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Abstract

Radiation induced DNA double-strand breaks (dsbs) were measured in CHO-K1 cells by means of an experimental approach involving constant field gel electrophoresis and densitometric scanning of ethidium-bromide stained gels. For X-irradiation, an induction efficiency of $36 \pm 5 \text{ dsbs}(\text{Gy} \times \text{cell})^{-1}$ was determined. With this setup, the induction of dsbs was investigated in CHO-K1 cells after irradiation with accelerated carbon ions with specific energies ranging from 2.7 MeV/u to 261 MeV/u. This set of particle beams covers the important LET range between 17 keV/ μm and 400 keV/ μm , where maximum efficiencies have been reported for other cellular endpoints like inactivation or mutation induction. For LETs up to 100 keV/ μm , RBEs around 1 have been determined, while efficiencies per unit dose decline for higher LET values. No RBE maximum with values much larger than 1 was found. Data are discussed together with published results on dsb induction in mammalian cells by radiations of comparable LET.

Introduction

The action of ionizing radiation on cultured mammalian cells has been studied extensively since several decades (Puck and Marcus (1956)). The prominent experimental endpoints investigated include cellular survival and recovery (Elkind (1984)), chromosome damage (Awa (1974)), mutation (Thacker (1986)) and DNA-strand breaks (Hagen (1994)).

DNA-lesions, especially DNA double-strand breaks, are looked upon as the dominant molecular effect of radiation action. Dsbs mark the beginning of a cascade of cellular processes that either result in complete repair of the DNA-damage or lead to deleterious stages such as mutation, transformation or even cell death. In the literature, correlations between the level of DNA dsbs induced by sparsely ionizing radiation and cell killing have been reported for cell lines with different intrinsic radiosensitivity (Radford (1986), Kelland *et al.* (1988), Schwartz *et al.* (1991)).

The radiosensitivity of cells in culture can be influenced by changing the radiation quality. Accelerated particles provide an excellent means of varying the ionization density of the test radiation. With ion beams, the molecular mechanisms underlying the biological consequences of high LET irradiation can be studied (Kraft (1987)) and biophysical models describing radiation action can be tested (Scholz and Kraft (1991)). These studies may prove especially useful for their application in clinical radiotherapy (Kraft and Gademann (1993)).

For many years it is known that the relative biological efficiency for cell killing rises with the ionization density expressed as linear energy transfer (LET [keV/ μm]) up to about 100 keV/ μm .

The RBE drops again to values far below unity for very high LET. This dependency was demonstrated with helium ions (Barendsen *et al.* (1963)). However, for heavier ions distinct RBE-LET curves exist for different ions, with RBE-maxima shifted to higher LET-values (Kraft and Scholz (1994)). When looking at the inactivation probability per particle or inactivation cross section σ , this separation of the LET-effect relationship is even more pronounced (Wulf *et al.* (1985)).

For the intracellular induction of DNA double-strand breaks, a similar σ -LET relationship was reported for heavy ions with $Z \geq 10$ (Heilmann *et al.* (1993)). Concerning the RBEs for dsb induction, literature data are controversial. While Kampf (1983) reported RBE values up to 5 for intracellular dsb induction, more recent investigations with either protons, helium- or α -particles in the low-LET regime and accelerated ions with LETs of a few hundred keV/ μ m indicated RBEs generally very near to or below unity (Belli *et al.* (1994), Prise *et al.* (1990), Jenner *et al.* (1992), Jenner *et al.* (1993), Weber and Flentje (1993), Heilmann *et al.* (1993)). Thus the correlation between dsb-induction and cell killing is still not clear for densely ionizing radiation in the important LET-regime around 100 keV/ μ m.

The variations in data on intracellular DNA double-strand breaks may accrue from the different experimental procedures used to detect dsbs. Established methods include centrifugation in sucrose gradients (Blöcher (1982)), neutral filter elution (Bradley and Kohn (1979)) and a number of variants applying gel electrophoresis to elute high molecular DNA-fragments from whole cellular DNA embedded in agarose (Ager *et al.* (1990), Wlodek and Olive (1992), Olive *et al.* (1992), Blöcher *et al.* (1989), Stamato and Denko (1990)). However it has been shown that all assays lead to very similar results (Iliakis *et al.* (1991)a).

