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Relationship between Aberration Yield and Mitotic Delay in Human Lymphocytes Exposed to 200 MeV/u Fe-ions or X-rays

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Human lymphocytes / Heavy ions / Time-course of aberrations / Calyculin A / Apoptosis

The time-course of Fe-ion (200 MeV/u, 440 keV/μm) and X-ray induced chromosomal damage was investigated in human lymphocytes. After cells were exposed in G_0 and stimulated to grow, aberrations were measured in first-cycle metaphases harvested 48, 60 and 72h post-irradiation. Additionally, lesions were analysed in G_2 and mitotic (M) cells collected at 48h using calyculin A-induced premature chromosome condensation (PCC). Following X-irradiation, similar aberration yields were found in all of the samples scored. In contrast, after Fe-ion exposure a drastic increase in the aberration frequency with sampling time was observed, i.e. cells arriving late at the first mitosis carried more aberrations than those arriving at earlier times. The PCC data indicate that the delayed entry of heavily damaged cells into mitosis observed after Fe-ion irradiation resulted from a prolonged arrest in G_2. Altogether these experiments provide further evidence that in the case of high-LET exposure cell-cycle delays of severely damaged cells have to be taken into account for any meaningful quantification of chromosomal damage and, consequently, for an accurate estimate of the RBE.

INTRODUCTION

Chromosome aberrations are regarded as being the most sensitive indicator of radiation-induced genetic alterations. In particular, the yields of aberrations in peripheral blood lymphocytes are used to estimate the dose to which an individual has been exposed (see[1,2] and references therein). According to the standard protocol, cytogenetic damage is analysed in first-cycle metaphases collected at one sampling time post-irradiation. When aberrations are investigated in human lymphocytes, metaphases are routinely harvested 48h after the initiation of the culture. In recent years, however, it has become increasingly apparent that high-LET radiation induces pronounced cell-cycle pertubations and mitotic delays, which interfere with the expression of aberrations[3–10]. For example, after exposure to particles with LET values above 100 keV/μm, a drastic increase in chromosomal damage with sampling time was observed, i.e. heavily damaged cells have been found to enter mitosis later than undamaged cells[3,5–8,10]. In contrast, following sparsely ionizing radiation, which has a less pronounced effect on the cell-cycle transition, only a slight rise in the aberration frequency with time, or a stable aberration yield, was detected[3,5,6,8].

This difference in the time-course of high and low-LET induced chromosomal damage in metaphase cells complicates the determination of meaningful RBE values. In particular, when aberration yields are measured at only one early sampling time post-irradiation, the true frequency of heavy ion-produced cytogenetic damage would be underestimated[3,10].

More reliable RBEs will be obtained when calculations are based on the total amount of damage induced within the initial cell population. This can be achieved by using multiple sampling times along with a mathematical approach[11]. Alternatively, the analysis of chromosomal damage by means of calyculin A-induced premature chromosome condensation is proposed[2]. Calyculin A condenses chromo-

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somes mostly in the G_2-phase, i.e. the method allows one to score chromosomal damage in cells which suffer a prolonged G_2-arrest, and thus escape conventional metaphase analysis at a single early sampling time[8,9].

In the present study, the expression of 200 MeV/u Fe-ion and X-ray induced chromosomal damage was investiagted in human lymphocytes. The aberration yields were determined in metaphases collected at 2 or 3 sampling times, and compared to the yields in G_2 and mitotic cells harvested at 48h by using calyculin A. In addition, radiation-induced cell death was measured.

MATERIALS AND METHODS

Lymphocytes isolated from the whole blood of a 40 years-old healthy female (non-smoker) were irradiated at room temperature with 200 MeV/u Fe-ions (LET: 440 keV/μm) or X-rays. Particle exposure was performed at HIMAC (Chiba, Japan) in polyethylene holders with a 1 mm thick well for the sample and 2 mm polyethylene between the cells and the beam, while X-ray exposure (200 kV, 20 mA, 0.5 mm Al and 0.5 mm Cu filtering) was done in 25 cm^2 flasks. Immediately after irradiation, the cells were seeded at a density of 3×10^5/ml in RPMI medium containing 5 μg/ml 5-bromo-2'-deoxyuridine and 1% phytohaemagglutinin, as described in detail by Durante et. al.[2].

