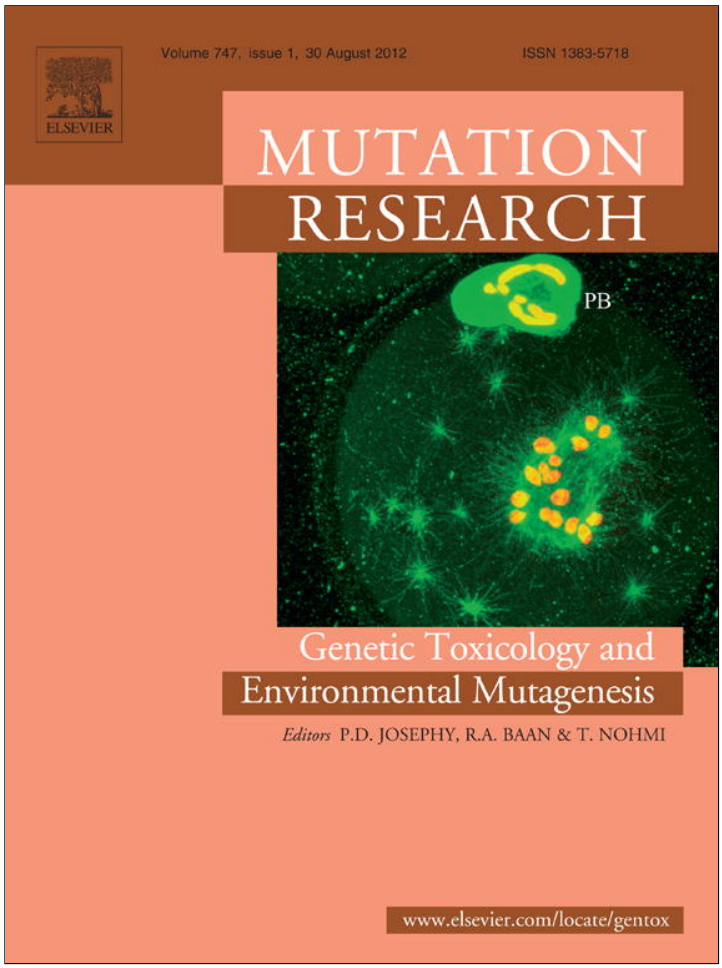

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Streptonigrin induces delayed chromosomal instability involving interstitial telomeric sequences in Chinese hamster ovary cells

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ABSTRACT

We analyzed the induction of chromosomal aberrations in Chinese hamster ovary (CHO) cells exposed to the radiomimetic compound streptonigrin (SN), in order to determine whether interstitial telomeric sequences (ITSs) are involved in the long-term clastogenic effect of this antibiotic. CHO cells were treated with a single concentration of SN (100 ng/ml), and the frequency of unstable chromosomal aberrations was determined at three times after treatment (18 h, and 6 and 15 days) by using PNA-FISH with a pan-telomeric probe. Cytogenetic analysis revealed a higher frequency of aberrations at 18 h and 6 days after treatment in SN-exposed cultures vs. untreated cultures. The percentage of damaged cells and the yield of SN-induced aberrations at 6 days after treatment increased on average twofold compared with the ones at 18 h after treatment. Moreover, a significant decrease in the frequency of aberrations was observed in SN-exposed cells at 15 days after treatment, resulting in a frequency of aberrations significantly lower than the frequency of aberrations observed in the corresponding control cultures. These data indicate that SN induces delayed chromosomal instability in CHO cells, and that the in vitro clastogenic effect of this compound persists for at least 6 days but less than 15 days after treatment. In addition, we found that SN induces delayed ITSs instability, cytogenetically detectable as additional FISH signals and centromeric breaks involving dissociation of the telomeric signal 6 days after treatment. We propose that the delayed effect of SN on ITSs results from breakage of heterochromatic centromeric ITSs blocks and further insertion of these sequences at the sites of mono- or isochromatid breaks occurring at G2 or G1-S phases of the cell cycle, respectively, since most of the additional FISH signals were present as single or double dots, and located at interstitial sites of the involved chromosomes.

