Research Articles

Long-Lasting Genomic Instability Following Arsenite Exposure in Mammalian Cells: The Role of Reactive Oxygen Species

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Previously, we reported that the progeny of mammalian cells, which has been exposed to sodium arsenite for two cell cycles, exhibited chromosomal instability and concurrent DNA hypomethylation, when they were subsequently investigated after two months of subculturing (about 120 cell generations) in arsenite-free medium. In this work, we continued our investigations of the long-lasting arsenite-induced genomic instability by analyzing additional endpoints at several time points during the cell expanded growth. In addition to the progressive increase of aneuploid cells, we also noted micronucleated and multinucleated cells that continued to accumulate up to the 50th cell generation, as well as dicentric chromosomes and/or telomeric associations and other complex chromosome rearrangements that began to appear much later, at the 90th cell generation following arsenite exposure. The increasing genomic instability was further characterized by an increased frequency of spontaneous mutations. Furthermore, the long-lasting genomic instability was related to elevated levels of reactive oxygen species (ROS), which at the 50th cell generation appeared higher than in stable parental cells. To gain additional insight into the continuing genomic instability, we examined several individual clones isolated at different time points from the growing cell population. Chromosomally and morphologically unstable cell clones, the number of which increased with the expanded growth, were also present at early phases of growth without arsenite. All genomically unstable clones exhibited higher ROS levels than untreated cells suggesting that oxidative stress is an important factor for the progression of genomic instability induced by arsenite. Environ. Mol. Mutagen. 00:000–000, 2011.

Key words: arsenite; genomic instability; reactive oxygen species

INTRODUCTION

Inorganic arsenic is a well known poison and is considered a carcinogen in humans [IARC, 1980; Rossman, 2003]. Important insights into the mechanisms that promote arsenic carcinogenesis have been provided by investigations on cultured cells exposed acutely or chronically to arsenic. The effects observed immediately after arsenite treatment are consistent with a nonmutagenic mode of action and are suggestive of indirect genotoxic mechanisms including aneuploidy, oxidative stress, and DNA methylation alterations [Klein et al., 2007]. Aneuploidy, regarded as one of the important forces driving carcinogenesis, has been suggested to be responsible for both chromosomal rearrangements and gene mutations [Fabarius et al., 2003, 2008]. DNA hypomethylation influences heterochromatin decondensation and may also promote chromosome instability [Karpinets and Foy, 2005]. In addition, chronic oxidative stress is another mechanism capable of disrupting the genomic integrity of cells [Limoli and Giedzinski, 2003]. Thus, many types of cellular changes can be involved in genomic instability, a major characteristic of tumor cells [Rodriguez et al., 2006; Franco et al., 2008].

Previously, we reported that Chinese hamster V79-Cl3 cells underwent early genetic instability when exposed to a 24-hr treatment with 10 μM sodium arsenite [Sciandrello et al., 2002]. We subsequently demonstrated that descendants of these surviving cells continued to be genetically unstable after about two months of subcultur-

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ing in arsenite-free medium, exhibiting gross aneuploidy and structural chromosome changes linked to DNA hypomethylation [Sciandrello et al., 2004]. To gain additional insight into the perpetuation of genomic instability induced by arsenic, we monitored the growing cell population and isolated several clones at three different time points during the expanded growth in arsenite-free medium. We tested the individual clones for chromosomal and genomic alteration frequencies and also for spontaneous gene mutation frequencies. The results reported here show that, in addition to arising immediately after the arsenite exposure, genomically unstable clones continued to accumulate with extended growth in the absence of continuous arsenite exposure, and that these clones were characterized by elevated mutation frequencies, as well as by levels of oxidative stress that exceeded those of untreated cells.

