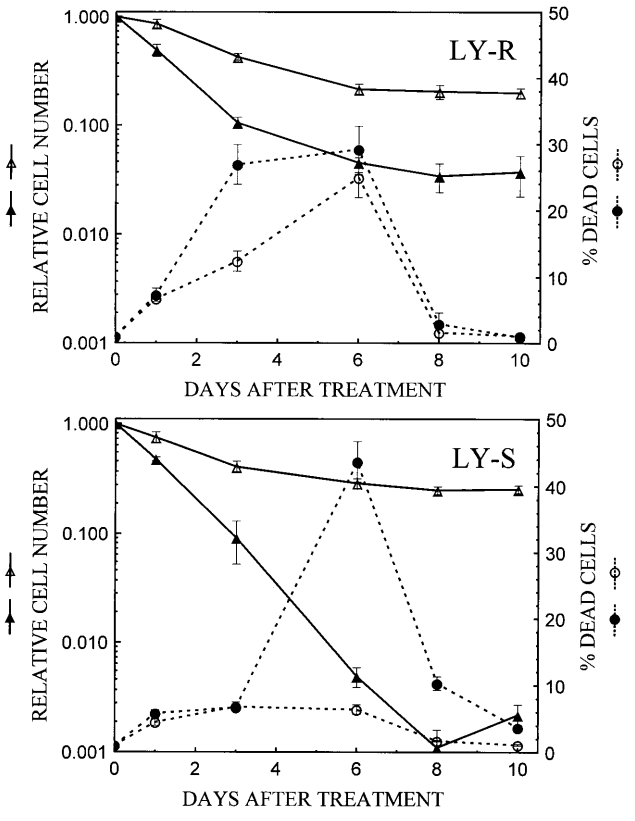


**Fig. 4.** Growth and % of dead cells after treatment of LY cells with 10 μM (open symbols) or 50 μM (closed symbols) BLM. Mean results from 2 experiments ± range.

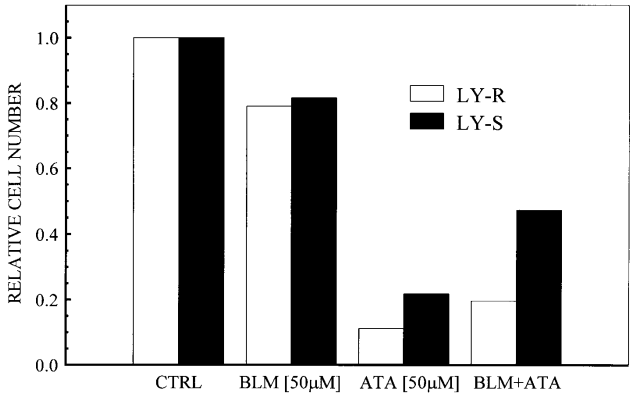


**Fig. 5.** Relative cell numbers in LY cell cultures treated with BLM and nuclease inhibitor, ATA. Mean results from 3 experiments ± SEM.

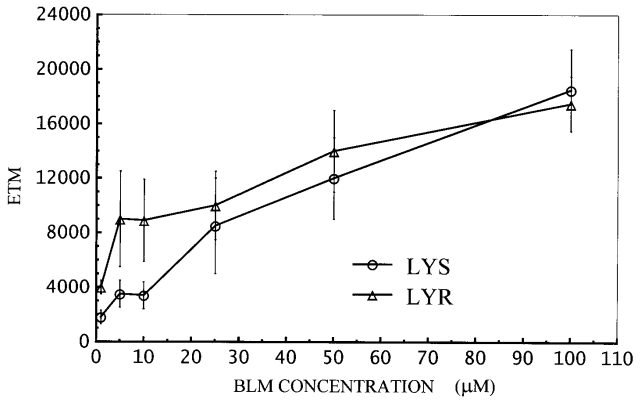


**Fig. 6.** Initial DNA damage in BLM-treated LY cells. See text for ETM definition. Mean results from 3 experiments ± SD.

ear (regression coefficients are 76.9 (LY-S) and 76.4 (LY-R); the difference is not statistically significant). The lack of difference between LY sublines in the initial DNA damage is consistent with the similarity of distribution of DNA damage in the studied cells (Fig. 8B-D, 8I-K).