Chromosomal damage was determined in metaphase cells collected at 48, 60 and 72h post-irradiation after a 3h colcemid treatment (400 ng/ml). Chromosomes were prepared according to standard techniques, stained with the Fluorescence-plus-Giemsa method[3,12] and for each dose and sampling time the yields of dicentrics, acentric and centric rings, interstitial deletions and chromosome- and chromatid-type breaks were determined in 100 first-cycle metaphases. Furthermore, aberrations were analysed in PCC-spreads obtained at 48h by the use of calyculin A[2]. Slides were stained with Giemsa, and aberrations were separately counted in G_2- and M-cells. Under the applied experimental conditions, G_2-cells could be discriminated from M-cells due to the lack of a visible centromere. The analysis of aberrations in PCC-samples was restricted to the number of chromosome fragments in excess of 46. The errors on the proportions of aberrant cells and the aberrations per cell were calculated by $\sqrt{A}$, where A is the frequency under consideration. In addition, at 1,14, 24, 48, 60, 72 and 96 h post-irradiation the number of apoptotic/necrotic cells was measured by dual-fluorescence staining with Hoechst 33342 and propidium iodide[13] and by trypan-blue exclusion[13]. For both assays, at least 500 cells were scored per dose and time-point.

RESULTS AND DISCUSSION

The analysis of lymphocytes reaching the first post-irradiation division revealed a strong influence of densely ionizing radiation on the time-course of chromosomal damage in mitosis. After Fe-ion exposure, the number of aberrant first-cycle cells increased more than twofold with sampling time and the frequency of aberrations per cell more than sevenfold (Fig. 1). In contrast, after X-irradiation stable yields of aberrant cells or aberrations per cell were found (Fig. 2). Similar differences in the time-course of high and low-LET induced chromosomal damage were recently observed in studies using human lymphocytes[6,8] or different

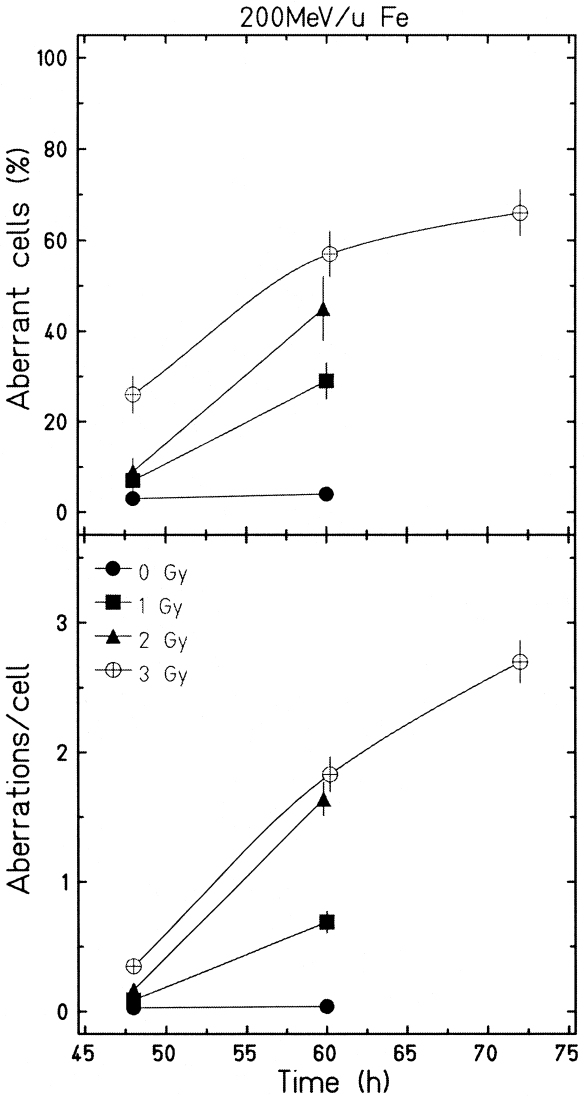

Fig. 1. Time-course of chromosomal damage in first-cycle metaphases after irradiation with 200 MeV/u Fe ions (conventional metaphase analysis).