MATERIALS AND METHODS

Cell Culture and Arsenite Treatment

V79-Cl3 Chinese hamster cells were cleansed of any preexisting 6-thioguanine resistant (6-TG^r) mutants by culturing in HAT medium (10^{-4} M hypoxantine, 3.2×10^{-7} M aminopterin, 10^{-5} M thymidine, Sigma-Aldrich, Milan, Italy) for 10–12 days [Sciandrello et al., 2003]. Cells were mutagenized with a moderately lethal dose of sodium arsenite (cell survival >70%), according to previously reported procedures [Sciandrello et al., 2004]. Briefly, cells in exponential growth were treated with 10 μM sodium arsenite (Sigma-Aldrich) for 24 hr; at the end of the treatment, rounded-up mitotic cells were harvested by shake-off and allowed to grow in arsenite-free medium. These cells were called ASO_2; they had a doubling time of 12–13 hr. During the expanded growth, aliquots of cells taken at the 6th, 50th, and 90th cell generation, called ASO_2-A, ASO_2-B, and ASO_2-C, respectively, were frozen in liquid nitrogen. For cloning, 500 cells from each of ASO_2-A, -B, or -C population were seeded in three 100-mm cell culture dishes. The cells were incubated for seven days, and then three colonies were randomly picked from each dish, propagated and then stored in liquid nitrogen. All cells were routinely subcultured every three days in D-MEM, supplemented with 5% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All chemicals were supplied by Invitrogen Corp., Italy unless otherwise specified. Cultures were maintained at 37°C in a 5% CO_2 humidified incubator.

Morphological and Cytogenetic Analysis

The exponentially growing ASO_2 cell population was examined weekly to detect nuclear abnormalities (micronuclei and multinucleated cells) and to assess chromosome number, according to the previously described protocols [Sciandrello et al., 2004]. Morphological analysis was performed on at least 1000 ASO_2 cells growing on glass cover-slips and on each isolated clone. Cytogenetic analyses were performed on at least 200 metaphases of each culture. Untreated V79-Cl3 cells were also examined.

Measurement of Reactive Oxygen Species by Flow Cytometry

Intracellular reactive oxygen species (ROS) levels were assessed in untreated parental V79-Cl3 cells, in arsenite-treated V79-Cl3 cells, in ASO_2-A, -B and -C cell populations and in clones isolated by flow cytometry using 5-(and-6)-chloromethyl-2′7′-dichlorodihydrofluorescein diacetate acetyl ester (CM-H_2DCFDA, Molecular Probes Inc., Eugene, OR) as a probe. The CM-H_2DCFDA penetrated into cells and was hydrolyzed by cellular esterase to 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCFH), which was further oxidized by intracellular ROS into a strong fluorescent compound, dichlorofluorescein, that could be dectected by Fluorescence Activated Cell Sorting (FACS). Exponentially growing cells were incubated for 1 hr with 5 μM CM-H_2DCFDA. After washing in Hank's Balanced Salt Solution (HBSS), the cells were harvested by trypsinization, resuspended at 1×10^6 cells/ml in HBSS and immediately subjected to FACScan flow cytometer (Beckman Coulter Inc., Fullerton, CA) with excitation and emission settings of 488 and 530 nm, respectively.

Mutation Assay at *Hprt* Locus

The *Hprt* mutation assay was performed on ASO_2-A, -B, -C cells and on their derived clones, as well as on parental V79-Cl3 cells to determine the spontaneous mutation frequency. Cells (5×10^5) were plated into each of sixteen 100-mm dishes in 10 ml of medium containing 30 μM 6-thioguanine. Concurrently, 300 cells were inoculated into each of three 60-mm dishes in nonselective medium to determine plating efficiency. The cells were incubated for 10 days, and were then fixed and stained with 0.1% methylene blue. Mutant frequencies were expressed as the number of mutants per 10^6 cells corrected for the plating efficiency.

Statistical Analysis

Nuclear abnormality and mutation frequencies and ROS levels in ASO_2-A, -B, -C cells, and their derived clones, were compared with those observed in untreated parent cells using the χ^2 test; $P < 0.05$ was considered significant.

The association between ROS levels and instability marker frequencies was determined on ASO_2-A, -B, -C cells and on their derived clones using the Pearson product moment correlation indexes and corresponding test. The test was performed using SPSS 13.0 software (Chicago, USA), with the level of significance set at $P < 0.01$.

