Mutation Research 731 (2012) 133–139

Contents lists available at SciVerse ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Bleomycin induces delayed instability of interstitial telomeric sequences in Chinese hamster ovary cells

Malena Vidal Bravo, Martha S. Bianchi, Alejandro D. Bolzán*

Laboratorio de Citogenética y Mutagénesis, Instituto Multidisciplinario de Biología Celular (IMBICE, CCT-CONICET La Plata-CICPBA), C.C. 403, 1900 La Plata, Argentina

ARTICLE INFO

Article history:
Received 1 November 2011
Accepted 21 December 2011
Available online 3 January 2012

Keywords:
CHO cells
Interstitial telomeric sequences
Bleomycin
Telomere PNA-FISH
Long-term clastogenic effect

ABSTRACT

We analyzed the behavior of interstitial telomeric sequences (ITSs) in the progeny of Chinese Hamster Ovary (CHO) cells exposed to the radiomimetic compound bleomycin (BLM) in order to determine if ITSs play some role in the long-term clastogenic effect of this antibiotic. To this end, CHO cells were treated with a single concentration of BLM (2.5 μg/ml), and the frequency of unstable chromosomal aberrations was determined at several times after treatment (18 h, and 6, 15 and 34/36 days) by using PNA-FISH with a pan-telomeric probe [(TTAGGG)*n* repeats]. Cytogenetic analysis revealed a higher frequency of aberrations at 18 h and 6 days after treatment in BLM-exposed cultures vs. untreated cultures, although the yield of BLM-induced aberrations decreased on average five times 6 days after treatment compared with the one induced 18 h after treatment. Moreover, no significant differences in the frequency of aberrations were observed between untreated and BLM-exposed cells at 15 or 34/36 days after treatment. These data indicate that, in terms of unstable aberrations, the in vitro clastogenic effect of BLM on CHO cells persists for at least 6 days but less than 15 days after exposure. In addition, we found that BLM induces ITSs instability, cytogenetically detectable as acentric fragments (18 h after treatment) or additional (new) FISH signals (6 days after treatment). We propose that the delayed effect of BLM on ITSs mainly results from breakage of heterochromatic ITSs blocks and further insertion of these sequences at the sites of monochromatid breaks occurring at G2 phase of the cell cycle, since most of the additional FISH signals were present as single dots and located at interstitial sites of the involved chromosomes.

1. Introduction

Several vertebrate species, including Chinese hamster, show large blocks of (TTAGGG)*n* telomeric repeats present in the centromeric or pericentromeric regions of most of their chromosomes [1–4], the so-called interstitial telomeric repeats or interstitial telomeric sequences (ITSs), which comprise not only those repeats located close to the centromeres, but also those ones found at interstitial sites, i.e., between the centromeres and the telomeres (see [5,6] for review).

Experimental evidence indicates that chromosomal regions containing ITSs are prone to spontaneous and mutagen-induced chromosome breakage, fragility and recombination (see [5–9] for review). However, most of the above studies focused on the involvement of ITSs in the short-term clastogenic effects of mutagens (i.e., aberrations observed in first metaphase cells after treatment). Only in a few studies, the role of ITSs on the long-term clastogenic effect of ionizing radiation has been investigated [10–14]. These studies suggest that ITSs are unstable and could lead to instability in adjacent sequences, promoting delayed chromosomal instability, the phenomenon of the formation of chromosomal aberrations at high frequency in cells several generations after exposure to a physical or chemical DNA damaging agent [15].

Since several chemical mutagens are usually employed as antineoplastic agents, the analysis of the long-term chromosomal instability produced by these mutagens, and the involvement of telomeric sequences (both terminal and interstitial) in this instability may be of great importance to understand the genomic instability associated with chemotherapy regimens used for the treatment of several cancers. Although it has been reported that, like ionizing radiation, some chemical mutagens have long-term effects on chromosomes, inducing delayed or persistent chromosomal instability [16–19], the role of ITSs in this instability has not been explored. The only data available to date on the involvement of ITSs on the chromosomal aberrations induced by chemical mutagens come from short-term studies (see [7–9] for review). Therefore, in the present work we analyzed the involvement of ITSs in the chromosomal aberrations present in the progeny of Chinese Hamster Ovary (CHO) cells exposed to the antibiotic bleomycin (BLM), in order to determine if ITSs play some role in the long-term clastogenic effect of this antibiotic. BLM is a compound extensively used in clinical chemotherapy for the treatment of several types

* Corresponding author. Tel.: +54 0221 4210112; fax: +54 0221 4253320.
E-mail address: abolzan@imbice.org.ar (A.D. Bolzán).

0027-5107/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2011.12.010

of cancer [20] which acts as an S-independent clastogenic agent, induces both single- and double-strand breaks in DNA, and is considered a radiomimetic drug [21,22]. For our present investigation, we used CHO cells because most of their chromosomes exhibit large blocks of heterochromatic ITSs at the centromeric regions, representing about 95% of the telomeric FISH signals in a metaphase cell [2,4,23]. Moreover, those very few telomeric signals located at one end of one or two chromosomes of the CHO karyotype represent former ITSs that had been converted into new telomeres, either by chromosome breakage or amplification mechanisms [13].

To accomplish our goal, we exposed CHO cells to a single pulse of BLM and determined the frequency of unstable chromosomal aberrations at several times after treatment, by using PNA-FISH with a pan-telomeric probe. Our results show that the in vitro clastogenic effect of BLM on CHO cells persists for at least 6 days after exposure and that this antibiotic induces ITSs instability, cytogenetically detectable as acentric fragments (at 18 h after treatment) or additional FISH signals (at 6 days after treatment).