1. Introduction

Experimental evidence indicates that chromosomal regions containing the so-called interstitial telomeric repeats or interstitial telomeric sequences (ITSs) are prone to spontaneous and mutagen-induced chromosome breakage, fragility and recombination (see [1–5] 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). Studies on the role of ITSs on the long-term clastogenic effect of ionizing radiation [6–10] 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 [11].

Since several chemical mutagens are usually employed as anti-neoplastic 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 [12–15], data on the role of ITSs in this instability are very scarce. The only data available to date on the involvement of ITSs on the chromosomal aberrations induced by chemical mutagens come from a recent study performed in our laboratory in which the relationship between ITSs and the chromosomal instability (in terms of unstable aberrations) in the progeny of CHO cells exposed to the radiomimetic compound bleomycin was investigated [16]. We found that bleomycin induces a persistent but not delayed clastogenic effect in CHO cells, and that the in vitro clastogenic effect of this antibiotic on CHO cells persists for at least 6 days but less than 15 days after exposure. In addition, we found that bleomycin induces ITSs instability, cytogenetically detectable

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as acentric fragments (18 h after treatment) or additional (new) FISH signals (6 days after treatment). We proposed that the delayed effect of bleomycin 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.

In order to gain further insight into the involvement of ITSs in the long-term clastogenicity of chemical mutagens, in the present work we analyzed the induction of chromosomal aberrations involving ITSs in the progeny of Chinese hamster ovary (CHO) cells exposed to the antibiotic streptonigrin (SN). Despite of being considered a radiomimetic compound, SN is an antibiotic capable of producing chromosome damage both by S-independent and S-dependent mechanisms [17]. In effect, like S-independent clastogens, SN produces chromatid aberrations in the G2-phase and in replicated S-phase chromatin, and chromosome-type aberrations in the G1-phase and in unreplicated S-phase chromatin. However, like S-dependent agents, SN also produces chromatid-type aberrations in the G1-phase, is an efficient inducer of SCEs, and has a delayed clastogenic effect [17–19].

To accomplish our goal, we exposed CHO cells to a single pulse of SN and determined the frequency of unstable chromosomal aberrations at three times after treatment, by using PNA-FISH with a pan-telomeric probe. 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 [20–23]. 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 [9]. Our results show that the in vitro clastogenic effect of SN on CHO cells persists for at least 6 days after exposure and that SN induces delayed instability of ITSs, cytogenetically detectable as additional FISH signals and centromeric breaks involving dissociation of the FISH signal.

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 [21–23]. 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 20 min pulse of 100 ng/ml of SN (Sigma, CAS No. 3930-19-6, dissolved in sterile Hank's balanced salt solution). Control cultures were set up in parallel but not exposed to SN. Time of exposure and concentration of SN were chosen according to previous studies carried out in our laboratory with CHO cells exposed to this compound [23,24]. At the end of the pulse treatment with SN, 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 7 passages or subcultures (15 days) 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, and 6 and 15 days after the end of treatments. These recovery times were selected taking into account our previous experience with another radiomimetic agent, bleomycin [16]. 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. Cultures were set up in duplicate.

2.2. Fluorescence in situ hybridization with the PNA pantelomeric 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 [16,22,23]. Briefly, after pretreatment 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) [16,21–23]. 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 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 SN-treated cells. We 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 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 [25]. 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 [16,21–23], 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 [21].