RESULTS

Morphological and Cytogenetic Analysis

Morphological and cytogenetic analyses were performed weekly for up to 120 cell generations (about 8 weeks) on ASO_2 cells exposed to 24-hr arsenite treatment followed by growth in drug-free medium. The frequency of unstable (micro- and multinucleated) ASO_2 cells, which was 8% immediately after the end of the brief arsenite treatment, declined to 3.5% by the 6th cell generation and continued to decrease eventually attaining the same level of nuclear stability as untreated V79-C13 cells (1.4%) by the 40th generation. Thereafter, the frequency of unstable ASO_2 cells reversed, rapidly increased and remained consistently high from the 50th cell generation onward (Fig. 1a). A similar pattern of cytogenetic stability was also observed in the ASO_2 cells. At the end of the 24-hr arsenite treatment, the frequency of unstable ASO_2 cells with deviations from the modal number of 21 chromosomes, was initially very high (80%) and decreased to 47% as the cells became more stable during the expanded

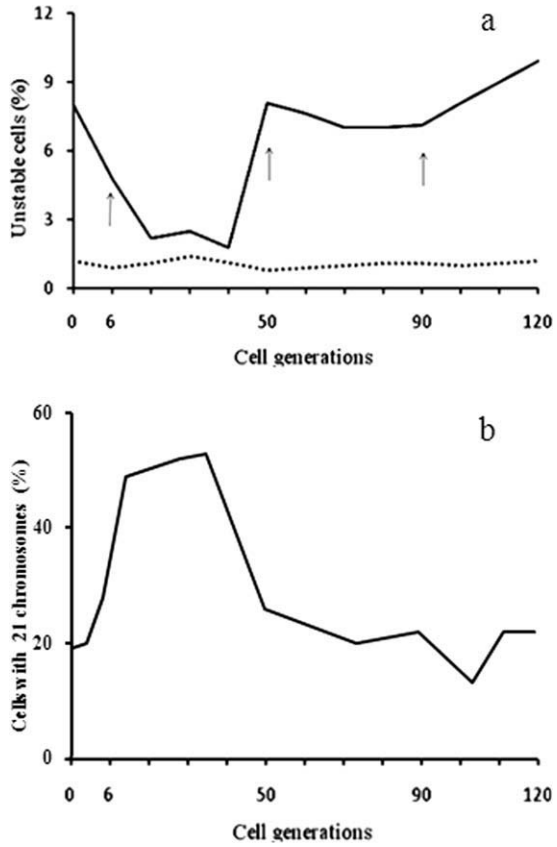

Fig. 1. Genomic instability in growing ASO_2. (a) Frequency of ASO_2 (—) and V79-Cl3 cells (.....) with nuclear abnormalities. Arrows indicate the three representative cell generations ASO_2-A, ASO_2-B, and ASO_2-C. (b) Frequency of ASO_2 cells with 21 chromosomes.

TABLE I. Frequencies of Cytogenetic and Nuclear Abnormalities

	Cells with			
Clones	21 chr (%)	Dicentric chr or tas (%)	Micronucleated cells (%)	Multinucleated cells (%)
V79-Cl3	61.00	0.00	0.80	0.50
ASO_2-A	38.00	0.00	1.75	0.01
-A1	29.00	0.00	1.90	0.01
-A2	29.00	0.00	1.80	0.00
-A3	21.00	0.00	0.29	0.00
-A4	27.00	0.00	0.80	0.00
-A5	19.00	6.00	2.20	0.25
-A6	35.00	0.00	2.10	0.05
-A7	49.00	1.00	2.90[a]	0.06
-A8	48.00	0.00	1.90	0.04
-A9	36.00	0.00	2.80	0.09
ASO_2-B	26.00	0.00	5.30[d]	1.00
-B1	14.00	0.00	1.49	0.04
-B2	34.00	2.00	5.00[d]	0.09
-B3	48.00	0.00	1.78	0.05
-B4	17.00	4.00	3.78[c]	0.17
-B5	13.00	4.00	2.87[a]	0.09
-B6	34.00	0.00	1.79	0.74
-B7	37.00	0.00	1.65	0.13
-B8	29.00	2.00	3.78[c]	0.18
-B10	26.00	3.00	3.98[c]	4.76[d]
ASO_2-C	22.00	1.00	3.68[b]	2.54[b]
-C1	5.00	11.00	2.46	3.63[d]
-C2	9.00	10.00	1.27	5.27[d]
-C3	21.00	8.00	2.72	3.21[d]
-C4	17.00	2.00	2.07	2.66[b]
-C5	23.00	5.00	1.39	2.78[b]
-C6	10.00	9.00	4.76[c]	5.42[d]
-C7	20.00	2.00	2.32	0.11
-C8	16.00	10.00	3.78[c]	4.76[d]
-C9	29.00	1.00	2.87[a]	3.03[c]