2. Materials and methods

2.1. Cell culture, drug treatments and cell harvesting

The CHO cell line was obtained from the Instituto Multidisciplinario de Biología Celular (IMBICE, La Plata, Argentina) cell repository and is an established immortalized cell line derived from Chinese hamster ovary cells, possessing on average $2n = 21$ chromosomes [4,23]. CHO cells were grown in Ham's F10 medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal calf serum, penicillin (100 U/ml) and streptomycin (100 μg/ml) at 37 °C and 5% CO_2 atmosphere. Cells were cultured as monolayer in TC25 Corning flasks containing 1×10^5 cells/ml. For each experiment, two flasks were set up, one for the control and one for the treated culture. During the log phase of growth the cells were treated with a 30 min pulse of 2.5 μg/ml of BLM (Blocamicina®, Laboratorios Gador, Argentina, CAS No. 9041-93-4, dissolved in sterile 0.9% NaCl). Control cultures were set up in parallel but not exposed to BLM. Time of exposure and concentration of BLM were chosen according to previous studies carried out in our laboratory with CHO cells exposed to this compound [4,23,24]. At the end of the pulse treatment with BLM, the cells were washed twice with Hank's balanced salt solution and kept in culture with fresh culture medium until harvesting. Cells were continuously maintained in culture during 15 passages or subcultures after treatment. Subcultivation was carried out whenever the cultures became confluent (approximately 5×10^6 cells/10 ml of culture medium). To estimate cell growth, at the time of subcultivation cells were collected by trypsinization, an aliquot of about 200 μl stained with 0.4% trypan blue, and the number of viable cells (cells not stained) was determined. Cells were then suspended in fresh culture medium and dispensed into new culture flasks containing 1×10^5 cells/ml to continue growing. The rest of the cells was discarded or dispensed in another flask for cytogenetic analysis, which was performed at 18 h, 6, 15, and 34 (experiment 1) or 36 (experiment 2) days after the end of treatments. To analyze chromosomal aberrations, colchicine (0.1 μg/ml) (Sigma, CAS No. 64-86-8) was added to cell cultures during the last 2.5 h of culture. Chromosome preparations were made following standard procedures. After harvesting, cells were hypotonically shocked, fixed in methanol:acetic acid (3:1), spread onto glass slides and processed for PNA-FISH. Two independent experiments were carried out.

2.2. Fluorescence in situ hybridization with the PNA pan-telomeric probe (PNA-FISH) and chromosome analysis

A Cy3-conjugated PNA pan-telomeric probe [Cy3-$(CCCTAA)_3$] obtained from DAKO Corporation (CA, USA) was used (DAKO Telomere FISH kit/Cy3). FISH was performed according to the instructions provided by the supplier and following previous experience in our laboratory with this kit [4,23,25]. Briefly, after pre-treatment with formaldehyde and a solution containing proteinase K for 10 min, the sample DNA was denatured at 80 °C for 4 min under a coverslip in the presence of the Cy3-conjugated probe. Hybridization (1 h at room temperature) was followed by two washes using solutions provided in the kit. Afterwards, slides were mounted in an antifade reagent containing DAPI (4,6-diamidino-2-phenylindole) as counterstain. Fluorescence microscopy was performed on a Nikon Eclipse 50i epifluorescence microscope equipped with an HBO 100 mercury lamp and filters for DAPI and Cy3 (Chroma Technology Corp, Rockingham, VT).

2.3. Scoring of aberrations

Chromosome analysis was performed on coded slides. For accurate observation and analysis, all images were digitalized and, when necessary, DAPI signals were enhanced for optimal contrast using the Adobe® Photoshop® CS2 Software. This allowed detection of even the smallest acentric fragments present in the damaged cells. Centromeres were identified using the DAPI filter, whereas telomeric signals were observed using the Cy3 filter. The total number of centromeres and telomeric signals was counted and all unstable chromatid- and chromosome-type aberrations were scored. In the CHO cell line available in our laboratory, telomeric repeats are predominantly localized in the centromeric regions of the chromosomes (see Section 3.1) (Fig. 1A) [4,23,25]. Since the position and number of centromeres could be easily determined with DAPI staining, all types of unstable aberrations were scored, namely: (1) dicentric chromosomes; (2) multicentrics (those chromosomes possessing three or more centromeres; for quantification, the number of centromeres present in the polycentric chromosomes minus one was used and scored as dicentric equivalents); (3) centric rings; (4) acentric fragments (without discrimination of their type); (5) chromatid-type aberrations: breaks, deletions, and exchanges (tri- and quadrirradials), and (6) centromeric breaks. When the size of the lesion is equal to or larger than the diameter of the chromatid, we consider the aberration a chromatid break (mono- and isochromatid type, i.e., affecting one or both chromatids of a chromosome). Gaps were excluded from the analysis. The frequencies of aberrations with and without telomeric signals and the number and distribution of telomeric repeats in each of the aberrations analyzed were determined in untreated (control) and BLM-treated cells. We both checked if telomeric signals were present on the aberrant chromosomes, and if these signals were localized at the breakpoint of the rearranged chromosomes. Thus, we both check ITSs instability and the instability of chromosomes with ITSs.

2.4. Statistical analysis of data

Statistical analysis of data was performed using GraphPad Prism version 4.00 software for Windows (GraphPad Software, San Diego, CA). Comparisons between control vs. exposed cultures in the percentage of damaged cells were carried out using the Chi-squared test. The significance of differences in aberration frequencies among different treatments was obtained by comparing the Z score of Poisson distributions of observed and expected values with 95% confidence intervals [26]. Differences were considered to be statistically significant at two-sided p values < 0.05.