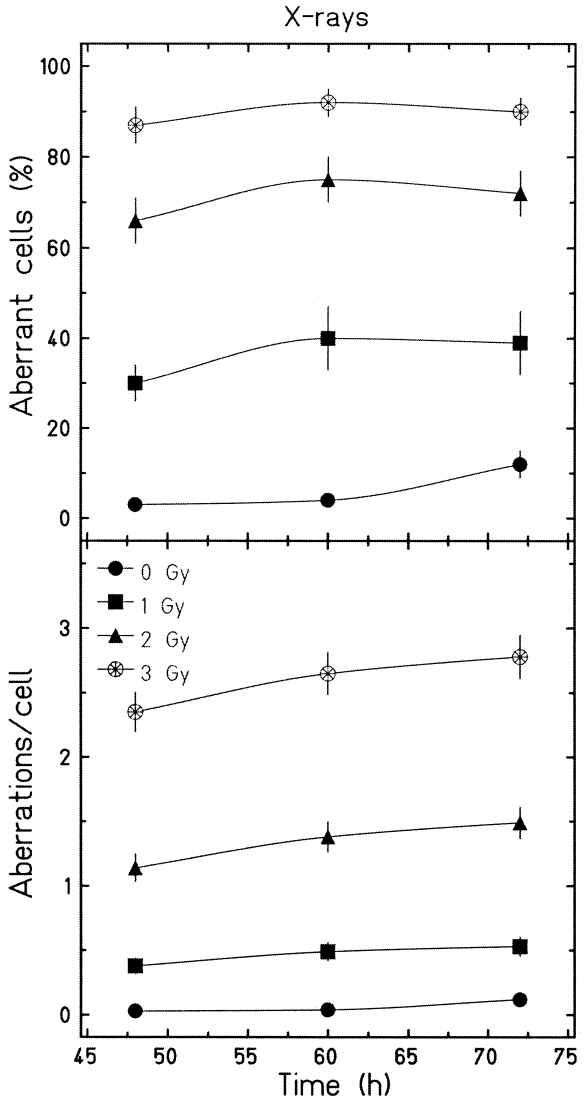

Fig. 2. Time-course of chromosomal damage in first-cycle metaphases after X- irradiation (conventional metaphase analysis).

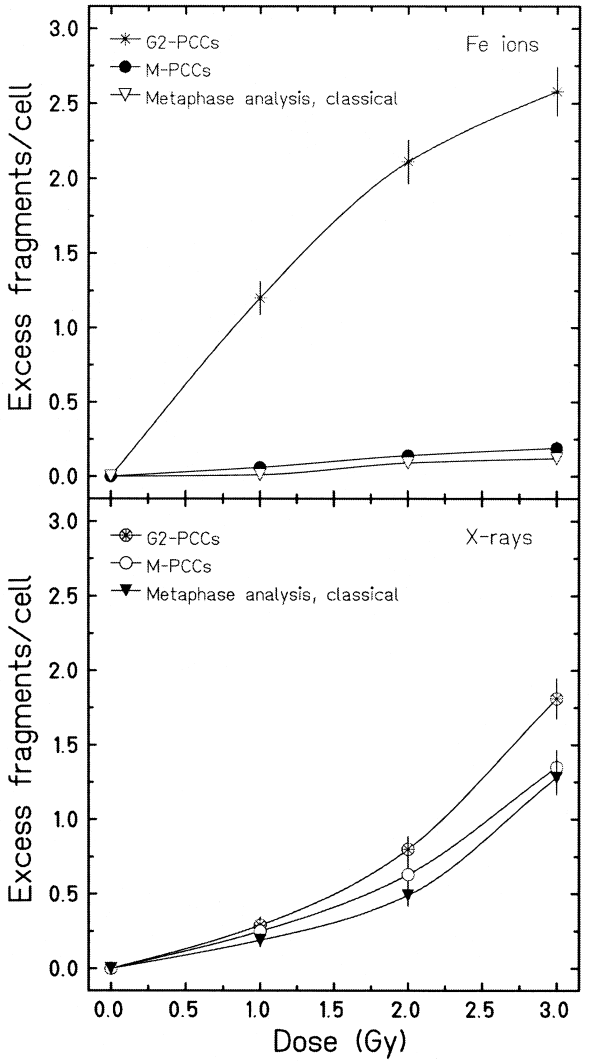

Fig. 3. Aberration yields in G_2-cells (G_2-PCCs) and M-cells (M-PCCs) 48h after irradiation with 200 MeV/u Fe ions (upper panel) or X-rays (lower panel). Premature chromosome condensation was induced by means of calyculin A. For comparison, the number of excess fragments found in conventional metaphase preparations at 48h is plotted (metaphases analysis, classical).