chr, chromosomes; tas, telomeric associations.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P << 0.001$, according to the χ^2test.

growth up to the 40th generation. Thereafter, similar to the nuclear instability noted above, cytogenetic instability rapidly increased (i.e., the number of cells with modal 21 chromosomes decreased) and the cytogenetic instability remained elevated for up to 120 cell generations in the absence of any further arsenite exposure (Fig. 1b). Chromosomal rearrangements including dicentric chromosomes and/or telomeric associations, appeared at the 90th cell generation post arsenite exposure.

The same indicators of genome instability as noted above were also measured for nine clones isolated from each of three representative post-arsenic exposure cell populations, namely population ASO_2-A (6th cell generation) and A series clones, population ASO_2-B (50th cell generation) and B series clones, and population ASO_2-C (90th cell generation) and C series clones. Results reported in Table I show that all three cells populations and derivative cell clones were unstable, exhibiting a lower frequency of cells with a modal number of 21 chromosomes than the 61% observed in untreated V79-C13 cells. The highest levels of instability were observed in the ASO_2-B and ASO_2-C populations and clones, in which the frequency of cells with the modal chromosome number of 21 ranged from 13 to 48% in ASO_2-B series clones and from 5 to 29% in ASO_2-C series clones. Two ASO_2-A clones, five ASO_2-B clones and all nine ASO_2-C clones showed dicentric chromosomes and/or telomeric associations in metaphase (Table I), as well as nucleoplasmic bridges in interphase (Fig. 2a). Furthermore, some of ASO_2-C clones exhibited complex chromosomal aberrations, namely radial configurations (Fig. 2b). Most of the clones derived from ASO_2-A, ASO_2-B, and ASO_2-C cells (7/9 for ASO_2-A, and ASO_2-C; 8/9 for ASO_2-B) had a percentage of micronucleated cells that was >2-fold that observed in untreated V79-Cl3 cells (Table I). The presence of multinucleated cells was a less effective indicator of instability in the ASO_2-A, -B, and -C populations and clones, deviating substantially (>2-fold) from the untreated V79-C13 cells primarily in the 90th cell generation population and in the series C clones.

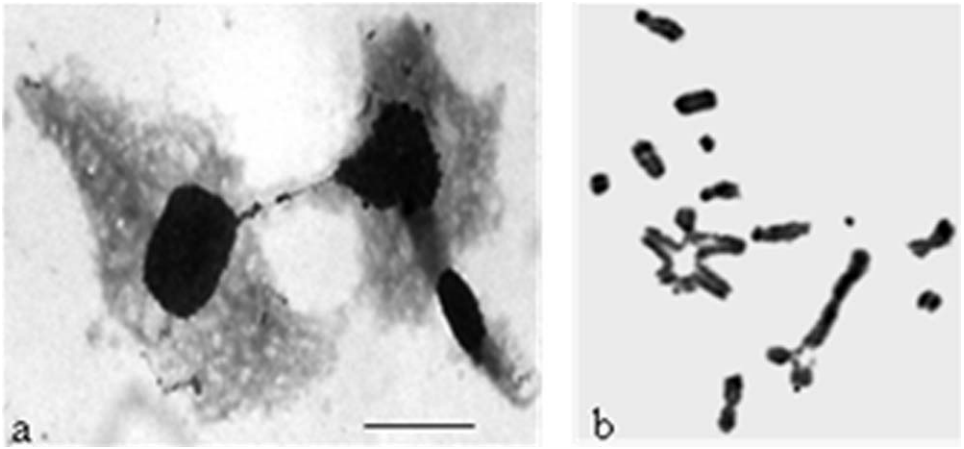

Fig. 2. Nucleoplasmic bridge (a) and radial chromosome configurations (b) in ASO_2-C cells. Bar represents 10 μm.