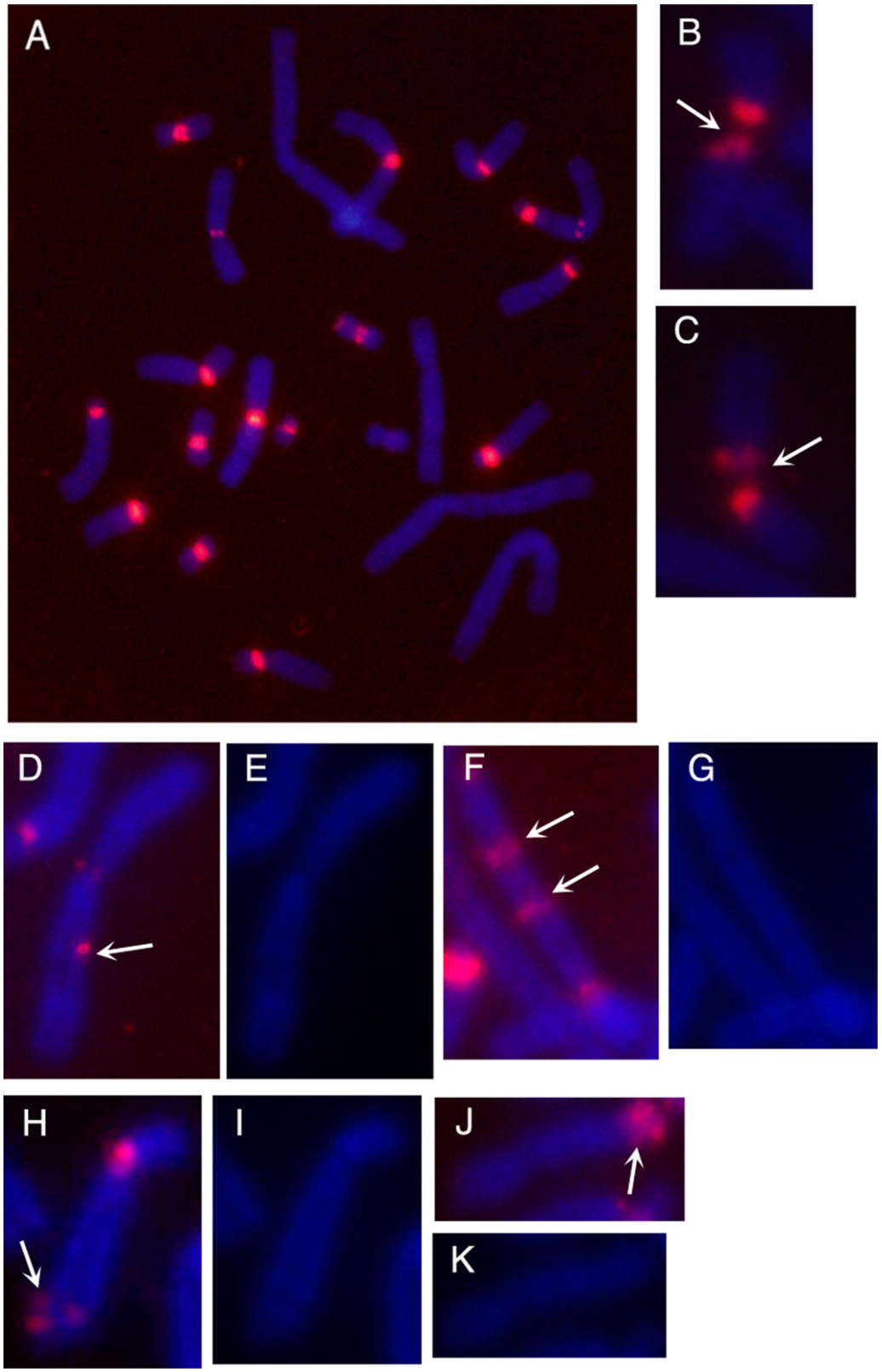

Fig. 1. Patterns of hybridization of (TTAGGG)*n* repeats in the metaphase spreads from untreated and SN-treated CHO cells. (A) Normal metaphase from untreated culture. Note the presence of telomeric signals at the centromeric regions of most of the chromosomes, one interstitial signal in the long arm of a large submetacentric chromosome, and terminal signals at one of the ends of a small metacentric chromosome; (B–K): Chromosomes from metaphase cells derived from a cell culture harvested 6 days after SN treatment; (B–C) chromosomes exhibiting a centromeric break involving dissociation of the telomeric FISH signal (DAPI + Cy3 images; arrows indicate the site of breakage); (D–K) chromosomes with (F–G and H–I) or without (D–E and J–K) telomeric signals at the centromere showing additional interstitial telomeric signals (indicated by arrows) (D, F, H and J: DAPI + Cy3 images; E, G, I and K: DAPI images). Chromosomes with one (D), two (F; in this case, the chromosome exhibits two pairs of double signals), three (H) and more than three (J) additional telomeric FISH signals are shown.

