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# No Evidence for a Different RBE between Pulsed and Continuous 20 MeV Protons

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**To obtain greater insight into the future potential of tumor radiotherapy using proton beams generated from high-intensity lasers, it is important to characterize the ionization quality of the new beams by measuring the relative biological effectiveness (RBE) under conditions where the full dose at one irradiation site will be deposited by a few proton pulses less than 1 ns in duration. HeLa cells attached to a Mylar foil were irradiated with 70 kV X rays to obtain a reference dose–response curve or with 3 Gy of 20 MeV protons at the Munich tandem accelerator (Garching), either using a continuous mode where a cell sample was irradiated within a 100-ms time span or using a pulsed mode where radiation was given in a single proton pulse of about 1 ns. After irradiation cytochalasin B was added; 24 h later cells were fixed and stained with acridine orange and micronuclei were counted. The X-ray dose–response curve for the production of micronuclei in HeLa cells followed a linear-quadratic model. The corresponding RBE values for 20 MeV protons in pulsed and continuous irradiation modes were 1.07 ± 0.08 and 1.06 ± 0.10 in the first proton experiment and 1.09 ± 0.08 and 1.05 ± 0.11 in the second, respectively. There was no evidence for a difference in the RBE for pulsed and continuous irradiation of HeLa cells with 20 MeV protons.** 

## INTRODUCTION

High-intensity lasers have demonstrated the potential for driving highly brilliant particle and photon beams whose unique properties will make a broad range of novel applications available. One possible application may be radiotherapy of malignant tumors by high-energy protons using laser-driven accelerators. Due to the inherent properties of the high-intensity laser source, the dose to a tissue voxel would be applied in a few or even only one single ion pulse of nanosecond duration whereas in conventional irradiation the dose is delivered in milliseconds or seconds. Thus it is mandatory to investigate the relative biological effectiveness (RBE) of the new beam quality before its use in radiotherapy is possible. The current subject of debate is whether pulsed irradiation with high-energy particles may induce either a different amount of damage in cells or changes in repair or apoptosis pathways relative to continuous irradiation. The short-pulse effects are the subject of our current investigations using pulsed ion beams at the SNAKE [Superconducting Nanoprobe for Applied nuclear (Kern) physics Experiments] microprobe of the Munich tandem accelerator, where $10^5$ high-energy protons can be bunched into a single nanosecond pulse at a beam diameter of about 100 μm. These beam parameters are sufficient to irradiate cell cultures or tissue with a dose of up to 5 Gy by a single pulse of protons.

The present experiments have been aimed at determining the RBE of continuous and pulsed irradiation with high-energy protons at 20 MeV in direct comparison with 70 kV X rays using the micronucleus test in HeLa cells. Since scoring of micronuclei in cells is an easy and fast procedure, this assay has been suggested as an alternative to scoring structural chromosome aberrations (*1*). Investigating such radiobiological effects at the Munich tandem accelerator is part of a series of research projects that are being performed in the framework of the DFG cluster of excellence ''Munich-Centre for Advanced Photonics'' (MAP).

## MATERIALS AND METHODS

### *Determination of the Chromosome Number of the HeLa Cells*

Prior to the radiation experiments, the chromosome number of nonexposed HeLa cells was examined. Slide cultures were set up in Quadriperm dishes (Heraeus, Germany). The cells were seeded at a density of $2 \times 10^5$ cells per slide in 5 ml RPMI 1640 medium and incubated for 24 h at 37°C in a humidified atmosphere containing 95% air/5% $CO_2$. During the last 3 h, colcemid (0.5 μg/ml) was

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present. Chromosome preparation was performed according to standard procedures (*2*). In brief, for hypotonic treatment the medium was removed and replaced by 5 ml of a prewarmed mixture of 1 volume Hanks solution and 3 volumes of water for 15 min at 37°C. A 5-ml aliquot of fixative (methanol-acetic acid, 3:1) was added drop by drop on each individual slide and left for 15 min. After two additional changes with fresh fixative (each for 15 min), the slides were air dried and stained with 2% acetic Orcein for 10 min. With this technique, the cytoplasm of the cells was well preserved and chromosome loss during preparation was avoided.