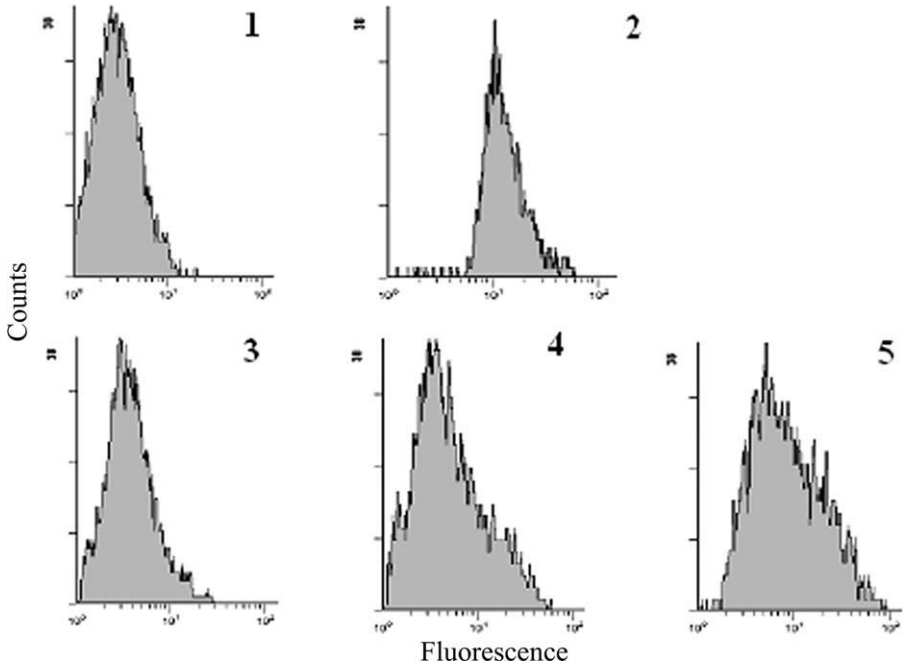

Fig. 3. Fluorescence-activated cell sorting (FACS) histograms showing oxidation of CM-H_2DCFDA in: (1) untreated V79-Cl3 cells; (2) 10 μM arsenite-treated V79-Cl3 cells; (3) ASO_2-A; (4) ASO_2-B; (5) ASO_2-C cell populations.

ROS Levels

We assessed ROS levels in untreated V79-Cl3 cells, in arsenite-treated V79-Cl3, in ASO_2 cell populations at the 6th, 50th, and 90th cell generations post arsenite exposure, and in isolated A, B, and C series clones, by measuring the oxidation product of CM-H2DCFDA, dichlorofluorescein, by flow cytometry. Compared with the FACS histogram for untreated parental cells, the histogram for V79-Cl3 cells examined immediately after 24-hr arsenite treatment showed a rightward shift, indicating a more extensive oxidation of the dye (11.8-fold increase in fluorescence) (Fig. 3, 1-2); thus confirming arsenic's direct capacity of inducing ROS. In comparison with the FACS histogram for untreated parental cells, those for the ASO_2-A (6th cell generation population) showed a small shift (1.5-fold increase in fluorescence); for ASO_2-B and ASO_2-C (50th and 90th cell generation populations) a greater shift was evident (2.8-fold and 5.2-fold increase in fluorescence, respectively) (Fig. 3, 3–5). Moreover, the number of individual clones with dichlorofluorescein levels higher than the level in untreated V79-Cl3 cells increased from the 6th to the 90th cell generation (Table II).