3. Results

3.1. Distribution pattern of telomeric sequences in untreated cells

In good agreement with previous reports [4,23,25], analysis of metaphase cells from untreated cultures of CHO cells after PNA-FISH with a pan-telomeric probe showed telomeric repeats predominantly localized in the centromeric regions of the chromosomes. The only exceptions were the largest pairs of metacentric chromosomes (i.e. chromosomes 1 and 2), which exhibited no telomeric signal, and the presence of telomeric signals at one of the ends of one small metacentric chromosome and in the long arm of a large submetacentric chromosome, although these signals were not always visible (Fig. 1A). A very few metaphases also exhibited a second chromosome with telomeric signals at one end, as previously reported [4].

3.2. Persistence of chromosome damage induced by BLM

As expected from previous reports [4,23], BLM induced a significant increase in the percentage of damaged or aberrant cells (i.e., cells showing at least one aberration) (Fig. 2) and in the frequency of chromosomal aberrations per cell (Fig. 3 and Table 1) compared with control cultures 18 h after treatment ($p < 0.05$). The enhanced effect of BLM with regard to the percentage of damaged cells and the frequency of aberrations was also observed in cell cultures 6 days after treatment, although these values were significantly lower than the ones at 18 h after treatment ($p < 0.05$). There was on average a fivefold decrease in the frequency of BLM-induced aberrations 6 days after treatment compared with 18 h after treatment (Fig. 3). In particular, the frequency of dicentrics and rings, acentric fragments and breaks (mono- and isochromatid ones) decreased five, seven and three times, respectively, at 6 days after treatment, compared with their frequencies 18 h after treatment (Table 1). However, at 6 days after treatment there was only a 10% less of damaged cells in BLM-exposed cells compared with those ones harvested at 18 h after treatment. Thus, the significant

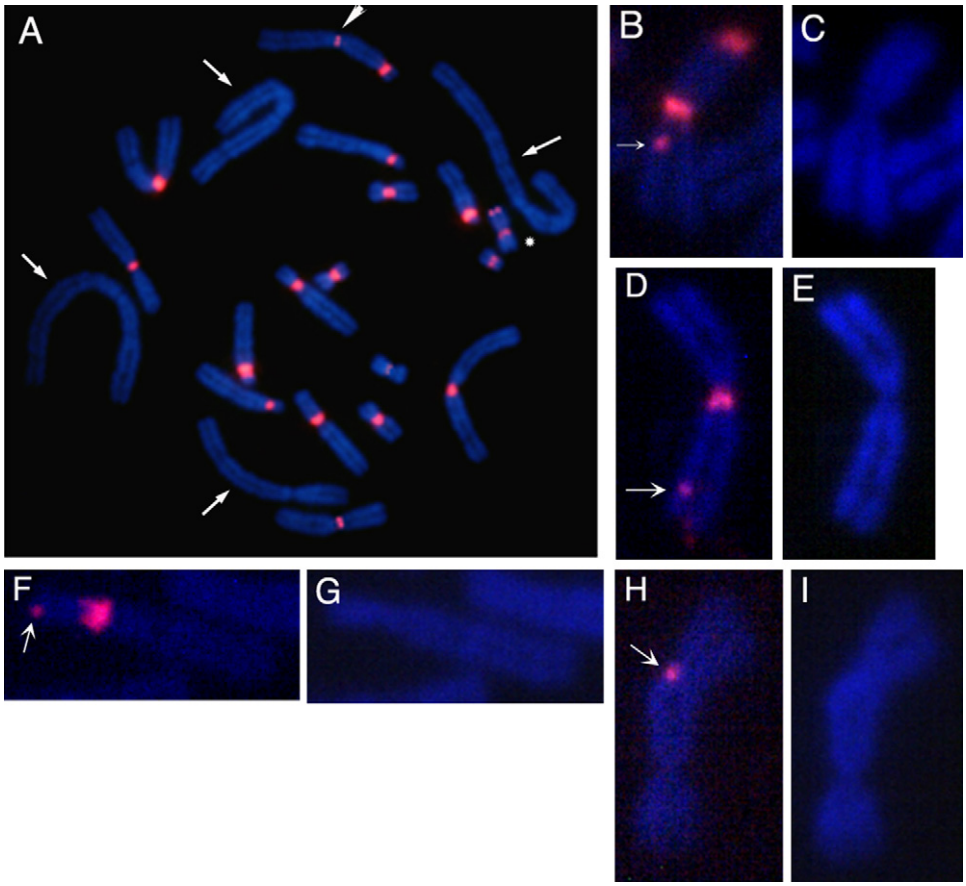

Fig. 1. Patterns of hybridization of (TTAGGG)*n* repeat in the metaphase spreads from untreated and BLM-treated CHO cells. (A) Normal metaphase from untreated culture. Note the presence of telomeric signals at the centromeric regions of most of the chromosomes, one interstitial signal in the long arm of a large submetacentric chromosome (arrowhead indicates the site of the interstitial signal), and terminal signals at one of the ends of a small metacentric chromosome (indicated with an asterisk); arrows indicate the four chromosomes exhibiting no telomeric signals; (B–I): Chromosomes from metaphase cells derived from a cell culture harvested 6 days after BLM treatment; (B–C) dicentric (dic) chromosome exhibiting two centromeric and one interstitial telomere FISH signals (B, DAPI + Cy3 image; C, DAPI image); (D–I) chromosomes with (D–E and F–G) or without (H–I) telomeric signals at the centromere showing an additional interstitial telomeric signal at one of the chromatids (indicated by arrows) (D, F, and H: DAPI + Cy3 images; E, G, and I: DAPI images).

decrease in the frequency of induced aberrations (fivefold on average) found in BLM-exposed cells 6 days after treatment cannot be solely explained by the decrease in the percentage of damaged cells, and strongly suggests that damaged cells exhibit heterogeneity in the number of aberrations.