### *Cell Culture for Proton Irradiation*

HeLa cells were grown as monolayer cultures in tissue culture flasks containing RPMI 1640 medium supplemented with 10% fetal calf serum, 100 units of penicillin and 20 μg of streptomycin per ml of culture medium. Cultures were incubated for 36 h at 37°C in a humidified atmosphere of 5% $CO_2$ in air. Shortly before irradiation, cells were trypsinized, seeded in irradiation containers, and allowed to adhere for 12 h. The construction of irradiation containers and the culture technique have been described in detail previously (*3, 4*); only a brief description is given here. Irradiation containers are built by stretching and clamping a 6-μm Mylar foil between two stainless steel plates. Cells to be irradiated are grown on this carrier foil, which is part of the container developed specifically for irradiation at SNAKE. For incubation, the containers were covered with the lid of a 9-cm tissue culture dish and containers plus covers were placed in 15-cm culture dishes. Immediately before irradiation, since the fixed horizontal beamline requires that the samples be held vertical to the beam axis, about half of the culture medium was removed and the containers were tightly closed with a second 6-μm Mylar foil glued to a stainless steel plate.

Cell irradiation experiments with 20 MeV protons were carried out in two independent proton experiments I and II during separate beam times, each with three replicates of 3 Gy in either continuous or pulsed irradiation mode.

### *Cell Irradiation with 20 MeV Pulsed Proton Beam*

A pulsed 20 MeV proton beam with up to $10^5$ protons per pulse focused into a spot of approximately $100 \times 100$ μm$^2$ is prepared for cell irradiation experiments using the ion microprobe SNAKE at the Munich tandem accelerator. The pulsed-beam preparation was described in detail by Dollinger *et al.* (*4*). By scanning the beam in regular pattern a close to homogeneous dose distribution is obtained, where the main dose is delivered by one proton pulse and a small additional dose is added by adjacent beam pulses. The homogeneity of the dose distribution is controlled by a CsI scintillator viewed by a CCD camera.

During irradiation the cell container with the cells attached on the carrier foil were mounted directly behind the beam exit nozzle. Thus the protons have to traverse the vacuum exit window, 7.5 μm Kapton, an air gap of approximately 30 μm, and the cell carrier foil, 6 μm Mylar. According to SRIM2003 (*5*), the energy loss for 20 MeV protons until they reach the cells is 0.11 MeV, resulting in a slightly increased LET in water of 2.661 keV/μm at the target in comparison to 2.648 keV/μm for 20 MeV protons (*5*).

The delivered energy dose *ED* can be calculated from

$$ED = \frac{N}{A}\frac{LET}{\rho}, \tag{1}$$

where *LET* is the linear energy transfer, ρ the target density (assuming water as a tissue equivalent) and *N*/*A* the number *N* of particles that impinge on the beam size area *A*. For dose control, the beam size *A* of the beam spot can be observed by the CsI scintillator (*4*). The irradiated area is well controlled with much better than 1% accuracy when scanning the beam by setting hundreds of individual beam spots side by side. The number of protons *N* per pulse is given by $N = \frac{I}{e \cdot r}$ and measured by the electrical beam current *I* for beam pulses repeating at a repetition rate $r = 5/128$ MHz and using the unity charge *e*. The systematic error of the beam current measurement through leakage currents and instrumental errors is estimated to be less than 5%. The largest error stems from beam fluctuations between current measurement at the Faraday cup situated in front of the target position and the application of the single pulses for cell irradiation. This statistical error of a single shot dose is estimated to be of the order of ±10% but should be averaged for the many pulses applied on one cell dish and by doing three independent irradiations for each experiment.

The pulse length was 1.6 ns in proton experiment I and 1.0 ns in proton experiment II. The area irradiated by a single pulse was $80 \times 123$ μm$^2$ in experiment I and $59 \times 95$ μm$^2$ in experiment II. The total size of the irradiation field was $1.5 \times 2.0$ mm$^2$ in both pulsed-mode experiments; it was obtained by scanning the beam in a regular pattern by a total of 304 pulses in experiment I and 546 pulses in experiment II. Thus most of the dose delivered to each cell was delivered by a single pulse.

The same setup is used for preparation of the pulsed and the continuous beam for proton irradiation. Thus many systematic errors can be compensated by direct comparison of results from the pulsed-beam and non-pulsed-beam experiments. In particular, the errors in the electrical beam current measurement by leakage currents and instrumental errors are compensated.

### *Cell Irradiation with Continuous 20 MeV Proton Beam*

A quadratic $A = 2.0 \times 2.0$ mm$^2$ beam spot is prepared with the installed micro slit system and the homogeneity is visualized and controlled on a CsI scintillator (*4*). The delivered dose is adjusted by the time *t* that is needed to irradiate the cells for the 3-Gy dose according to Eq. (1) by using the measured Faraday cup current (I): $N = \frac{I \cdot t}{e}$. In continuous mode, too, the dose error is dominated by beam fluctuation, resulting in a dose error of less than ±10% for any continuous-beam experiment.