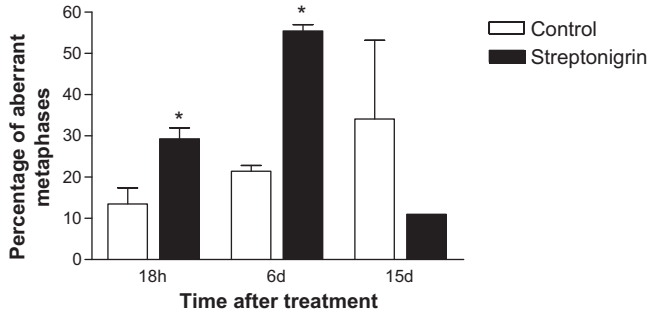

Fig. 2. Percentage of aberrant metaphases (i.e., metaphases containing at least one chromosome aberration) in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with SN (100 ng/ml). For each treatment, mean ± S.E. is indicated, except for SN-exposed cells at 15 days after treatment, in which only one cell culture was analyzed. Chi-squared test indicated significant differences (*) between control and exposed cultures at 18 h and 6 days after treatment ($p < 0.05$).

3.2. Persistence of chromosome damage induced by SN

As expected from previous reports [21,23,24], SN induced a significant increase in the percentage of aberrant cells (i.e., metaphases 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 SN with regard to the percentage of damaged cells and the frequency of aberrations was also observed in cell cultures 6 days after treatment, and these values were significantly higher than the ones at 18 h after treatment ($p < 0.05$) (Figs. 2 and 3). However, both the percentage of damaged cells and the frequency of aberrations decreased dramatically at 15 days after treatment (Figs. 2 and 3) There was on average a twofold increase in the percentage of damaged cells and in the frequency of SN-induced aberrations 6 days after treatment compared with 18 h after treatment (Figs. 2 and 3). As can be noted in Table 1, this increase was due to the increase in the frequency of breaks. Notably, both the percentage of damaged cells and the frequency of aberrations in SN-exposed cells markedly decreased 15 days after treatment (Figs. 2 and 3). Moreover, we observed an increase in both the frequency of aberrations and the percentage of damaged cells in control cells with time in culture, which indicates that the CHO cell line exhibits spontaneous chromosomal instability (Figs. 2 and 3).

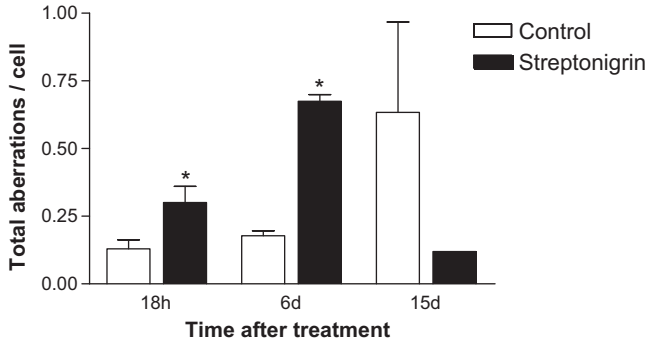

Fig. 3. Changes in the frequency of chromosome-type aberrations (dicentrics, rings, acentric fragments, breaks and chromatid exchanges) in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with SN (100 ng/ml). For each treatment, mean ± S.E. is indicated, except for SN-exposed cells at 15 days after treatment, in which only one cell culture was analyzed. The *Z* score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by SN at 18 h and 6 days after treatment compared with untreated (control) cultures ($p < 0.05$).

Table 1
Chromosome damage observed at various subculture times in untreated and SN-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%)
SN 100 ng/ml	18 h (S1)	184	4 (3)	16* (5)	33* (1)	2 (2)	56*,† (0.30)*	11 (0.06)/(20%)
SN 100 ng/ml	6 d (S3)	184	1 (1)	19 (7)	77* (0)	27 (23)	124* ((0.67)*	31 (0.17)/(25%)
SN 100 ng/ml	15 d (S7)	91	1 (0)	1 (1)	3 (0)	6 (4)	11 (0.12)	5 (0.05)/(42%)

The total number of aberrations and the number (between brackets) of aberrations exhibiting one or more FISH signals are indicated. 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.