## Discussion

BLM is considered to be a radiomimetic; hence; its effects should closely mimic the effects of low LET radiation. The results reported in this paper, however, indicate marked differences between the effects of the two DNA damaging factors.

The difference in clonogenic survival between LY sublines after BLM treatment is in good agreement with that found for irradiated cells. $D_{10}$ for BLM differs twofold, similarly as the mean lethal dose for X or γ rays. The latter depends on DNA repair defect in the radiation sensitive LY-S subline: the rejoining of DNA double strand breaks and chromatid breaks in this subline is markedly slower than in LY-R subline [22-24]. Since double strand break is considered to be the lethal lesion both in the case of low LET radiation and BLM and in vitro rejoining of BLM-induced double strand breaks proceeds to an extent and with kinetics similar to those observed with radiation-induced double strand break [3], it seems reasonable to assume that the same molecular defect underlies the high sensitivity of LY-S cells to both damaging agents. Cross-sensitivity to BLM and X-rays was reported for Chinese hamster ovary mutant cell lines with DNA double strand break repair defect [6].

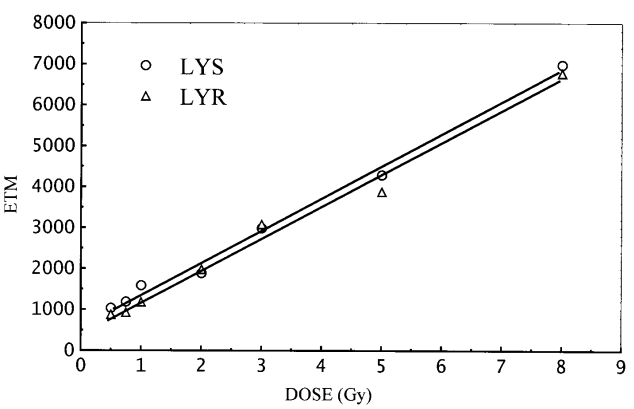


**Fig. 7.** Initial DNA damage in γ-irradiated cells. See text for ETM definition. Mean results from 2 experiments ± range. Data taken from [20].

One factor that modifies sensitivity to BLM may be the MDR system [13], which has a broad specificity to small molecular weight hydrophobic drug molecules. The influence of this system on the BLM sensitivity difference in the LY model system is negligible, as shown in Fig. 2.

The response to BLM and low LET radiation differs when the end point analyzed is growth disturbance. The decline in relative cell number after BLM treatment starts later (3–6 days) than after irradiation (24 h, cf Fig. 4). The period of slow growth after BLM treatment involves elimination of dead cells and is followed by restoration of normal growth rate. After X-irradiation elimination of dead cells is often followed by a period of normal viability and slow growth indicating the presence of heritable proliferation disturbances [2, 8]. It should be noted that such disturbances were observed both after high (6 Gy) [8] and low doses of radiation; slow growth after irradiation with 1 or 3 Gy are shown in [2]. X-irradiated LY cells show delayed apoptosis, followed by secondary necrosis (to be published elsewhere). This mode of death operates in BLM-damaged LY cell populations, as indirectly indicated by experiments with ATA.

Biological effects of BLM can be explained at the molecular level. BLM is active in the presence of iron ions. These are more numerous in LY-R nuclei (7.7 ± 1.8 ng/$10^6$ cells) than in LY-S nuclei (3.1 ± 0.9 ng/$10^6$ cells) [20]. It is plausible to assume that this is the reason for a higher initial DNA damage in LY-R than in LY-S cells in the low dose range of BLM (Fig. 6). In the higher dose range, the DNA damage distribution in both sublines is similar and much less uniform than in the case of γ-irradiated cells (Fig. 8).