Techniques applying pulsed field gel electrophoresis (PFGE) are proven to be very sensitive, allowing reproducible measurements with X-ray doses of a few Gy. It has been shown that pulsed field gel electrophoresis can be substituted by constant field electrophoresis (CFGE) in these assays without any loss in sensitivity (Wlodek *et al.* (1991), Schneider *et al.* (1994)).

In this article, data on dsb-induction after irradiation with X-rays and carbon-ions in a LET-range from 14 keV/ μ m to 400 keV/ μ m are presented. Constant field gel electrophoresis (CFGE) was employed together with densitometric analysis of ethidium-bromide stained gels. The fraction of DNA retained was taken as quantitative measure to calculate absolute yields of induced DNA double-strand breaks. Besides details on the sensitivity and limits of CFGE, the data on dsb-induction with carbon-ions are compared with results already published in this LET-range of special interest.

Materials and Methods

Cell Material

Monolayer-cultures of Chinese hamster ovary cells (CHO-K1) were grown in Ham's F12-medium (Flow) containing 10% fetal bovine serum and 2 mmol/l glutamine. Cells were seeded in plastic culture dishes (petri dishes 35 mm in diameter or 75 cm² culture flasks, Greiner) and were incubated at 37°C in water-saturated air supplemented with 5% CO₂. Exponential growth was maintained by biweekly passages. Cells were cloned regularly and checked for mycoplasma-contamination using an ELISA-based commercial detection kit (Boehringer). Mycoplasma-free cell clones were stored in liquid nitrogen.

Irradiation and Embedding in Agarose

Asynchronous cells were irradiated either in culture dishes at a density of about 1×10^5 cells/cm² or embedded in agarose, depending on the penetration depth of the test-radiation. Agarose-plugs were obtained by harvesting cells from culture flasks by trypsinization with 2 ml enzyme solution containing 0.05% trypsin. After addition of 8 ml medium and transfer of the suspension into centrifuge-tubes, cells were spun down and the pellet resuspended in cold

PBS to a final cell concentration of 5×10^6 cells/ml. Then an equal volume of prewarmed low melting point agarose in PBS (1%, Type VII, Sigma) was added to the cell suspension. This final mixture was filled into a plug-mold (Beckman) after vortexing shortly. The volume of the resulting agarose plugs was 160 μ l. Cells irradiated in petri dishes were embedded likewise after removing them from the culture-surface with cell-scrapers (Greiner).

For X-irradiation a Siemens Stabiliplan generator (Type TR300F) with 1 mm Al- and 1 mm Cu-filtering was used. At 250 kV and 20 mA, a dose rate of 7 Gy/min was achieved. The UNILAC facility at the Gesellschaft für Schwerionenforschung in Darmstadt (GSI) provided the low energetic ions up to 15 MeV/u. One irradiation with 5.4 MeV/u C-ions was performed at the tandem-accelerator located in Heidelberg at the Max-Planck-Institut für Kernphysik. The synchrotron facility (SIS) at GSI produced the high energetic ions with energies from 100 to 400 MeV/u. Irradiation procedures and dosimetry are described comprehensively elsewhere (Kraft *et al.* (1980), Haberer *et al.* (1993)).

Cell Lysis

After irradiation and eventually embedding of the cells irradiated at low particle energies, the agarose plugs were transferred into ice-cold lysis buffer (0.4 mol/l EDTA, 2% sarcosyl, 1 mg/ml proteinase K). After equilibration for 1 h at 0°C, protein digestion was allowed for 20 h at 50°C. The plugs were then transferred into TE-buffer (10 mmol/l Tris, 1 mmol/l EDTA, pH 8.0) on ice and used directly for electrophoresis.

Constant Field Gel Electrophoresis (CFGE)

Gels containing 0.5% agarose (Ultrapure, DNA-grade, Biorad) in $0.5 \times$ TBE (45 mmol/l boric acid, 45 mmol/l Tris, 2 mmol/l EDTA, pH 8.3) were prepared and plug pieces containing about 1.2×10^5 cells were loaded into the gel slots. Gel size was 14 cm $\times$ 14 cm. Electrophoresis was performed in $0.5 \times$ TBE at 25 V for 24 h at room-temperature. Gels were stained for at least 3 h in ethidium bromide (2 μ g/ml) and destained overnight in distilled water at 4°C.