No significant differences were found between unexposed and BLM-exposed cultures at 15 or 34/36 days after treatment, neither in the percentage of damaged cells nor in the frequency of chromosomal aberrations ($p > 0.05$) (Table 1, and Figs. 2 and 3).

Table 1
Chromosome damage observed at various subculture times in untreated and BLM-exposed CHO cells after PNA-telomere FISH scoring.

Treatment	Time after treatment (passage)	No. of cells analyzed	Dicentrics and Centric rings	Acentric fragments	Breaks	Breaks at centromeric regions	Total aberrations (frequency/cell)	Total aberrations with telomeric signal (frequency/cell)/ (percentage)
Control	18 h (S1)	202	2 (1)	8 (2)	9 (1)	7 (5)	26 (0.13)	9 (0.04)/(31%)
Control	6 d (S3)	192	3 (3)	14 (5)	4 (0)	13 (13)	34 (0.18)	21 (0.11)/(61%)
Control	15 d (S7)	194	14 (12)	51 (8)	9 (3)	47 (22)	121 (0.62)	45 (0.23)/(37%)
Control	34/36 d (S15)	201	5 (4)	22 (15)	16 (0)	30 (15)	73 (0.36)	34 (0.17)/(47%)
BLM 2.5 μg/ml	18 h (S1)	180	38[a] (30)	139 (26)	39 (3)	14 (8)	230 (1.28)*	67 (0.37)*/(29%)
BLM 2.5 μg/ml	6 d (S3)	182	8[b] (7)	19 (9)	14 (0)	7 (5)	48 (0.26)*	21 (0.11)/(42%)
BLM 2.5 μg/ml	15 d (S7)	201	9 (7)	63 (17)	15 (0)	30 (15)	117 (0.58)	39 (0.19)/(33%)
BLM 2.5 μg/ml	34/36 d (S15)	200	9[c] (8)	12 (7)	22 (1)	27 (26)	70 (0.35)	42 (0.21)/(60%)

The total number of aberrations and the number (between brackets) of aberrations exhibiting one or more FISH signals are indicated. Results of two independent experiments were pooled. (*) Significantly different from the respective control value ($p < 0.05$, Poisson distribution). For statistical analysis of data, the frequency of aberrations per cell was taken into account (for a more detailed analysis see Fig. 3). In the case of dicentrics and centric rings, this refers to centromeric signals, whereas in the case of breaks (which includes mono- and isochromatid ones), this refers to the presence of signal at the site of breakage. Breaks at centromeric regions showing telomeric signal includes those ones with and without dissociation of the signal. No chromatid exchanges were observed.

[a] Includes a tricentric chromosome and one centric ring.
[b] Includes two centric rings.
[c] Includes one centric ring.

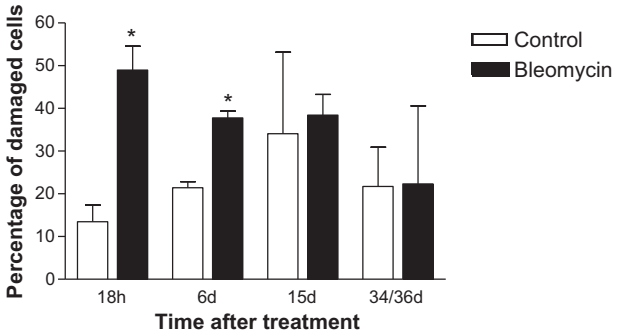

Fig. 2. Percentage of damaged cells in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with BLM (2.5 μg/ml). Data represent pooled values from two independent experiments. For each treatment, mean ± S.E. is indicated. Chi-squared test indicated significant differences (*) between control and exposed cultures at 18 h and 6 days after treatment ($p < 0.05$).

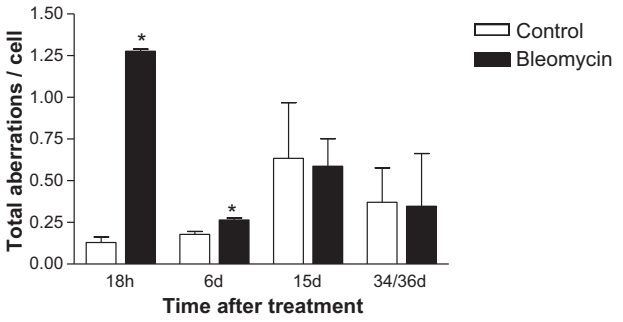

Fig. 3. Changes in the frequency of chromosome-type aberrations (dicentrics, rings, acentric fragments and breaks) in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with BLM (2.5 μg/ml). Data represent pooled values from two independent experiments. For each treatment, mean ± S.E. is indicated. The Z score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by BLM at 18 h and 6 days after treatment compared with untreated (control) cultures ($p < 0.05$).

Analysis of the number of live cells in each subculture or passage showed that the number of live cells per ml of culture medium was very similar in control vs. BLM-treated cultures for each subculture analyzed (Fig. 4). Thus, there was no significant effect of BLM on cell survival, and no BLM-induced delayed cell death was observed in CHO cells.