In continuous mode the sample was irradiated for 100 ms in the first experiment and for 23 ms in the second experiment. Such irradiation times are typically used to irradiate a certain voxel of a tumor in common treatment plans of raster scanned beams.

### *Irradiation for Dose–Response Reference Curve*

To obtain a reference dose–response curve, HeLa cells were irradiated in a first reference experiment A with doses in the range of 0 to 10 Gy and in a second reference experiment B with doses of 0 to 4 Gy with 70 kV X rays (Philips RT100; Philips Medical Systems, Eindhoven, The Netherlands) at a dose rate of approximately 1 Gy per minute (10 mA, 2.0 mm aluminum, HVL = 1.9 mm aluminum) and a source–cell distance of 30 cm using a field of 20 cm × 20 cm.

### *Detection of Micronuclei*

After irradiation, the remaining culture medium was replaced with fresh medium containing 10 μg/ml Cytochalasin B and the cells were incubated for 24 h. Cells were fixed with 2% paraformaldehyde and stained with acridine orange.

The irradiated regions were identified by immunostaining of the phosphorylated histone H2AX (γ-H2AX) as described by Greubel *et al.* (*6*). Applying the cytokinesis block, the frequency of micronuclei could be detected precisely in the first division cycle after irradiation and easily identified from their binucleate appearance (*7*). From each sample (unirradiated or irradiated) at least 300 binucleate cytokinesis block cells with well-preserved cytoplasm were analyzed. The number

of micronuclei was counted only if cytokinesis-blocked cells contained detached micronuclei in the cytoplasm.

*Determination of RBE Value*

The RBE of 20 MeV protons (pulsed or continuous) is calculated as the ratio between the dose, $D_\gamma$, of the reference radiation (70 kV X rays) and the dose, $D_p$, of protons (20 MeV) that produced equal response, $y$: RBE = $D_\gamma/D_p$. To calculate $D_\gamma$ the measured dose–response curve is parameterized by a linear-quadratic function, $y = c + \alpha D + \beta D^2$, fitted to determine the parameters $c$, $\alpha$ and $\beta$ and inverted.

Because the fit parameters $c$, $\alpha$ and $\beta$ depend on each other, a Monte Carlo simulation-based Bayesian data analysis is performed. In a first step the three-dimensional probability density is calculated as a function of $c$, $\alpha$ and $\beta$ for measuring the data set used for the dose–response curve. In a second step, assuming a Gaussian probability density for $y$, the probability density for $D_\gamma$ is calculated leading to the confidence interval for $D_\gamma$. From this and from the error of the proton dose measurement the error of the RBE value is calculated by Gaussian error propagation.

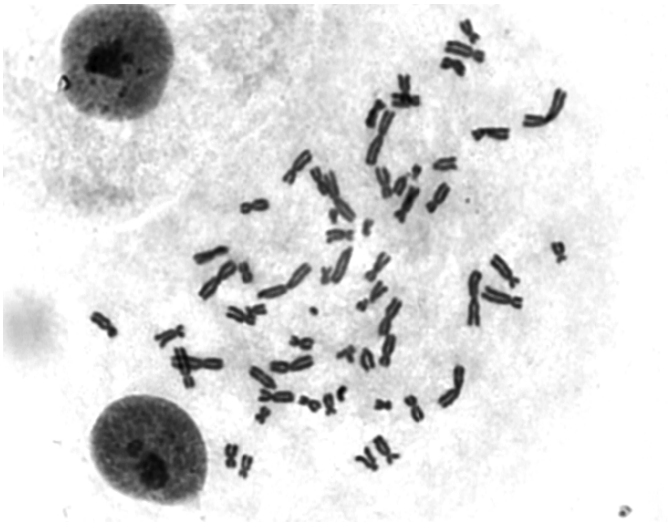

**FIG. 1.** Example of a HeLa cell with 62 chromosomes.

## RESULTS

*Chromosome Number of HeLa Cells*

There are many isolates of HeLa cells grown in different laboratories all over the world, but all HeLa cells are descended from the same tumor cells removed from Ms. Lacks in 1951. To characterize the HeLa subline used in the present experiment, the number of chromosomes was examined in a total of 200 metaphases with preserved cytoplasm. Since there was no substantial variation between the findings from three replicates, the data were pooled. The chromosome analysis showed a modal number of 62 chromosomes (71% of analyzed cells), whereas 29% of the analyzed cells had a loss or gain of chromosomes (4% with 60 chromosomes, 10% with 61 chromosomes, 13% with 63 chromosomes, 2% with 64 chromosomes). A HeLa karyotype containing 62 chromosomes is demonstrated in Fig. 1.