Experiments with λ -DNA

To investigate the fate of very small DNA-fragments in the course of the assay, λ -DNA cleaved with the restriction enzyme *Hind* III (Boehringer) was embedded in agarose plugs. These plugs were either used directly for electrophoresis or subjected to the same protocol as cell plugs including incubation in lysis buffer and equilibration with TE. Electrophoresis conditions were either as described in the last paragraph for dsb-detection or were chosen to separate the λ -fragments (1% agarose, 0.5 TBE, 30 V, 12 h). For comparison, samples containing cellular DNA were added on both types of gels.

Densitometric Scanning

The fluorescence pattern of DNA in ethidium-bromide stained gels was visualized on a transilluminator and digitized using a commercial video-system (Cybertech, Berlin) equipped with a highly sensitive CCD-camera (Fairchild). Images were stored and analyzed using an IBM-compatible computer and both commercial (Cybertech) and own software. Fluorescence intensities in the gel lanes and in the agarose plugs were determined and fractions of DNA retained were calculated. For effect curves, the fraction of DNA retained in the wells was plotted as function of radiation dose or particle fluence.

DNA-Detection with [methyl-³H]-Thymidine

To validate the densitometric scanning, constant field electrophoresis was performed using ³H-labelled cellular DNA. CHO-cells were grown in medium with the addition of 2×10^{10} Bq/ml [methyl-³H]-thymidine (NEN). Cells were harvested, sealed into agarose plugs and irradiated as already described. After electrophoresis and staining, the gel was first scanned with the CCD-camera system and then cut lane by lane into agarose pieces of appropriate size. Agarose plugs were melted in 100 μ l 1 mol/l HCl. After neutralization with 1 mol/l NaOH and addition of scintillation cocktail (NEN), the activity in the samples was determined using a Beckman 7500 liquid scintillation counter. The fraction of activity retained in the plugs was then calculated.

Results

Radiolabeling and Densitometric Scanning

The quantitative determination of the DNA-content via fluorescent dyes poses numerous problems upon the techniques applied. These include background fluorescence of the gel matrix, differences in the fluorescence depending on DNA-conformation, and nonlinearity of the video-system. Therefore, results obtained by densitometric scanning and liquid scintillation counting of ³H-labelled cellular DNA were compared in several experiments performed with X-rays. The same gel was first photographed with the video system for densitometric analysis and then prepared for liquid-scintillation counting immediately afterwards. An example is given in Fig. 1, demonstrating that both methods of DNA-detection result in almost identical dose-effect curves.

Determination of DSB Yields

Most electrophoretic protocols described in the literature for the quantification of DNA double-strand breaks do not lead to a separation of large radiation induced DNA-fragments by size. The assay described here separates only DNA-molecules smaller than about 30 kbp. However, the total amount of small DNA-pieces induced in the relevant dose range is negligible (Fig. 4a). Large DNA-fragments smaller than the exclusion size are eluted into the gel and move together in one broad band in the electric field.

It has been shown that the application of mathematical formalisms derived from polymer chemistry to X-ray induced DNA-fragmentation fits very well to experimental data (Blöcher (1990)). Only two parameters, the mean size of the molecules, i.e. the chromosomes (n), and the exclusion size of the gel (k) need to be known. The fraction of DNA retained (F_{ret}) can then be calculated as

$$F_{ret} = e^{(-\alpha D \frac{k}{n})} \left(1 + \alpha D \frac{k}{n} \left(1 - \frac{k}{n} \right) \right)$$

where α represents the mean number of DNA double-strand breaks in one chromosome per unit dose (D). The strand break yield per cell per unit dose is then given by $\alpha \times 22$, the number of chromosomes per cell. The use of this formalism results in curvilinear dose-effect curves, although the strand break yield is independent of dose, i.e a linear induction of dsbs with dose is assumed.

While the mean chromosome size for hamster cells can be estimated quite well to about 245 Mbp from literature data (Sontag *et al.* (1990), Blöcher (1990)), the exclusion size for the method described here was estimated to 6 Mbp from the literature (Blöcher (1990), Stenerlöv *et al.* (1994), Schneider *et al.* (1994)).