3.3. Short- and long-term effect of BLM on ITSs

Table 1 shows that both in control and BLM-treated cells, chromosomal aberrations exhibiting telomeric signals represent between 30 and 60% of the total aberrations observed. These percentages are higher than expected, taking into account the

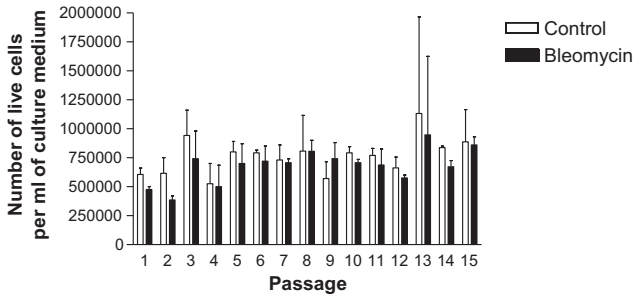

Fig. 4. Variation in the number of live cells observed in CHO cells with time in culture after treatment with BLM (2.5 μg/ml). Data represent pooled values from two independent experiments. For each treatment, mean ± S.E. is indicated.

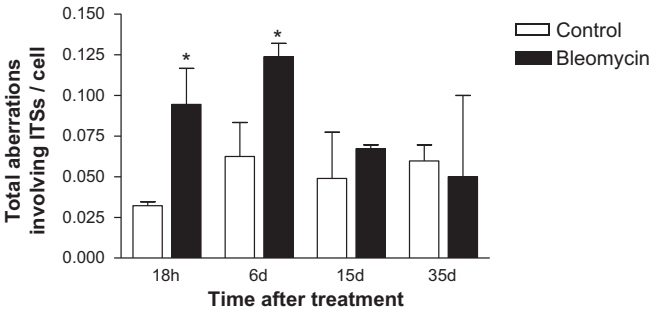

Fig. 5. Changes in the frequency of chromosomal aberrations directly involving ITSs (i.e., ITSs instability; see also Table 2) in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with BLM (2.5 μg/ml). Data represent pooled values from two independent experiments. For each treatment, mean ± S.E. is indicated. The Z score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by BLM at 18 h and 6 days after treatment compared with untreated (control) cultures ($p < 0.05$).

percentage of the CHO karyotype occupied by telomeric sequences (i.e., 3% on average) [4]. At 18 h and 15 days after treatment, the percentage of aberrations with telomeric signals was very similar for control and BLM-treated cells, but at 6 days after treatment, the percentage of aberrations with telomeric signals was significantly higher in control (61%) than in BLM-exposed (42%) cells, and at 34/36 days after treatment this percentage was higher in BLM-treated (60%) than in control (47%) cells. However, the frequency of aberrations exhibiting telomeric signal was very similar for control and BLM-exposed cells and no significant differences were found ($p > 0.05$), except for cells harvested 18 h after treatment, where the frequency of aberrations with telomeric signals was significantly higher in BLM-exposed than in control cells ($p < 0.05$) (Table 1).

On the other hand, we determined the frequency of chromosomal aberrations directly involving ITSs (breaks at centromeric regions containing ITSs blocks, acentric fragments and mono- and isochromatid breaks exhibiting telomeric signal) and determined the position and number of telomeric FISH signals in the metaphases studied, in order to analyze the instability of ITSs. We found that BLM induces ITSs instability in CHO cells, mainly evidenced as acentric fragments at 18 h and new (de novo) telomeric signals at 6 days after treatment (Table 2 and Fig. 5; see Fig. 1B–I for illustrations of additional FISH signals) ($p < 0.05$) (Table 2). To determine the increase in the frequency of additional signals, we took into account the normal pattern of distribution of telomeric signals in CHO cells (see Section 3.1 of this manuscript; additional telomeric signals due to the presence of a second chromosome with terminal repeats were excluded from this analysis). Since the appearance of additional (new) telomeric signals suggests possible amplification or translocation of ITSs, they were considered as aberrations in order to estimate the instability of ITSs and their relationship with the genomic instability (as determined by unstable aberrations). Most of the additional signals (about 90%) were single signals localized at one chromatid of one chromosome arm and they exhibited a random distribution, being localized in different chromosomes. Only in one case, the signal was located at one of the chromatids of a dicentric chromosome (Fig. 1B, C). The presence of additional signals was not accompanied by the lack of one or more of the original signals (i.e., those ones belonging to the standard karyotype of CHO cell line). In all cases, DAPI images showed that the chromosome did not exhibit any discontinuity at the site in which the additional signal is located (Fig. 1C, E, G, and I).

The observed instability of ITSs was about three times higher at 18 h and two times higher at 6 days after treatment in BLM-treated than in control cells. In addition, we found that ITSs involvement in the aberrations induced by BLM (14%) is lower than in control cells

Table 2
ITSs instability observed at various subculture times in untreated and BLM-exposed CHO cells after PNA-telomere FISH scoring.