* 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).

† Chromatid exchange (without FISH signal). For each treatment, results from two replicated cultures were pooled, except in the case of SN 100 ng/ml at 15 days after treatment (one of the cultures was contaminated).

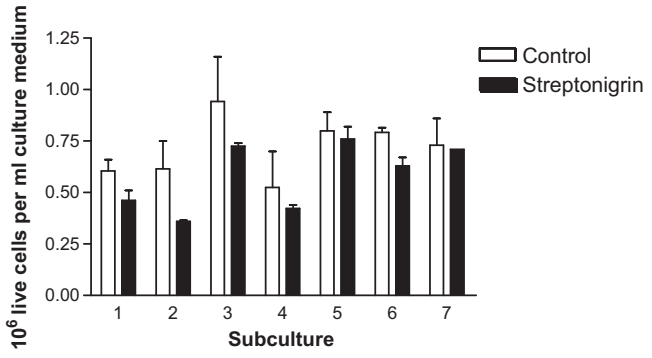

Fig. 4. Variation in the number of live cells observed in CHO cells with time in culture after treatment with SN (100 ng/ml). For each treatment, mean ± S.E. is indicated, except for SN-exposed cells at 15 days after treatment, in which only one cell culture was analyzed.

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. SN-treated cultures for each subculture analyzed (Fig. 4). Thus, there was no significant effect of SN on cell survival, and no SN-induced delayed cell death was observed in CHO cells.

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

Table 1 shows that both in control and SN-treated cells, chromosomal aberrations exhibiting telomeric signals represent between 20 and 60% of the total aberrations observed. These percentages are higher than expected, taking into account the percentage of the CHO karyotype occupied by telomeric sequences (i.e., 3% on average) [21]. At 18 h and 6 days after treatment, the percentage of aberrations with telomeric signals was higher in control than in SN-treated cells, but at 15 days after treatment, the percentage of aberrations with telomeric signals was very similar in both control and SN-exposed cells.

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 SN induces ITSs instability in CHO cells, evidenced as new (de novo) telomeric signals and centromeric breaks involving dissociation of the FISH signal 6 days after treatment (Table 2 and Fig. 5; see Fig. 1B and C for examples of chromosomes with centromeric breaks, and Fig. 1D–K 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 (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 strongly suggests amplification and/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 located at one chromatid of one chromosome arm and exhibited a random distribution, being located in different chromosomes. 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. 1E, G, I, and K).

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

Treatment	Time after treatment (passage)	No. of cells analyzed	Additional (new) FISH signals -/·/··/>...	Breaks at centromeric regions	Acentric fragments	Breaks	Total aberrations (frequency/cell)	ITSs involvement
Control	18 h (S1)	202	1/7/0/0 (8)	2	2	1	13 (0.06)	34/13 (38%)
Control	6 d (S3)	192	9/3/0/0 (12)	7	5	0	24 (0.12)	46/24 (52%)
Control	15 d (S7)	194	1/1/0/0 (2)	6	8	3	19 (0.10)	123/19 (15%)
SN 100 ng/ml	18 h (S1)	184	7/6/0/0 (13)	0	5	1	19 (0.10)	69/19 (28%)
SN 100 ng/ml	6 d (S3)	184	7/14/1/2 (24)*	15*	7	0	46 (0.25)*	148/46 (31%)
SN 100 ng/ml	15 d (S7)	91	0/4/0/0 (4)	1	1	0	6 (0.07)	15/6 (40%)

For each treatment, the number of aberrations exhibiting FISH signal are indicated. Additional FISH signals: Number of new telomeric FISH signals observed as single (·), double (··), triple (···); or more than three (>···) 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). For each treatment, results from two replicated cultures were pooled, except in the case of SN 100 ng/ml at 15 days after treatment (one of the cultures was contaminated).

* 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. 5).