Figure 1: Dose effect curves obtained by elution of radiation-induced DNA-fragments from cellular DNA into agarose gels by constant field gel electrophoresis. Compared are fluorescent detection of DNA (●) and quantification via ³H-labeling (○) performed with one single electrophoresis gel. The fraction of DNA retained in the gel slots was calculated and plotted as function of X-ray dose. Both types of measurement are in excellent agreement. Typical errors for identically treated samples in experiments with X-rays were $\pm 5\%$ (data not shown). The two methods of evaluation are very well within these limits of error.

With these values, the experimental data from Fig. 2 were fitted using the equation given above. A yield of 36 ± 5 DNA double-strand breaks per Gy per cell was calculated for X-rays. Data points up to 50 Gy match very well with the theoretical curve. For higher doses, a flattening in the dose-response was observed (data not shown). This phenomenon has been reported by others, too (Stamato and Denko (1990), Warters and Lyons (1990), Whitaker and McMillan (1992)). Possible explanations are replication forks that are responsible for the high degree of DNA-retention especially in S-phase cells (Iliakis *et al.* (1991)b) and complex types of damage such as protein-DNA or DNA-DNA crosslinks.

The same fitting procedure was applied to the heavy ion data (Fig. 3b). Within the limits posed upon accelerator experiments, good agreement was achieved here, too. Thus, in this assay, the shapes of response curves do not differ for sparsely and densely ionizing radiation. As already explained, the electrophoretic assay applied here is very sensitive with respect to the fragment sizes of the DNA molecules. Only DNA fragments smaller than the exclusion size of 6 Mbp are eluted into the gel matrix. The mode of elution is not affected by a non-uniform distribution of the ionization density, as the methods average the radiation damage in several million individual chromosomal DNA-molecules per lane. A randomly induced inhomogeneity of DNA strand breaks in individual chromosomes does not affect the resulting overall fragment distribution in a large number of molecules. Therefore, effect curves obtained with heavy ions could be fitted with the equation given above in the same way as X-ray data. Typical experimental results from irradiations with carbon-ions are shown in Fig. 3b and summarized in Table 1.

Figure 2: Experimental data of DNA-dsb detection in CHO-K1 cells after X-irradiation. Data points and error bars give the mean and standard deviations from 5 independent experiments. The curve was fitted with the equation given in the text and parameters $n=245$ Mbp, and $k=6$ Mbp. Very good agreement is achieved in the entire dose region up to about 50 Gy.

Loss of Small DNA Fragments During the Assay

Restriction of λ -DNA with *Hind* III results in DNA-fragments ranging from 23 kbp to about 500 bp. Restricted DNA was embedded in LMP-agarose as already described. When the DNA in these agarose-plugs was subjected to CFGE as established for dsb-detection, only DNA-fragments smaller than 6.5 kbp were eluted completely out of the gel (Fig. 4a).

Another test was designed to monitor the effect of proteinase-K incubation and TE-equilibration on small DNA molecules in agarose plugs. Electrophoresis was performed to separate the restriction fragments after treatment of the λ -plugs in all buffers according to the protocol for the preparation of cellular DNA. All fragments were visible except for the smallest after proteinase-K treatment (Fig. 4b). The fluorescence intensities of fragments smaller than 6.5 kbp were considerably lower after extensive incubation at 50°C.

However, fragments of this size are lost anyway when applying standard CFGE conditions. Assuming a homogeneous length-distribution of DNA-fragments at least after X-irradiation i.e. equal probabilities for all possible fragment lengths to occur, the loss of DNA sums up to far less than 1 % of the total DNA eluted in the dose range applied here. This loss can be easily estimated using the equation given above, and inserting $k=6500$ bp instead of 6 Mbp. More than 5000 dsbs per chromosome, i.e. a dose of at least 3000 Gy would then be necessary to elute 1 % of the cellular DNA completely out of the gel matrix. Thus, this systematic error is significantly lower than the total experimental error as can be seen from Fig. 2.

Figure 3: a) Typical fluorescence pattern in an electrophoresis gel obtained after irradiation of CHO-K1 cells with 261 MeV/u carbon ions. Particle fluences ranged from 0.6×10^8 to 20×10^8 Particles/cm² (lanes 1-9). In lane 10, the fluorescence signal of DNA from an untreated control sample is shown.

b) Fluence effect curves for DNA double-strand break induction in CHO-K1 cells by carbon ions. The left graph shows curves obtained with 2.8 MeV/u ($\bullet$) and 5.4 MeV/u ($\square$) carbon ions. High energetic carbon beams with energies of 186 MeV/u ($\triangle$) and 261 MeV/u ($\diamond$) were applied in the experiments shown in the right graph. Error bars cannot be given, as experiments were all performed only once due to the mode of accelerator operation. However, from comparisons we estimate an error of approximately $\pm 20\%$.