Treatment	Time after treatment (passage)	No. of cells analyzed	Additional (new) FISH signals single/double	Breaks at centromeric regions	Acentric fragments	Breaks	Total aberrations	ITSs involvement (ITSs instability related to genomic instability) (frequency/cell)
Control	18 h (S1)	202	1/7 (8)	2	2	1	13 (0.06)	34/13 (38%)
Control	6 d (S3)	192	9/3 (12)	7	5	0	24 (0.12)	46/24 (52%)
Control	15 d (S7)	194	1/1 (2)	6	8	3	19 (0.10)	123/19 (15%)
Control	34/36 d (S15)	201	1/2 (3)	6	15	0	24 (0.12)	76/24 (31%)
BLM 2.5 μg/ml	18 h (S1)	180	1/4 (5)	0	26*	3	34 (0.19)*	235/34 (14%)
BLM 2.5 μg/ml	6 d (S3)	182	30/4 (34)*	2	9	0	45 (0.25)*	82/45 (55%)
BLM 2.5 μg/ml	15 d (S7)	201	1/6 (7)	3	7*	0	27 (0.13)	124/27 (22%)
BLM 2.5 μg/ml	34/36 d (S15)	200	1/1 (2)	10	7	1	20 (0.10)	72/20 (28%)

For each treatment, the number of aberrations exhibiting FISH signal are indicated. Results of two independent experiments were pooled. For statistical analysis of data, the frequency of aberrations per cell was taken into account (for a more detailed analysis see Fig. 5). Additional FISH signals: Number of new telomeric FISH signals observed (as double or single dots); the total number of signals observed is indicated between brackets. Breaks at centromeric regions mean those breaks involving dissociation of the FISH signal in chromosomes exhibiting centromeric ITSs. Acentric fragments mean those fragments partially or totally labeled with the telomeric probe. Breaks mean mono- and isochromatid breaks exhibiting telomeric FISH signal at the site of breakage. ITSs involvement is expressed as the percentage of aberrations directly involving ITSs related to the total amount of aberrations observed (i.e., those aberrations indicated in Table 1 plus the additional FISH signals indicated in the present table).

* Significantly different from the respective control value ($p < 0.05$, Poisson distribution).

(38%) 18 h after treatment, but is very similar at 6, 15, and 34/36 days after treatment.

Overall, data from Tables 1 and 2 show that BLM does not induce an increase in the involvement of ITSs in the chromosomal aberrations, but significantly increases the frequency of aberrations with telomeric signals at 18 h after treatment (Table 1), and the frequency of aberrations directly involving ITSs at 18 h and 6 days after treatment (Table 2) compared with control (unexposed) cells.

4. Discussion

As a first approach to determine if ITSs play some role in the long-term chromosomal instability induced by chemical mutagens, we analyzed the relationship between chromosome damage and ITSs in the progeny of CHO cells exposed to BLM. Telomere PNA-FISH analysis revealed a higher frequency of chromosomal aberrations at 18 h and 6 days after treatment in BLM-exposed cultures vs. untreated cultures, which shows that the in vitro clastogenic effect of BLM persists for at least 6 days after exposure. However, the yield of BLM-induced aberrations decreased on average five times 6 days after treatment compared with the one induced 18 h after treatment, very likely because of the instability of the chromosome lesions analyzed (i.e., dicentrics, rings, acentric fragments and chromosome breaks). In addition, no significant differences in the frequency of aberrations were observed between untreated and BLM-exposed CHO cells at 15 and 34/36 days after treatment. Therefore, our study shows that the in vitro clastogenic action of BLM, in terms of unstable aberrations, decreases with time in culture and persists for less than 15 days after treatment. Since we found that BLM did not impair cell survival, the possibility that the absence of a clastogenic effect by BLM at late subculturing times (i.e., 15 and 34/36 days after treatment) may be due to the death of heavily damaged cells should be discarded.

Although previous reports indicate that BLM induces delayed chromosomal instability in human cells in the form of chromosomal rearrangements [16], micronuclei and aneuploidy [17], our present data show that in CHO cells this antibiotic induces a persistent but not delayed effect (no increase in the frequency of BLM-induced aberrations was found at 6 days after treatment compared with 18 h after BLM exposure). These discrepancies could be due to differences in the type of cells employed in the studies and in the concentration of BLM used. Moreover, our data agrees well with the finding by Cho et al. in human fibroblasts, indicating that the clastogenic action of BLM (determined by the micronucleus test) persists for at least 10 days after treatment [17].

On the other hand, our present data show that BLM does not induce an increase in the involvement of ITSs in the chromosomal aberrations, but significantly increases the frequency of the chromosomal aberrations involving telomeric signals and ITSs instability in CHO cells exposed to the antibiotic, 18 h and 6 days after treatment. In effect, we found that this compound induces a significant increase in the frequency of chromosomal aberrations directly involving ITSs, and that this effect persists for at least 6 days after treatment. According to our data, BLM-induced ITSs instability is mainly due to the formation of acentric fragments containing ITSs (at 18 h after treatment) and the appearance of additional (new) FISH signals (at 6 days after treatment). In a recent work, we found that BLM induces breakage, translocation and amplification of ITSs in CHO cells [23], but this is the first report of a delayed effect of this compound on ITS. According to current knowledge about ITSs, an increase in the number of telomere FISH signals may be the result of amplification and/or translocation of telomeric repeats. Amplification of telomeric sequences (i.e., increase in the number of TTAGGG repeats) can be cytogenetically visualized as an increase in the size, intensity or number of telomeric FISH signals compared with the normal pattern of hybridization of the cell being analyzed (see [7–9] for review, and [29] for a recent report showing amplification of ITSs in Chinese hamster cells in the form of additional FISH signals), whereas translocation of telomeric repeats is usually visualized as a change in the position of one or more of the telomeric signals originally present in the cell or, less frequently, as an increase in the number of telomeric FISH signals accompanied by a decrease in the intensity of some of the other signals (due to the partial loss of telomeric repeats) taking into account the normal pattern of hybridization of the cell being analyzed (see [7–9] for review). In our present investigation, we found that about 90% of the additional signals present in the chromosomes of BLM-exposed cells 6 days after treatment were single dots distributed at random (i.e., signals were located in different chromosomes and different chromosome arms). Since almost 100% of the telomeric FISH signals in a CHO metaphase cell corresponds to ITSs and the chromosomes (one or, less frequently, two) with terminal signals were always present in those cells possessing additional signals, we can assume that the telomeric sequences involved in the chromosomal aberrations induced by BLM which directly involve ITSs come from broken ITSs. This is in good agreement with our present results showing a significant increase in the frequency of acentric fragments exhibiting telomeric signal 18 h after treatment in BLM-exposed cells compared with control cells, with our previous observation

that BLM induces breakage at chromosomal sites containing ITSs [23] and with the fact that ITSs, especially those ones located at centromeric regions of Chinese hamster chromosomes, are prone to breakage, fragility and recombination (see [5–9] for review, and [29]).