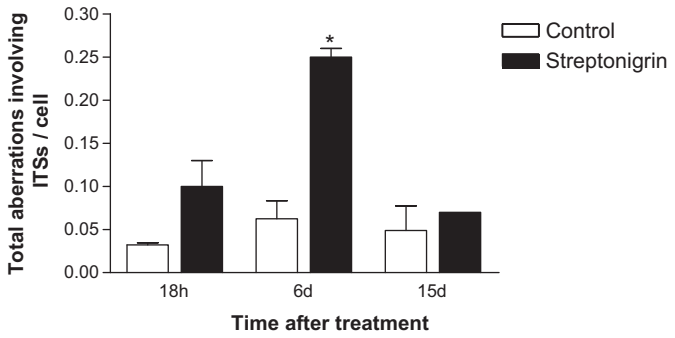

Fig. 5. Changes in the frequency of chromosomal aberrations directly involving ITSs (i.e., ITSs instability; see also Table 2) in continually subcultured (proliferating) CHO cells as a function of time (days) after treatment with SN (100 ng/ml). For each treatment, mean ± S.E. is indicated, except for SN-exposed cells at 15 days after treatment, in which only one cell culture was analyzed. The *Z* score of Poisson distribution indicated significant increase (*) in the frequency of chromosomal aberrations induced by SN at 6 days after treatment compared with untreated (control) cultures ($p < 0.05$).

The observed instability of ITSs 6 days after treatment was about two times higher in SN-treated than in control cells. In addition, we found that ITSs involvement in the aberrations induced by SN is lower than in control cells 18 h and 6 days after treatment, but higher than in control cells at 15 days after treatment (Table 2). Overall, data from Tables 1 and 2 show that SN does not induce an increase in the involvement of ITSs in the chromosomal aberrations, but significantly increases the frequency of aberrations directly involving ITSs at 6 days after treatment (Table 2) compared with control (unexposed) cells.

4. Discussion

Telomere PNA-FISH analysis of CHO cells revealed a higher frequency of chromosomal aberrations 18 h and 6 days after treatment in SN-exposed cells vs. untreated cells. Moreover, the yield of SN-induced aberrations increased on average two times 6 days after treatment compared with the one induced 18 h after treatment. In particular, there was a significant increase in the yield of chromosome breaks with time in culture, and a delayed induction of breaks at centromeric regions of CHO chromosomes 6 days after SN treatment. These findings show that the in vitro clastogenic effect of SN persists for at least 6 days after exposure and that this compound induces delayed chromosomal instability in CHO cells. These observations are in good agreement with previous data reported by Testoni et al. [18,19] indicating that SN has a persistent clastogenic effect on CHO cells that lasts for at least three cell cycles after the end of treatment. On the contrary, we previously found that bleomycin, another radiomimetic drug, induces a persistent but not delayed clastogenic effect in CHO cells, since the yield of aberrations decreases dramatically 6 days after treatment [16]. These discrepancies could be due to differences between SN and bleomycin in their mode of action [17,22,23,26,27]. The persistence of the clastogenic action of SN may be due to the formation of a stable complex between SN and the DNA molecule, which may induce chromosome damage through a persistent cyclic redox process and the resulting generation of active oxygen species, as suggested by Testoni et al. [19].

In addition, a significant decrease in the frequency of aberrations was observed in SN-exposed CHO cells at 15 days after treatment, the yield of aberrations being lower than in control cells. This may be due to the induction of DNA repair mechanisms by the antibiotic itself. This assumption is supported by the finding of Testoni et al. [19], who found that a pulse treatment of CHO cells with SN elicited a triphasic response characterized by repair–damage–repair of DNA. Thus, the low level of aberrations found at 15 days after treatment could correspond to a phase of delayed DNA repair induced by SN. Alternatively, the unstable nature of the aberrations induced could be the cause for the low rate of aberrations observed in SN-treated cells 15 days after treatment. Since we found that SN did not impair cell survival, the possibility that the absence of a clastogenic effect by SN at 15 days after treatment may be due to the death of heavily damaged cells seems unlikely. The increase in the frequency of chromosomal aberrations in control cultures seems to be due to spontaneous instability of the CHO cell line and selective advantage for cells with aberrations, but the cause of this instability remains to be determined.