Chinese hamster cell lines[3,5,7,10].

The non-uniform expression of high-LET induced chromosomal damage can be related to the stochastics of particle exposure (for a more detailed discussion see[3]). For example, for human lymphocytes with a nuclear cross section of about 25 μm^2 as determined by confocal microscopy[6], exposure to 2 Gy Fe ions (3×10^6 particles/cm^2) results in a mean number of 0.8 direct particle hits per cell nucleus. From Poisson statistics, it follows that under these irradiation conditions 45% of all cell nuclei receive no direct hit, 36% are hit once, 14% are hit twice and 5% are hit three times or more. Consequently, cells with quite different numbers of aberrations and cell cycle transition times are produced within the same cell population. In contrast, when cells are exposed to sparsely ionizing radiation, the energy is fairly uniformly deposited leading to a more homogeneous distribution of aberrations and delay times within the exposed cell population.

To gain further insights into the relationship between the aberration burden of cells and the cell-cycle delay, calyculin A was used to induce premature chromosome condensation in lymphocytes 48h post-irradiation. In these PCC-samples, the frequencies of excess fragments were separately determined for G_2- and M-cells. As shown in Fig. 3 (upper panel), following Fe-ion exposure high aberration yields were found in G_2-cells, but not in M-cells, indicating that an

arrest of heavily damaged cells in G_2 was the main cause for their delayed entry into mitosis. As expected, following X-irradiation, similar aberration yields were found in G_2-cells and in M-cells collected from PCC-samples (Fig. 3, lower panel). Noteworthy is that the aberration frequencies in M-cells from PCC-samples and in metaphases collected by conventional harvesting were similar (Fig. 3), confirming that Giemsa staining allows one to discriminate G_2- from M-cells.

To investigate, wheather interphase death interferes with the expression of aberrations in metaphase cells, the proportion of apoptotic/necrotic cells was determined at several culture times by dual-fluorescence staining and by trypan-blue staining. Both assays showed that the frequencies of apoptotic/necrotic cells tend to be higher in Fe and X-ray irradiated cultures than in unirradiated control. For 0,1,2 and 3 Gy Fe-ions the time-averaged frequencies of apoptotic/necrotic cells detected by fluorescence staining amounted to $5.2 \pm 1.4\%$, $9.0 \pm 2.5\%$, $9.6 \pm 3.5\%$ and $10.2 \pm 4.3\%$ (mean $\pm$ SD), while the same doses of X-rays resulted in $6.3 \pm 2\%$, $8.5 \pm 4.2\%$, $10.0 \pm 4.0\%$ and $10.9 \pm 4.9\%$, respectively. The proportions observed after trypan- blue staining were even lower (data not shown), because this test detectes only late apototic cells with permeable membranes and necrotic cells. Thus, under the applied irradiation conditions the influence of cell death on the aberration yield can be ruled out. To the best of our knowledge, data for the induction of interphase death in human lymphocytes exposed to high-LET radiation and stimulated to grow are not available. For other cell systems, conflicting results are reported. For example, in experiments using Chinese hamster ovary cells, particles with an LET of about 400 keV/μm were found to be two-times more effective in the induction of apoptosis than X-rays[14], while data from Aoki et al. for V79 Chinese hamster cells[15] indicate an effectiveness of close to 1. Thus, there is a need for further studies to investigate radiation-induced cell death and its dependence on LET as well as on the cell type under study.