It has been proposed that gene amplification driven by breakage within large ITSs blocks might be causing the delayed chromosomal instability observed in ionizing radiation-exposed cells [15]. In particular, Day et al. [14] using a human–hamster hybrid cell line demonstrated that interstitial telomere bands (or ITSs) function as recombinational hotspots that participate in the delayed chromosomal instability observed in the progeny of cells surviving X-ray-exposure at multiple generations after irradiation. Thus, recombination involving ITSs may be a major driving force in ionizing radiation-induced genomic instability. In addition, several studies performed in radiation-exposed cells showed de novo telomeric signals at derived chromatid breaks observed several hours following irradiation, and suggest that these signals may be the result of amplification of interstitial telomeric sites in the first cell cycle and spontaneous breakage of these sites in subsequent cell cycles [13,27,28]. Taking into account the above reports and our present results, we propose that the delayed effect of BLM on ITSs (cytogenetically visualized as de novo telomeric signals) mainly results from breakage and further insertion of heterochromatic ITSs at the sites of chromatid breaks occurring at G2 phase of the cell cycle. In good agreement with this assumption, we previously found that BLM induces mainly monochromatid breaks at G2 in CHO cells [23,Sánchez, unpublished]. Alternatively, the new telomeric FISH signals present in BLM-exposed CHO cells could be the result of telomere capture events [28] or the "healing" by telomerase [13] of monochromatid breaks induced by BLM. Telomere capture is a telomerse-independent process which involves the addition of telomeres at the site of DSB by subtelomeric cryptic translocations, undetectable by classical cytogenetic techniques [8]. Since CHO cells possess only one or, less frequently, two chromosomes exhibiting telomeric signals at the ends (and only one end), we think that telomere capture events cannot explain the telomeric sequences instability observed in BLM-exposed cells (i.e., in case telomeres were transferred, this will not be detectable). We may assume that telomerase is not involved in the delayed instability of ITSs induced by BLM, since telomerase products are usually below the resolution of FISH [28], and previous work in our laboratory showed that this antibiotic did not induce telomerase activity in CHO cells [4]. In addition, the possibility that breakage-fusion-bridge (BFB) cycles play some role in the delayed instability of ITSs (i.e., causing amplification of telomeric repeats) seems unlikely, since BFB cycles involve the formation of dicentric chromosomes, and the frequency of these aberrations decreases with time in culture in BLM-exposed CHO cells.

In summary, our present findings show that the in vitro clastogenic effect of BLM on CHO chromosomes, in terms of unstable aberrations, persists during at least 6 days after treatment, and that this compound promotes delayed instability of ITSs in CHO cells in the form of an increase in the number of telomeric signals, as detected after PNA-FISH. The reason why the observed delayed ITSs instability in BLM-exposed cells no longer exists in CHO cells 15 days after treatment remains to be established. According to a very recent report, ITSs instability is not only related to the cell cycle phases and the occurrence of breaks within ITSs, but also to the functions of DNA-PKcs, and the DNA double-strand breaks (DSBs) repair pathways recruited in accordance with the chromatin state of the exposed cells [29]. Therefore, it would be interesting to explore the possible role of DNA-PKcs and the DSBs repair pathways in the ITSs instability induced by BLM.

Future studies on this area of research should aim to confirm our present assumptions concerning the mechanisms underlying the delayed instability of ITSs by BLM, and to investigate the long-term effects of other chemical mutagens on ITSs in order to determine if they also induce the same ITSs instability as BLM.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgements

This work was supported by grants from CONICET (PIP No. 0316) and CICPBA of Argentina. We wish to thank César Horgan and Daniel Castrogiovanni for technical assistance, Dr. Jorge López Camelo for assistance with statistical analysis of data, and Alejandro Portela (Lab. Gador, Argentina) for providing us the bleomycin samples.