Table 1: Parameters of the particle beams used and strand break yields as calculated from the fluence effect curves. Particle fluences or doses at fraction 0.7 of DNA retained ($F_{0.7}$ and $D_{0.7}$ respectively) are given as convenient parameter to compare the experimental data with results published by other groups. Efficiencies per particle as conceptual equivalents of inactivation cross sections, and efficiencies per unit dose are given. The latter values were taken to calculate the RBEs of dsb-induction (Fig. 5). Doses were calculated from particle fluences:

$$\text{Dose (Gy)} = \text{Fluence (P/cm}^2\text{)} \times \text{LET (keV/}\mu\text{m)} \times 1.602 \times 10^{-9}.$$

Ion	Energy (MeV/u)	LET (keV/ μ m)	$F_{0.7}$ (P/cm ²)	dsb/Particle/Cell	dsb/Gy/Cell
C	2.8	400	1.27×10^8	7.65×10^{-6}	11.9
	5.4	228	1.89×10^8	5.14×10^{-6}	14.1
	10.9	154	1.54×10^8	6.31×10^{-6}	25.6
	18.1	103	1.61×10^8	6.04×10^{-6}	36.6
	186	17	8.22×10^8	1.05×10^{-6}	38.7
	261	14	1.26×10^9	7.71×10^{-7}	34.6
X-Rays (250 kV, 20 mA)		2	$D_{0.7}$: 28 Gy	1.12×10^{-7}	36 ± 5

Figure 4: a) Ethidium-bromide stained CFGE-gel loaded with *Hind* III restricted λ -DNA (lanes 1–4) and cellular DNA irradiated with 60 Gy X-rays (lane 5). Restricted λ -DNA was embedded in LMP-agarose and subjected to electrophoresis directly (lane 1) after 24 h incubation in TE-buffer (lane 2), after 20 h incubation in lysis buffer at 50°C with (lane 3) or without proteinase K (lane 4). The 6.5 kbp fragment can still be seen in the gel, while the smaller fragments were eluted out of the gel during electrophoresis.

b) 1.0% agarose gel showing *Hind* III restricted λ -DNA directly after plug-preparation (lanes 1 and 8), after 24 h storage (lane 2), following TE-equilibration at room temperature for 24 h (lane 3) and 48 h (lane 4) and after incubation in lysis buffer at 50°C with (lane 5) or without proteinase K (lane 6). Lane 7 contains DNA from cells irradiated with 60 Gy X-rays. During DNA-preparation, only fragments smaller than 6.5 kbp partially diffuse out of the agarose plugs. However, these fragments are lost anyway in the CFGE-assay, as seen from the gel on the left.

Discussion

In this article, data on intracellular DNA double-strand break induction for carbon-ions of various energies covering an LET-range from 14 keV/ μ m to 400 keV/ μ m are presented. For the detection of dsbs, constant field gel electrophoresis in combination with densitometric scanning of ethidium-bromide stained gels was applied.

With CFGE, DNA-fragments smaller than the exclusion size of the gel matrix move in the direction of the electric field with a velocity almost independent of the fragment length. In most protocols involving pulsed field gel electrophoresis, the same principle is made use of and the eluted molecules are not separated by size. Therefore, both types of electrophoresis are equally suitable for the elution of DNA-fragments that result from double-strand breakage in intact mammalian DNA (Wlodek *et al.* (1991), Schneider *et al.* (1994)). However, less expensive equipment can be used and electrophoresis times are reasonably shorter, making the whole assay far less time consuming (total time approx. 48 h).

For the quantification of the DNA in the gel lanes and in the gel slots, two different approaches are established; densitometric scanning of gels stained with a fluorescent dye (Blöcher *et al.* (1989)) and detection of radionucleotides incorporated into cellular DNA, either via liquid scintillation counting (Ager *et al.* (1990)) or by phosphor imaging (Story *et al.* (1994)). While the latter methods are very accurate and reliable, fluorescence detection has the advantage of avoiding the production of radioactive waste. Together with the latest generation of CCD-cameras, direct densitometric scanning of ethidium-bromide stained gels is equivalent to the determination of ^3H -labeled DNA in the gel, as demonstrated here (Fig. 1).