*Reference Data from 70 kV X Rays*

The detailed results for micronuclei and their intercellular distribution in HeLa cells found in reference experiment A with 70 kV X rays are shown in Table 1 together with the corresponding background frequencies. This experiment with doses up to 10 Gy was carried out to determine the most appropriate dose range for the reference dose–response curve. At doses of 2 and 5 Gy, the intercellular distribution of micronuclei was overdispersed, whereas at the highest dose (10 Gy) the corresponding distribution was underdispersed. This result is seen from the dispersion ratio (variance/mean, $\sigma^2/y$), which should be 1 for a Poisson distribution. The significance of the observed excess is assessed in terms of the test quantity $u$ (*8*), which approximates to a unit normal deviation; a value of $u > 1.96$ indicates an overdispersion at the 5% level of significance and a value of $u < -1.96$ an underdispersion.

The dispersion ratio decreased with increasing dose from 2 to 10 Gy. Therefore, since the appearance of a saturation effect, i.e. several chromosomal fragments in a single micronucleus, could not be excluded, additional micronucleus data were acquired in reference experiment B to allow improved evaluation of the dose response in the lower-dose range from 0.5 to 4 Gy. The corresponding results for micronuclei and their intercellular distribution for exposure of HeLa cells to 70 kV X rays are given in Table 2. At the five doses from 0.5 to 4 Gy, the intercellular distribution was significantly overdispersed compared to Poisson. Moreover, within this

**TABLE 1**
**Reference Experiment A: Distribution of Micronucleus Frequencies in Cytokinesis-Blocked Binucleate HeLa Cells Induced by 70 kV X Rays (dose range 0–10 Gy)**

| Dose (Gy) | Cells analyzed | Micronuclei per cytokinesis-blocked cell | Micronuclei per cell | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | $\sigma^2/y$[a] | $u$ value[b] |
| 0 | 1000 | 0.029 | 973 | 25 | 2 | 0 | 1.11 | 2.50 |
| 2 | 1000 | 0.195 | 846 | 121 | 25 | 8 | 1.31 | 6.95 |
| 5 | 1000 | 0.394 | 698 | 220 | 72 | 10 | 1.12 | 2.69 |
| 10 | 1000 | 0.748 | 430 | 403 | 156 | 11 | 0.76 | −5.37 |

[a] Dispersion ratio (variance/mean).
[b] Test quantity.

**TABLE 2**
**Reference Experiment B: Distribution of Micronucleus Frequencies in Cytokinesis-Blocked Binucleate Cells Induced by 70 kV X rays (dose range 0–4 Gy)**

| Dose (Gy) | Cells analyzed | Micronuclei per cytokinesis-blocked cell | Micronuclei per cell | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | $\sigma^2/y^a$ | *u* value[b] |
| 0 | 1000 | 0.032 | 968 | 32 | 0 | 0 | 0.97 | −0.68 |
| 0.5 | 1000 | 0.070 | 939 | 53 | 7 | 1 | 1.22 | 4.95 |
| 1 | 1000 | 0.098 | 914 | 75 | 10 | 1 | 1.17 | 3.82 |
| 2 | 1000 | 0.145 | 880 | 99 | 17 | 4 | 1.26 | 5.83 |
| 3 | 1000 | 0.247 | 811 | 141 | 38 | 10 | 1.30 | 6.72 |
| 4 | 1000 | 0.355 | 729 | 204 | 50 | 17 | 1.22 | 4.92 |

[a] Dispersion ratio (variance/mean).
[b] Test quantity.

dose range there was little change in the dispersion coefficient and no indication of a correlation with increasing dose. The control value of 0.032 ± 0.006 obtained for micronuclei in this experiment B did not differ significantly from the corresponding value of 0.029 ± 0.005 that was determined in reference experiment A.

A weighted least-squares approximation was applied to fit the data for micronuclei obtained in reference experiment B with the linear-quadratic function, $y = c + \alpha D + \beta D^2$, where $y$ is the micronucleus yield, $D$ is the radiation dose, $c$ is the background frequency of micronuclei, and $\alpha$ and $\beta$ are coefficients. Reciprocal variances of the mean (total number of cells analyzed, $n$, divided by variance, $\sigma^2$) were used as weighting factors. The coefficients (±SE) of the dose–response relationship are: $c = 0.0352 \pm 0.0057$, $\alpha = 0.0497 \pm 0.0119$ Gy$^{-1}$ and $\beta = 0.0071 \pm 0.0034$ Gy$^{-2}$. The resulting curve for induction of micronuclei in HeLa cells by 70 kV X rays is shown in Fig. 2. This dose–response curve for X rays served as the reference for calculation of the RBE of 20 MeV protons.