On the other hand, our present data show that SN induces delayed instability of telomeric sequences in CHO cells in the form of centromeric breaks involving dissociation of the telomeric signal and additional telomeric signals. 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 (at only one end) were always present in those cells possessing additional signals, is valid to assume that the delayed instability of telomeric sequences induced by SN corresponds to ITSs and not to terminal telomeric sequences. We previously found that in first mitosis cells after treatment, most of the chromosome breaks involving telomeric repeats induced by SN in CHO cells were located outside the centromeric regions rich in ITSs, and observed no induction by SN of centromeric breaks involving dissociation of the telomeric signal 18 h after treatment, even with a concentration of SN higher than the one employed in the present study [23]. Therefore, the induction of centromeric breaks involving dissociation of the telomeric signal seems to be a delayed effect of SN. Our present findings partially agree with our previous observations showing a delayed induction of ITSs instability in bleomycin-exposed CHO cells [16], since bleomycin also induced the appearance of additional telomeric signals but not of centromeric breaks involving dissociation of the telomeric signal 6 days after treatment. Thus, delayed induction of ITSs instability in the form of additional telomeric signals seems to be a common feature of the long-term clastogenic action of radiomimetic compounds.

An increase in the number of telomere FISH signals may be the result of amplification and/or translocation of telomeric repeats [3–5]. It is well-known that ITSs, especially those ones located at centromeric regions of Chinese hamster chromosomes, are prone to breakage, fragility and recombination (see [1–5] for review). 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 [9,28,29]. In our present investigation, we found that the majority of the additional signals present in the chromosomes of SN-exposed cells 6 days after treatment were single or double dots distributed at random (i.e., signals were located in different chromosomes and different chromosome arms) and that SN produces a delayed induction of centromeric breaks exhibiting dissociation of the FISH signal 6 days after treatment. We previously found that SN induces breakage at chromosomal sites containing ITSs and also induces translocation and amplification of ITSs [23]. Taking into account the above reports and our present results, we propose that the delayed effect of SN on ITSs results from breakage of centromeric ITSs and further insertion of these sequences at the sites of chromatid or chromosome breaks occurring at late-S/G2 (single signals) or G1/early S (double signals) phases of the cell cycle, respectively. Our previous finding that SN is a good inducer of mono- and isochromatid breaks in CHO cells [18,19,23] also supports the above assumption. Alternatively, the new telomeric FISH signals present in SN-exposed CHO cells could be the result

of telomere capture events [29] or the "healing" by telomerase [9] of mono- or isochromatid breaks induced by SN. Telomere capture is a telomerase-independent process which involves the addition of telomeres at the site of DSB by subtelomeric cryptic translocations [4]. Since the chromosomes with terminal signals were always present in those cells possessing additional signals, it seems that the telomeric sequences instability observed in SN-exposed cells cannot be the result of telomere capture events. We may assume that telomerase is not involved in the delayed instability of ITSs induced by SN, since telomerase products are usually below the resolution of FISH [29], and previous work in our laboratory showed that this antibiotic did not induce telomerase activity in CHO cells 18 h after treatment [21].

In summary, our present findings confirm that SN has a persistent clastogenic action on mammalian cells, show that the in vitro clastogenic effect of SN on CHO chromosomes increases with time in culture and persists for at least 6 days after treatment, and that this compound promotes delayed instability of ITSs in CHO cells in the form of additional telomeric signals and breaks at centromeric regions rich in ITSs. The reason why the observed delayed ITSs instability in SN-exposed cells no longer exists in CHO cells 15 days after treatment remains to be established, but taking into account previous reports [19] it can be speculated that this could be due to a stimulation of the DNA repair machinery by SN. Since 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 [30], it would be interesting to explore the possible role of DNA-PKcs and the DSBs repair pathways in the ITSs instability induced by SN.

Future studies on this area of research should aim to confirm our assumptions regarding the mechanisms involved in the delayed instability of ITSs induced by radiomimetic compounds in CHO cells, 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 bleomycin or SN.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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