Taking an exclusion size of 6 Mbp, as described in the results section, and an average chromosome size for CHO-cells of 245 Mbp (Blöcher (1990)), an induction rate of 36 ± 5 dsbs/(cell $\times$ Gy) $^{-1}$ or 6×10^{-9} dsb/(bp $\times$ Gy) $^{-1}$ can be calculated for X-rays from experimental data using the equation given above. This value corresponds very well to X-ray data presented by several other groups ranging from $\approx 3.2 \times 10^{-9}$ – 8×10^{-9} dsb/(bp $\times$ Gy) $^{-1}$ (Ager *et al.* (1990), Belli *et al.* (1994), Blöcher (1988), Iliakis *et al.* (1991)b, Löbrich *et al.* (1994), Prise *et al.* (1990), Stenerlöv *et al.* (1994), Weber and Flentje (1993)).

Yields of DNA double-strand break induction were also determined for the particle irradiations and relative biological efficiencies were calculated.

In Fig. 5 RBE values are plotted as function of LET. RBEs reported in this LET range by several other groups are shown, too. Measurements were performed with a variety of particles ranging from protons, α -particles and He-ions to C, O, Ne and Fe.

Generally, RBE-values are very close to 1 in the LET-range up to 50 keV/ μ m. Most data collected in this range are from protons, or helium-particles. The RBEs generally vary between 0.7 and 1.3. The investigators themselves stated that within the experimental errors, RBEs are around unity for both protons and alpha particles in the LET-regime covered (Prise *et al.* (1990), Jenner *et al.* (1993)). For higher LETs, relative biological efficiencies drop down to 0.5. RBEs between 0.75 and 1 were reported for Ne-ions with LET-values between 315 keV/ μ m and 395 keV/ μ m (Heilmann *et al.* (1993)). In an experiment performed by another group using the same accelerator facility, an RBE of 0.4 was calculated for an 310 keV/ μ m Ne-beam (Weber and Flentje (1993)). According to the well known separation of the LET-effect dependency for the different ion species, RBEs for dsb-induction with Ne-ions are likely to drop at higher LET-values than with C-ions. The general systematics described here for the RBE of DNA dsb-induction in mammalian cells was also observed using a slightly different CFGE-assay (Taucher-Scholz *et al.* (1995)). This study covers predominantly the low and very high LET regime with accelerated charged particles from deuterons to uranium ions.

All RBEs published fit together well except for the results reported for helium ions by Kampf (Kampf (1983)). These data indicate a steep increase for the RBE of dsb-induction from 10 keV/ μ m to 160 keV/ μ m with a maximum greater than 4 at 160 keV/ μ m. The discrepancies of this dataset to the general outline of the RBE-LET relationship are especially high. For

Figure 5: RBEs for the induction of intracellular DNA double-strand breaks as function of LET. Carbon data (except for the point at 360 keV/ μ m) are own measurements, the other values for ions with $Z \leq 10$ were taken from the literature (Aufderheide *et al.* (1987), Belli *et al.* (1994), Blöcher (1988), Heilmann *et al.* (1993), Jenner *et al.* (1993), Kampf (1983), Prise *et al.* (1990), Rydberg *et al.* (1994), Stenerlöw *et al.* (1994), Weber and Flentje (1993)). A set of data for protons and He-ions at 20 keV/ μ m and 23 keV/ μ m was not integrated in the figure (Jenner *et al.* (1992)), but would fit nicely to the proton-data in this LET-regime. The dashed line indicates the systematics of the He-data published by Kampf, while the solid line follows the RBEs for C-ions. Both lines were fitted by eye. Except for the data reported by Kampf, all other RBEs are only slightly above or smaller than unity.