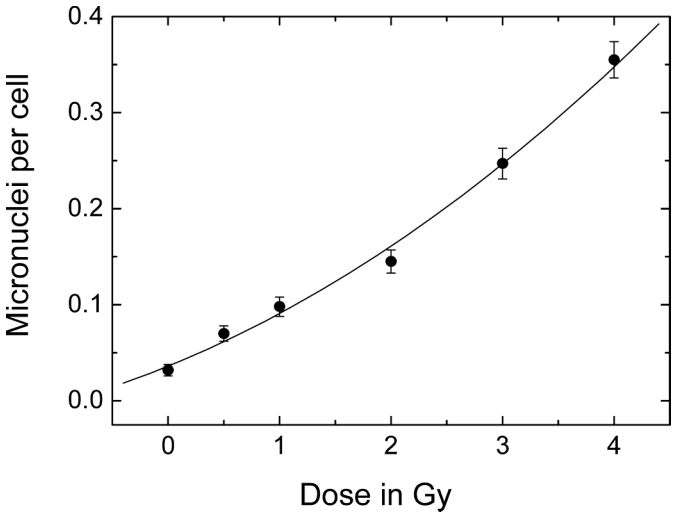


**FIG. 2.** Linear-quadratic dose–response relationship for the yields of micronuclei induced in HeLa cells by 70 kV X rays, based on reference experiment B.

*Data from Proton Experiments*

Tables 3 and 4 present the basic data for micronuclei from the two independent proton experiments, each with three replicates of exposure of HeLa cells to 3 Gy of 20 MeV protons at pulsed or continuous irradiation modes. Since no major differences between the results of the replicates were observed, the data were pooled. These pooled data are also shown as mean values (±SE) in Tables 3 and 4. The mean values of 0.0287 ± 0.0030 and 0.0330 ± 0.0059 for spontaneous induction of micronuclei under control conditions are consistent with the control values determined in the experiments using 70 kV X rays.

In proton experiment I (October 2007) with a proton dose of 3 Gy at pulsed and continuous irradiation modes, mean numbers of micronuclei per cytokinesis block cell of 0.2689 ± 0.0128 and 0.2633 ± 0.0102 were obtained (Table 3). In proton experiment II (from May 2008), also with a proton dose of 3 Gy at pulsed and continuous irradiation modes, mean numbers of micronuclei per cytokinesis-blocked cell were 0.2723 ± 0.0081 and 0.2625 ± 0.0141 (Table 4). Within the accuracy of measurement the values for the yield of micronuclei for both pulsed and continuous irradiation mode were the same. Thus the difference in the yield of micronuclei is less than the accuracy of measurement of about 5%.

The RBE for 20 MeV protons was calculated using the dose of the reference radiation that produced the same response as 3 Gy of protons. In proton experiment I (Table 3), the pooled frequencies of micronuclei of 0.2689 ± 0.0128 (pulsed irradiation mode) and 0.2633 ± 0.0102 (continuous irradiation mode) corresponded to the values found for 3.22 and 3.17 Gy of X rays, resulting in RBE values of 1.07 ± 0.08 and 1.06 ± 0.10, respectively. In proton experiment II (Table 4), the pooled frequencies of 0.2723 ± 0.0081 (pulsed irradiation mode) and 0.2625 ± 0.0141 (continuous irradiation mode) correspond to the frequencies for 3.27 and 3.16 Gy of X rays, resulting in RBE values of 1.09 ± 0.08 and 1.05 ± 0.11, respectively.

**TABLE 3**
**Proton Experiment I (Munich Tandem Accelerator; October 2007): Distribution of Micronucleus Frequencies in Cytokinesis-Blocked Binucleate Cells Induced by 3 Gy of 20 MeV Protons at Pulsed (P) or Continuous (C) Irradiation Modes**