Mutation Assay at *Hprt* Locus

A gene mutation assay was employed as another method of assessing genomic instability. Table II reports the frequency of 6-TG^r mutants assessed in untreated V79-Cl3 cells, in ASO_2 cell populations at the 6th, 50th, and 90th cell generation post-arsenite treatment, and in isolated A, B and C series clones. The mutation frequency at the *Hprt* locus was the same in parental V79-Cl3 cells and in ASO_2-A cells at the 6th generation, and declined to lower than untreated cells at the 50th cell generation in ASO_2-B cells. The mutant frequency rose to fourfold higher than untreated cells in ASO_2-C cells at the 90th cell generation post-arsenite exposure. Investigations on the individual clones showed that two of seven ASO_2-A series clones had a mutation frequency $\geq$3-fold more than in V79-Cl3 cells; three of eight ASO_2-B series clones and six of nine ASO_2-C series clones had also a higher mutation frequency ($\geq$2-fold more) compared to untreated V79-C13 cells. Some of the clones in the A, B and C series exhibited 6-TG^r mutant frequencies that were either unmeasurable or lower than that of the parental V79-C13 cells.

TABLE II. Frequency of 6-TG^r Mutants and ROS Levels

Clones	Cloning efficiency	Mut. frequency ($\times 10^{-6}$)	ROS
V79-Cl3	89.0	1.4	4.1
ASO_2-A	74.6	1.4	6.2
-A1	80.0	2.0	9.3[a]
-A2	88.0	1.4	4.1
-A3	32.0	1.3	4.1
-A4	48.0	1.1	4.1
-A5	81.0	0.5	18.4[d]
-A8	86.0	12.4[d]	13.0[d]
-A9	82.4	5.2[c]	11.4[d]
ASO_2-B	73.8	0.2	11.5[d]
-B1	76.0	0.0	n.d.
-B2	60.0	0.0	15.3[d]
-B3	40.5	1.9	4.1
-B4	52.3	0.8	7.4
-B5	43.0	0.0	19.1[d]
-B6	86.0	9.5[d]	19.5[d]
-B8	68.3	5.1[b]	11.5[d]
-B10	56.0	3.7	13.8[d]
ASO_2-C	86.6	5.7[d]	21.3[d]
-C1	70.0	4.0[a]	9.6[b]
-C2	51.0	0.0	7.3
-C3	61.0	0.0	5.4
-C4	46.0	3.6	14.1[d]
-C5	78.9	0.0	6.3
-C6	87.0	7.1[d]	36.3[d]
-C7	61.0	3.8[a]	5.3
-C8	83.7	5.9[d]	18.8[d]
-C9	31.0	3.0	10.0[b]

[a]$P < 0.05$.
[b]$P < 0.01$.
[c]$P < 0.001$.
[d]$P << 0.001$, according to the χ^2test.

The Pearson's correlations between detected ROS levels and the various genomic instability marker frequencies indicated that ROS correlated positively ($r = +0.81$) and significantly ($P < 0.005$) only with the mutation frequency of ASO_2-C cell population and its clones (Table III).

DISCUSSION

In previous studies, we found that genome instability arose after a brief arsenite treatment [Sciandrello et al., 2002] and we further characterized the progeny of arsenite-treated cells that were maintained in arsenite-free medium for many cell generations after the initial 24-hr exposure [Sciandrello et al., 2004]. Here we further analyzed genomic instability at periodic intervals up to 120 cell generations after the brief (24 hr) arsenite treatment to investigate the ongoing process of genomic instability and to determine its persistence in the absence of arsenite. Cell populations were thus monitored weekly for several months. This kind of serial investigations allowed us to determine that arsenic-induced nuclear abnormalities and the marked degree of aneuploidy, evident in early phases of expanded population growth, unexpectedly declined within six cell generations after treatment such that the ASO_2 cell

TABLE III. Correlation Between ROS Levels and Instability Markers in ASO_2 Populations and Their Clones