α -particles with a LET of 110 keV/ μ m an RBE of 1.6 was reported (Blöcher (1988)), the RBE published by Kampf is twice as high. Both investigators used sedimentation techniques for the detection of double-strand breaks. A more recent measurement with 120 keV/ μ m α -particles reports RBEs of 0.85 and 0.68 detected using either sedimentation or neutral elution (Jenner *et al.* (1993)). The RBE for dsb-induction measured by us with carbon ions of 400 keV/ μ m is well below unity, while Kampf reported a RBE of 2.4 for 360 keV/ μ m carbon ions. These differences cannot be attributed to the separation of the LET-RBE function for different Z, as results obtained with the same ion species and at similar LETs are compared. The experimental protocol applied as well as differences in the cell material may be responsible for this anomaly. However, the vast majority of literature data covering this LET-range is clearly in favour of a relative biological efficiency for dsb-induction near unity up to LET-values of about 100 keV/ μ m, followed by a decrease in RBE down to about 0.5.

Obviously, the rate of DNA double-strand break induction does not correlate with cell killing in the LET-range where cell killing is in its maximum. To understand this, it has to be kept in mind that all methods currently in use for dsb-detection are incapable of differentiating between DNA lesions of varying complexity. A clear cut of the DNA double helix induced by the action of a restriction enzyme cannot be distinguished experimentally from a complex high-LET lesion resulting in small deletions.

Damage induced by ionizing radiation is almost always mediated by energetic δ -electrons. They lose their energy through interactions with the target atoms. The higher the local density of the δ -electrons, the higher is the chance to induce multiple damage in nano- or subnanometre regions (Goodhead (1994)). At the sites of such complex lesions small fragments of 100 bp to 2 kbp can also be produced (Chatterjee and Holley (1994)). The assays currently in use do all at best detect complex lesions as a single double-strand break. This holds true also for the method described here, as the lower limit of DNA-fragments trapped in the gel matrix (6.5 kbp) is still larger than the size of the expected small fragments. The contribution of these fragments to the total amount of eluted DNA would be insignificantly small. The property of our assay as well as of the techniques used by others may lead to an underestimation of both the yields of DNA dsbs and the RBEs, if complex damage is involved. Besides these limitations, the experimental results are useful to test models describing radiation action in this LET-regime and to relate molecular damage and cell killing.

The enhanced complexity of the lesions as proposed by several authors (Goodhead (1994), Brenner and Ward (1992), Prise (1994)) are made responsible for the high efficiency in cell killing seen with particle radiation around 100 keV/ μ m. Computer simulations scoring ionization clusters have been applied to model the RBE dependence of dsb-induction as function of LET (Brenner and Ward (1992)). Depending on the cluster sizes chosen, the calculated RBE-LET dependence does not show up with any clear RBE maximum, nor do the RBE values rise significantly above unity. Our experimental data compiled here are also in favour of RBEs around or below unity for high-LET radiation.

Indirect evidence for the existence of clustered DNA damage comes from studies on double-strand break rejoining. There is agreement in several reports that particle irradiation results in slow rejoining kinetics and an increase of residual strand breaks compared to X-irradiation (Ritter *et al.* (1977), Aufderheide *et al.* (1987), Weber and Flentje (1993), Heilmann *et al.* (1993), Taucher-Scholz *et al.* (1995)). The resulting hypothesis calls for clustered DNA-damage as origin of insufficient damage repair, leading to a reduction in the viability of the cells carrying these residual lesions. Consequently, the lack of correlation between dsb-induction and cell killing may be explained by the occurrence of complex DNA-lesions that cannot be adequately resolved by current assays. For the direct detection of complex DNA-damage, techniques need to be developed that are capable of detecting minute quantities of small DNA molecules down to the size of oligonucleotides (Rydberg *et al.* (1994)). Systematic studies on DNA-rejoining and repair are also feasible to monitor the intermediate stages from damage induction to damage fixation.

In this paper it was demonstrated that constant field gel electrophoresis in combination with direct densitometric analysis of ethidium bromide stained gels provides a fast and accurate

system to detect DNA fragmentation via dsb induction. Yields of DNA double-strand breaks were determined with accelerated carbon-ions covering the important LET range between 14 keV/ μ m and 400 keV/ μ m. Our data, as well as results obtained by others in this LET regime, do clearly show that by applying the experimental approaches established to date, the detected efficiencies of dsb-induction per unit dose do not differ significantly from the values obtained with X-irradiation. By contrast, RBEs drop in the regime of around 100 keV/ μ m, where the RBE of cell killing reaches its maximum. The investigation of DNA lesions with respect to a change in their complexity and repairability should be the focus of future research.

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