| Dose (Gy) | Cells analyzed | Micronuclei per cytokinesis-blocked cell | Micronuclei per cell | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | $\sigma^2/y^a$ | $u$ value[b] |
| 0 | 1029 | 0.027 | 1001 | 28 | 0 | 0 | 0 | 0.97 | −0.69 |
| 0 | 1109 | 0.030 | 1076 | 33 | 0 | 0 | 0 | 0.96 | −0.88 |
| 0 | 1031 | 0.029 | 1001 | 30 | 0 | 0 | 0 | 0.97 | −0.65 |
| 3 (P) | 396 | 0.268 | 321 | 53 | 14 | 7 | 1 | 1.51 | 7.17 |
| 3 (P) | 758 | 0.274 | 612 | 98 | 36 | 10 | 2 | 1.48 | 9.31 |
| 3 (P) | 497 | 0.262 | 399 | 74 | 16 | 8 | 0 | 1.36 | 5.63 |
| 3 (C) | 1107 | 0.277 | 877 | 168 | 47 | 15 | 0 | 1.32 | 7.62 |
| 3 (C) | 691 | 0.236 | 574 | 82 | 25 | 9 | 1 | 1.48 | 8.91 |
| 3 (C) | 758 | 0.268 | 614 | 96 | 37 | 11 | 0 | 1.42 | 8.24 |
| $\sum$ 0 | 3169 | 0.0287 ± 0.0030 | 3078 | 91 | 0 | 0 | 0 | 0.97 | −0.88 |
| $\sum$ 3 (P) | 1651 | 0.2689 ± 0.0128 | 1332 | 225 | 66 | 25 | 3 | 1.45 | 12.94 |
| $\sum$ 3 (C) | 2556 | 0.2633 ± 0.0102 | 2065 | 346 | 109 | 35 | 1 | 1.39 | 13.95 |

*Note.* Pooled data are given as means ± SE for three replicates.
[a] Dispersion ratio (variance/mean).
[b] Test quantity.

## DISCUSSION

### *Results for the RBE of Protons*

A number of radiobiological studies have been performed with different proton energies using a continuous irradiation mode, as summarized by the ICRP (*9*). However, most of these experiments were carried out without data for a reference radiation obtained under the same experimental conditions. Data on the dose–response relationship for proton-induced damage in cells or tissues as well as the estimation of the RBE have been obtained predominantly with protons of energies higher than 50 MeV [for reviews, see e.g. refs. (*10, 11*)]. Although these RBE values are affected in part by the different biological systems and end points or differences in the reference radiation used for comparisons, these high-energy protons have generally been considered to be low-LET radiations with biological effects much like X rays or γ radiation. Data for proton energies lower than 50 MeV are scarce. Bettega *et al.* (*12*) reported RBE values from 0.5 to 1.6 for 12 MeV protons at doses up to 7 Gy, which were determined by formation of micronuclei in a heteroploid cell line with epitheloid morphology (EUE line with a modal number of 60 chromosomes), using γ rays as the reference. However, these findings were based on a rather poor fit to the data for γ-ray-induced micronuclei. Further experiments by Bettega *et al.* (*13*) resulted in RBE values of 1.7, 1.3 and 1.0 for proton beams of 8, 12 and 31 MeV,

**TABLE 4**
**Proton Experiment II (Munich tandem accelerator; May 2008): Distribution of Micronucleus Frequencies in Cytokinesis-Blocked Binucleate Cells Induced by 3 Gy of 20 MeV Protons at Pulsed (P) or continuous (C) Irradiation Modes**

| Dose (Gy) | Cells analyzed | Micronuclei per cytokinesis-blocked cell | Micronuclei per cell | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | $\sigma^2/y^a$ | $u$ value[b] |
| 0 | 548 | 0.0274 | 533 | 15 | 0 | 0 | 0 | 0.97 | −0.46 |
| 0 | 771 | 0.0389 | 745 | 22 | 4 | 0 | 0 | 1.23 | 4.50 |
| 0 | 620 | 0.0306 | 602 | 17 | 1 | 0 | 0 | 1.08 | 1.41 |
| 3 (P) | 524 | 0.2753 | 419 | 76 | 21 | 6 | 2 | 1.44 | 7.07 |
| 3 (P) | 556 | 0.2626 | 448 | 79 | 22 | 5 | 2 | 1.41 | 6.87 |
| 3 (P) | 749 | 0.2777 | 599 | 109 | 28 | 9 | 4 | 1.48 | 9.38 |
| 3 (C) | 621 | 0.2689 | 501 | 87 | 23 | 6 | 4 | 1.49 | 8.72 |
| 3 (C) | 687 | 0.2460 | 564 | 88 | 26 | 7 | 2 | 1.45 | 8.43 |
| 3 (C) | 776 | 0.2719 | 627 | 101 | 35 | 12 | 1 | 1.46 | 9.08 |
| $\sum$ 0 | 1939 | 0.0330 ± 0.0059 | 1880 | 54 | 5 | 0 | 0 | 1.12 | 3.76 |
| $\sum$ 3 (P) | 1829 | 0.2723 ± 0.0081 | 1466 | 264 | 71 | 20 | 8 | 1.45 | 13.52 |
| $\sum$ 3 (C) | 2084 | 0.2625 ± 0.0141 | 1692 | 276 | 84 | 25 | 7 | 1.47 | 15.28 |

*Note.* Pooled data are given as means ± SE for three replicates.
[a] Dispersion ratio (variance/mean).
[b] Test quantity.

respectively, determined from data on chromosome aberrations induced in the same cell line by doses up to 5 Gy.