In summary, our data provide further evidence that there is a pronounced mitotic delay of heavily damaged cells after high-LET exposure. This prolonged delay would lead to an underestimation of the damage, when the aberration yield is measured at only one early sampling time post-irradiation, as it is routinely done. For a meaningful quantification of particle-induced chromosomal damage and consequently, an accurate estimate of the RBE, modified protocols must be developed taking the cell cycle delays of heavily damaged cells into account[2,8,11]. This particularly applies to the biodosimetry of space radiation, because astronauts are exposed to a mixed radiation field including high LET radiation.

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REFERENCES

1. Kanda, R. (2000) Improvement of accuracy of chromosome aberration analysis for biological radiation dosimetry. J. Radiat. Res. **41**: 1–8.
2. Durante, M., Furusawa and Gotoh E. (1998) A simple method for simultaneous interphase-metaphase chromosome analysis in biodosimetry. Int. J. Radiat. Biol. **74**: 325–331.
3. Ritter, S., Nasonova, E., Scholz, M., Kraft-Weyrather, W. and Kraft G. (1996) Comparison of chromosomal damage induced by X-rays and Ar ions with an LET of 1840 KeV/μm. Int. J. Radiat. Biol. **69**: 155–166.
4. Durante, M., Furusawa, Y., George, K., Gialanella, G., Greco, O., Grossi, G., Matsufuji, N., Pugliese, M. and Yang, T. C. (1998) Rejoining and misrejoining of radiation-induced chromatin breaks. IV. Charged particles. Radiat. Res. **149**: 446–454.
5. Nasonova, E., Ritter, S., Fomenkova, T. and Kraft G. (1998) Induction of chromosomal damage in CHO-K1 and their repair-deficient mutant xrs5 by X-ray and particle irradiation. Adv. Space Res. **22**: 569–578.
6. Anderson, R. M., Mardsen, S. J., Wright, E. G., Khadim, M.A., Goodhead, D.T. and Griffin, C.S. (2000) Complex chromosome aberrations in peripheral blood lymphocytes. Int. J. Radiat. Biol. **76**: 31–42.
7. Ritter, S., Nasonova, E., Gudowska-Nowak, E., Scholz, M. and Kraft, G. (2000) High LET induced chromosome aberrations in V79 cells analysed in first and second post-irradiation metaphases. Int. J. Radiat. Biol. **76**: 149–161.
8. George, K., Wu, H., Willingham, V., Furusawa, Y., Kawata, T., Cucinotta, F.A. (2001) High and low LET induced chromosome damage in human lymphocytes: a time-course of aberrations in metaphase and interphase. Int. J. Radiat. Biol **77**: 175–183.
9. Wu, H., George, K., Kawata, T., Willingham, V. and Cucinotta, F. A. (2001) Comparison of F-ratios generated from interphase and metaphase chromosome damage induced by high doses of low and high LET radiation. Radiat. Res. **155**: 57–62.
10. Ritter, S., Nasonova, E. and Gudowska-Nowak, E. (2002) Effect of LET on the yield and quality of chromosomal damage in metaphase cells: a time-course study. Int. J. Radiat. Biol. **78**: 191–202.
11. Scholz, M., Ritter, S. and Kraft, G. (1998) Analysis of chromosome damage based on the time-course of aberrations. Int. J. Radiat. Biol. **74**: 457–462.
12. Perry, P. and Wolff, S. (1974) New Giemsa method for the

differential staining of sister chromatids. Nature **251**: 156–158.

13. Meijer, A. E., Bäckbro Saeidi, A. B., Zelenskaya, A., Czene, S., Granath, F. and Harms-Ringdahl, M. (1999) Influence of dose rate, post-irradiation incubation time and growth factors on interphase cell death by apoptosis and clonogenic survival of human peripheral lymphocytes. Int. J. Radiat. Biol. **75**: 1265–1273.
14. Sasaki, H., Yatagai, F., Kanai, T., Furusawa, Y., Hanaoka, F., Zhu, W.-G. and Mehnati, P. (1997) Dependence of induction of interphase death of Chinese hamster ovary cells exposed to accelerated heavy ions on linear energy transfer. Radiat. Res. **148**: 449–454.
15. Aoki, M., Furusawa, Y. and Yamada, T. (2000) LET dependency of heavy ion induced apoptosis in V79 cells. J. Radiat. Res. **41**: 163–175.

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