	Mutation frequency ($\times 10^{-6}$)	Dicentric chrs or tas	Micro-nucleated cells	Multinucleated cells
ROS Pearson's Correlation ASO_2-A	0.338	−0.367	−0.126	0.275
Sig. (2 tailed)	0.413	0.372	0.765	0.509
N	8	8	8	8
ROS Pearson's Correlation ASO_2-B	0.319	0.145	0.65	0.261
Sig. (2 tailed)	0.441	0.732	0.878	0.532
N	8	8	8	8
ROS Pearson's Correlation ASO_2-C	0.809	0.125	−0.389	−0.463
Sig. (2 tailed)	0.005[a]	0.732	0.266	−0.178
N	10	10	10	10

chr, chromosomes; tas, telomeric associations.
[a]Correlation is significant at the 0.01 level.

population apparently regained the growth and cytogenetic phenotype of parent V79-Cl3. Thereafter, at about 50 cell generations the markers of genome instability progressively increased and in later phases (after the 90th cell generation) remained constantly high with the overt presence of chromosome rearrangements.

One of the main causes of genomic instability is thought to be the alteration of oxygen metabolism which can give rise to increased levels of reactive products, such as lipid peroxides, hydroxyl radicals, and hydrogen peroxide that may act as clastogenic factors and thus produce chromosome aberrations and mutations [Emerit, 1994; Valko et al., 2005]. As arsenic is known to produce reactive oxygen species [Liu et al., 2003; Rossman, 2003; De Vizcaya-Ruiz et al., 2009], we focused our study on the possible relationship between ROS production and long-lasting genomic instability by examining three cell populations (ASO_2-A, -B, and -C) collected at periodic intervals during the expanded growth of arsenite-treated cells in the absence of arsenite. Our results demonstrated that ROS levels were very high just after the treatment with arsenite and returned to values similar to those of untreated parent cells within a short period of time after exposure, but progressively increased as the population aged. This trend was comparable to what we also observed for several genome instability markers.

To gain insight into this unusual pattern of genomic instability progression, our investigations were further extended to examine clones isolated from the three representative cell generations by evaluating instability markers, gene mutation frequencies and ROS levels. Unstable clones were already present at the 6th cell generation following arsenite exposure and they were heterogeneous exhibiting nuclear abnormalities and/or dicentric chromosomes sometimes associated with high gene mutation frequency and high ROS levels. Depending on the examined endpoint some clones appeared normal and others were aberrant, but no clone was normal with respect to ploidy. It noteworthy that four out of seven analyzed ASO_2-A clones had high ROS levels (Table II), suggesting that oxidative metabolism alterations had already occurred in some cells at the beginning of cell proliferation following the brief arsenite exposure.

The progressive increase of genome instability was underlined by increasing number of unstable clones isolated from the ASO_2-B and -C cell populations. Interestingly, high ROS levels characterized these same unstable clones.

For all the clones, ROS levels always correlated positively with the mutation frequency but not with other measured parameters, such as chromosome rearrangements or morphological abnormalities. In particular, for ASO_2-C cells and their clones the Pearson's correlations between ROS levels and mutation frequencies were positive and significant. Furthermore, subculturing the clones highlighted the fact that the ASO_2-C clones showed transformed phenotypes, as demonstrated by loss of contact inhibition and acquisition of capability of growing in soft agar (data not shown). Thus, oxidative stress seems to be responsible, in part, for changes in genes involved in the control of cell proliferation.

In conclusion, our investigations showed that genomic instability arises in a few cells capable of sustaining the damage induced by arsenite treatment which results in severe aneuploidy, chromosomal and nuclear abnormalities, gene mutations and ROS production. These unstable cells, after a latent period in which they accumulate further changes possibly due to epigenetic factors, namely changes in methylation levels [Sciandrello et al., 2004], and to gene mutations induced by the high ROS levels, acquire selective advantage and can proliferate, fostering perpetuated genomic instability. In this scenario, cells that are the distant progeny of the cells exposed to arsenic may exhibit memory of genome changes that can lead to later exacerbation of the transformed phenotype.

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