Using the Munich tandem accelerator, where the present proton experiments were carried out at 20 MeV with a dose-averaged LET of 2.66 keV/μm, Schmid *et al.* (*14*) irradiated human lymphocytes in a multilayer array with 16.5 MeV protons with a dose-averaged LET of 3.1 keV/μm and found a linear-quadratic dose–response relationship for dicentrics. To determine the RBE of the 16.5 MeV protons with respect to photons, the comprehensive data set for photon radiation qualities compiled by the ICRP (*9*) and the BEIR VII Committee (*15*) were used. This procedure was justified because the published data on the induction of dicentrics and acentric fragments by photons with mean energies from 5.4 keV to 1.2 MeV and by 16.5 MeV protons were all obtained using human lymphocytes from blood samples from the same donor using identical culture and chromosome aberration scoring conditions (*14*). Based on the reference data set for 60 kV X rays (*16*), an RBE of 1.15 was calculated for 3 Gy of 16.5 MeV protons. This means that the RBE determined in the present study for 20 MeV protons based on measurement of micronuclei is in line with the RBE value resulting from chromosome aberration analysis, even though different cell types and different end points were used.

### *Micronucleus Assay as an End Point for RBE Calculation*

Scoring of micronuclei has been suggested as a less time-consuming alternative to chromosome analysis for quantifying chromosomal damage (*1*). Micronuclei are separate, smaller nuclei present in the cytoplasm of a cell in addition to the main nucleus. Since they are not manifest until the first postirradiation interphase of the cell cycle, their quantitative analysis is highly dependent on cell proliferation kinetics. An important step in solving the kinetic problem of the micronucleus assay was certainly the introduction of the cytokinesis block method (*17*), which allows quantitative analysis of micronuclei to be carried out under cell cycle-controlled conditions. Micronuclei originate from acentric fragments or whole chromosomes that have not been included into daughter nuclei during mitotic division. Using immunofluorescence staining to detect centromeres, only 3 to 11% of radiation-induced micronuclei revealed a kinetochore fluorescence, indicating that the majority of micronuclei had originated from acentric fragments (*18, 19*). This result was in agreement with the interpretation of curve-fitting data for radiation-induced acentric fragments and micronuclei by Littlefield *et al.* (*20*), suggesting that owing to the estimates of curve-fitting coefficients, all acentric fragments are recorded as micronuclei, at least at lower radiation doses. From this point of view, it is not surprising that the present RBE values for 20 MeV protons obtained by analysis of micronuclei do not differ from the RBE value for 16.5 MeV protons obtained by scoring dicentrics or acentric fragments in human cells, taking into consideration that in the study with 60 kV X rays (*16*), used for reference for the 16.5 MeV protons, similar linear-quadratic dose–response relationships for dicentrics and for acentric fragments were reported. However, an important prerequisite for obtaining such corresponding results is that the linear-quadratic dose–response curves for the photon radiations used as references in both studies (70 and 60 kV X rays, respectively) were derived at lower doses. Since in the present study the dispersion ratio for the data for micronuclei was smaller at 5 and 10 Gy of 70 kV X rays than at 2 Gy (reference experiment A, Table 1), the dose–response relationship was determined only from the micronucleus data for doses up to 4 Gy (reference experiment B, Table 2). As already mentioned, the decreased dispersion ratio observed at 5 and 10 Gy may reflect a saturation effect. Such an effect could be explained by a combination of different phenomena. First, one may assume a depression of proliferation in highly damaged cells at doses higher than 4 Gy. Some cells, especially the damaged ones with more micronuclei, may be sufficiently delayed and have not reached the binucleate cytokinesis-blocked cell stage. This will influence the frequency of binucleate cells. Second, although a large number of binucleate cells were scored, one could argue that cells containing several acentric fragments in one or more micronuclei might have already died during the first karyokinesis and thus prior to the end of cytokinesis. Such badly damaged cells would escape scoring as micronucleated cytokinesis-blocked cells.