References

[1] J. Meyne, R.L. Ratliff, R.K. Moyzis, Conservation of the human telomere sequence (TTAGGG)*n* among vertebrates, Proc. Natl. Acad. Sci. U.S.A. 86 (1989) 7049–7053.
[2] A.S. Balajee, H.J. Oh, A.T. Natarajan, Analysis of restriction enzyme-induced chromosome aberrations in the interstitial telomeric repeat sequences of CHO and CHE cells by FISH, Mutation Res. 307 (1994) 307–313.
[3] P. Slijepcevic, P.E. Bryant, Absence of terminal telomeric FISH signals in chromosomes from immortal Chinese hamster cells, Cytogenet. Cell Genet. 69 (1995) 87–89.
[4] A.D. Bolzán, G.L. Páez, M.S. Bianchi, FISH analysis of telomeric repeat sequences and their involvement in chromosomal aberrations induced by radiomimetic compounds in hamster cells, Mutat. Res. 479 (2001) 187–196.
[5] K.W. Lin, J. Yan, Endings in the middle: current knowledge of interstitial telomeric sequences, Mutation Res. 658 (2008) 95–110.
[6] A. Ruíz-Herrera, S.G. Nergadze, M. Santagostino, E. Giulotto, Telomeric repeats far from the ends: mechanisms of origin and role in evolution, Cytogenet. Genome Res. 122 (2008) 219–228.
[7] A.D. Bolzán, M.S. Bianchi, Telomeres, interstitial telomeric repeat sequences, and chromosomal aberrations, Mutat. Res. 612 (2006) 189–214.
[8] A.D. Bolzán, Cytogenetic evaluation of telomere dysfunction: chromosomal aberrations involving telomeres and interstitial telomeric sequences, in: L. Mancini (Ed.), Telomeres: Function, Shortening and Lengthening, Nova Science Publishers Inc., New York, 2009, pp. 133–185.
[9] A.D. Bolzán, Chromosomal aberrations involving telomeres and interstitial telomeric sequences, Mutagenesis 27 (2012) 1–15.
[10] B.A. Marder, W.F. Morgan, Delayed chromosomal instability induced by DNA damage, Mol. Cell. Biol. 13 (1993) 6667–6677.
[11] L. Bertoni, C. Attolini, L. Tessera, E. Mucciolo, E. Giulotto, Telomeric, nontelomeric, (TTAGGG)*n* sequences in gene amplification and chromosome stability, Genomics 24 (1994) 53–62.
[12] T.K. Pandita, D. DeRubis, Spontaneous amplification of interstitial telomeric bands in Chinese hamster ovary cells, Cytogenet. Cell Genet. 68 (1995) 95–101.
[13] P. Slijepcevic, Y. Xiao, I. Dominguez, A.T. Natarajan, Spontaneous and radiation-induced chromosomal breakage at interstitial telomeric sites, Chromosoma 104 (1996) 596–604.
[14] P. Day, C.L. Limoli, W.F. Morgan, Recombination involving interstitial telomere repeat-like sequences promotes chromosomal instability in Chinese hamster cells, Carcinogenesis 19 (1998) 259–266.
[15] S.D. Bouffler, W.F. Morgan, T.K. Pandita, P. Slijepcevic, The involvement of telomeric sequences in chromosomal aberrations, Mutat. Res. 366 (1996) 129–135.
[16] C.L. Limoli, M.I. Kaplan, J.W. Phillips, G.M. Adair, W.F. Morgan, Differential induction of chromosomal instability by DNA strand-breaking agents, Cancer Res. 57 (1997) 4048–4056.
[17] Y.H. Cho, H.K. Jeon, H.W. Chung, Effects of extremely low-frequency electromagnetic fields on delayed chromosomal instability induced by bleomycin in normal human fibroblast cells, J. Toxicol. Environ. Health A. 70 (2007) 1252–1258.
[18] G. Sciandrello, F. Caradonna, M. Mauro, G. Barbata, Arsenic-induced DNA hypomethylation affects chromosomal instability in mammalian cells, Carcinogenesis 25 (2004) 413–417.
[19] G. Sciandrello, M. Mauro, I. Catanzaro, M. Saverini, F. Caradonna, G. Barbata, Long-lasting genomic instability following arsenite exposure in mammalian cells: the role of reactive oxygen species, Environ. Mol. Mutagen. 52 (2011) 562–568.
[20] J. Chen, J. Stubbe, Bleomycins: towards better therapeutics, Nat. Rev. Cancer 5 (2005) 102–112.
[21] L.F. Povirk, M.J.F. Austin, Genotoxicity of bleomycin, Mutation Res. 257 (1991) 127–143.
[22] L.F. Povirk, Biochemical mechanisms of chromosomal translocations resulting from DNA double-strand breaks, DNA Rep. (Amst) 5 (2006) 1199–1212.

[23] J. Sánchez, M.S. Bianchi, A.D. Bolzán, Effect of bleomycin on interstitial telomeric sequences of immortalized Chinese hamster cells, Mutat. Res. 669 (2009) 139–146.
[24] M.L. Larramendy, D.M. López-Larraza, L. Vidal-Rioja, N.O. Bianchi, Effect of the metal chelating agent *o*-phenanthroline on the DNA and chromosome damage induced by bleomycin in Chinese hamster ovary cells, Cancer Res. 49 (1989) 6583–6586.
[25] J. Sánchez, M.S. Bianchi, A.D. Bolzán, Relationship between heterochromatic interstitial telomeric sequences and chromosome damage induced by the radiomimetic compound streptonigrin in Chinese hamster ovary cells, Mutat. Res. 684 (2010) 90–97.
[26] R.A. Fisher, F. Yates, Statistical Tables for Biological, Agricultural and Medical Research, 5th ed., Oliver & Boyd, London, 1957, p. 61.
[27] P. Slijepcevic, Y. Xiao, A.T. Natarajan, P.E. Bryant, Instability of CHO chromosomes containing interstitial telomeric sequences originating from Chinese hamster chromosome 10, Cytogenet. Cell Genet. 76 (1997) 58–60.
[28] P. Slijepcevic, A.T. Natarajan, P.E. Bryant, Telomeres and radiation-induced chromosome breakage, Mutagenesis 13 (1998) 45–49.
[29] D. Revaud, L.M. Martins, F.D. Boussin, L. Sabatier, C. Desmaze, Different DNA-PKcs functions in the repair of radiation-induced and spontaneous DSBs within interstitial telomeric sequences, Chromosoma 120 (2011) 309–319.