Induction of a broad range of DNA damages and the presence of heavily damaged cells is a characteristic feature of BLM, as shown previously by other authors [1, 15]. This lack of uniformity is also reflected in chromosomal aberrations: after treatment with 100 μg/ml BLM [10] there are 2 classes of cells with aberrations – "normal" (up to 30 aberrations per cell) and heavily damaged (over 30 aberrations per cell). Other authors [4] lower the limit to 10 aberrations per cell (after 10 μg/ml BLM) but also discern these 2 classes of damaged cells. In comparison, the distribution of aber-

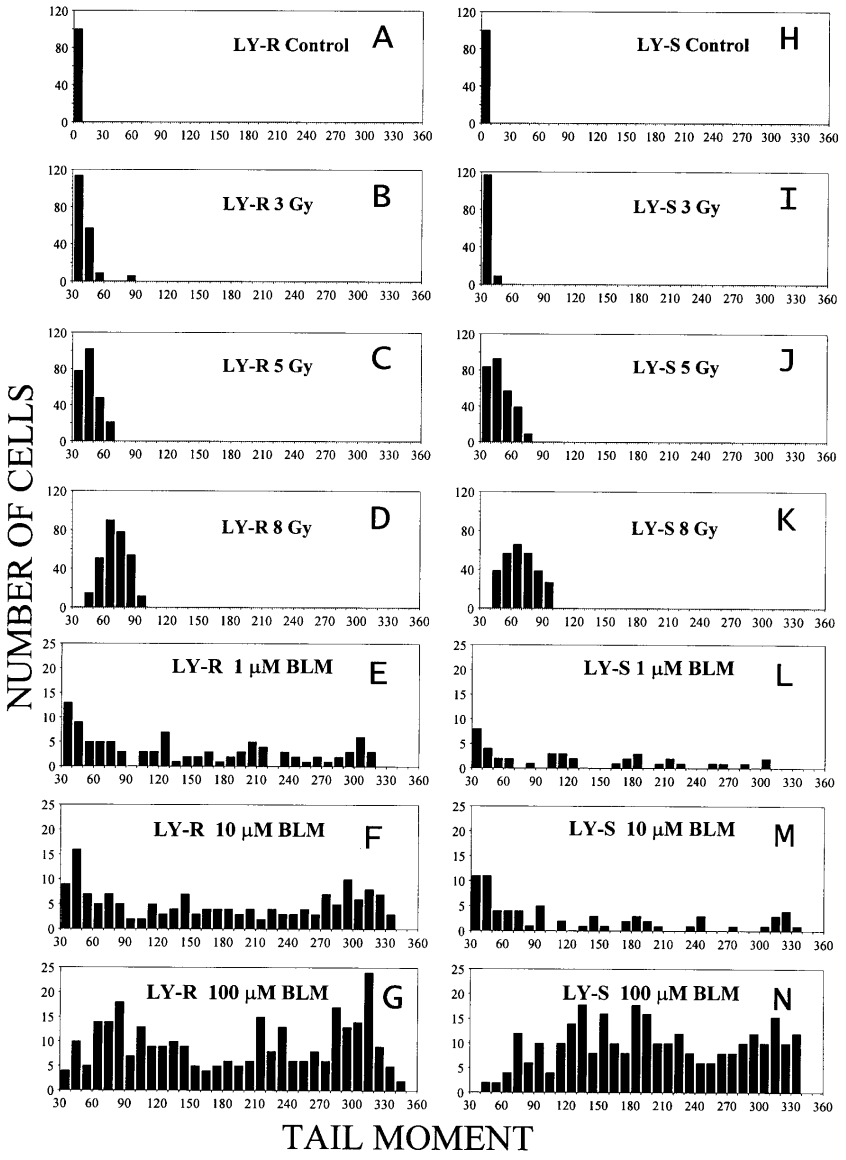


**Fig. 8.** Distribution of tail moment values in LY-R and LY-S cells treated with γ-rays (panels B–D and I–K) or BLM (panels E–G and L–N) and control cells (panels A, H). In panels B–G and I–N cells exhibiting tail moments below 30 are left out. Results pooled from 3 independent experiments (100 comets each).

rations in irradiated cells reflects a more random distribution of DNA damage. Numerous hypotheses concerning the action of BLM have been postulated (recently reviewed by McLeod *et al.*[10]) none of them, however, has been experimentally verified as yet.

## Conclusions

Bleomycin is a well known anticancer drug widely used in the clinic that mimics the effects of low LET radiation. Its mode of action differs, however, from that of ionizing radiation, since BLM toxicity is critically dependent on the presence of iron ions. In spite of the higher iron content and the higher initial DNA damage at the lower BLM concentrations in LY-R cells than in LY-S cells, the drug is more toxic for the latter cells. Our results suggest that other cellular features, such as the ability to repair BLM-induced DNA damage and/or proneness to apoptosis play a crucial role in BLM toxicity.

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