### *Effects of Dose Delivery in High-Intensity Pulses on RBE*

Radiation damage in cells is influenced by the dose, dose rate and quality of the radiation, because the biological effectiveness depends on the spatial and temporal distribution of the energy imparted and the LET of the ionizing particles. Therefore, differences in the dose rate can affect the dose–response relationship for radiation-induced damage in cells, because its quadratic component is dependent on time, whereas the linear component increases with increasing LET. It is well known that the biological effectiveness increases with radiation dose rate in the range below several Gy/min (*15*). However, comparisons of RBEs for single high-intensity pulses of radiation with those for continuous irradiation are scarce and conflicting. Such RBE studies have been carried out in the past predominantly by examining the clonogenic survival of cultured mammalian cells and comparing single high-intensity pulses with continuous irradiation by electrons and X rays.

Reports on survival curves for HeLa cells irradiated under various initial oxygen conditions with nanosecond

pulses of electrons from a linear accelerator (*21*) as well as from a field emission source (*22*) indicate no significant differences in corresponding findings obtained with continuous irradiation. A comparable result could also be observed for an ultrahigh-dose-rate irradiation of Chinese hamster ovary (CHO) cells with electrons delivered as a single 3-ns pulse from a field emission source (*23*).

However, when the mortality of mice after exposure to pulsed X rays from a linear accelerator (single pulse of 3.3 μs) was analyzed, RBE values of 1.11 and 1.28 were found relative to $^{60}$Co γ rays and conventional 180 kV X rays, respectively (*24*). These significantly increased RBE values for X rays given using a pulsed irradiation mode were confirmed in studies using survival of cultured mouse L5178Y cells (*24*). In contrast, there was evidence of a reduction in dicentric yield by a factor of about 2 for exposure of human lymphocytes to ultrahigh-dose X rays delivered in a 2-ns pulse relative to conventional low dose rates (*25*). However, these conflicting results for the biological effectiveness may be due to a lack of cell cycle-controlled analysis as well as the very low number of cells analyzed. This assumption agrees well with a study of Purrott *et al.* (*26*) that indicated no significant difference in the induction of chromosome aberrations in human lymphocytes by 15 MeV electrons delivered in microsecond pulses and at a conventional dose rate.

### *High-Intensity Pulses Derived from Laser-Driven Accelerators*

Recent progress in laser technology has led to extremely intense X-ray sources that are based on laser-produced plasmas. The survival of V79 Chinese hamster lung fibroblast cells using irradiated with X rays from laser-generated plasmas that produced ultrahigh peak absorbed dose rates of $\sim 10^{11}$ Gy/min was not markedly different from that after exposure to conventional X rays (*27*). A similar result was reported for irradiation of cultured mouse L5178Y cells with ultrahigh-dose-rate X rays (single pulse $\leq$1 ps) emitted from laser-produced plasmas (*28*).

To our knowledge, RBEs for protons from a single or a few high-intensity pulses, as could be generated by laser-driven accelerators and used for radiotherapy in the years to come, have not been reported so far. For 20 MeV protons with a dose-averaged LET of 2.66 keV/μm, our data revealed no significant difference in the RBE for pulsed irradiation compared with conventional irradiation. The good reproducibility of our results in two independent experiments with different beam access times demonstrates the stability of the experimental system and the validity of these findings. Our data support the idea that the amount of chromosomal damage inflicted is not markedly affected by the ultrahigh dose rate of the pulsed delivery compared to irradiation times close to a second.

## CONCLUSION

The results of our experiments provided no evidence for a different RBE for micronucleus induction in HeLa cells by pulsed and continuous irradiation with 20 MeV protons. Our results are consistent with most of the earlier investigations on the radiation-induced biological effects obtained. However, it should be taken into account that in the literature, only electrons and photons were applied for experiments with single, short beam pulses of radiation. Therefore, our results using pulsed proton beams add a new element to the available knowledge. Before clinical use of pulsed proton or heavy-ion beams generated by high-intensity lasers is possible, further experiments are necessary to evaluate different radiobiological end points reflecting different aspects of radiation damage, repair, apoptosis and tissue reaction.

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## REFERENCES

1. F. Verhaegen and A. Vral, Sensitivity of micronucleus induction in human lymphocytes to low-LET radiation qualities: RBE and correlation of RBE and LET. *Radiat. Res.* **139**, 208–213 (1994).
2. IAEA, *Biological Dosimetry: Chromosomal Aberration Analysis for Dose Assessment*. Technical report no. 260, IAEA, Vienna, 1986.
3. A. Hauptner, S. Dietzel, G. A. Drexler, P. Reichart, R. Krücken, T. Cremer, A. A. Friedl and G. Dollinger, Microirradiation of cells with energetic heavy ions. *Radiat. Environ. Biophys.* **42**, 237–245 (2004).
4. G. Dollinger, A. Bergmaier, V. Hable, R. Hertenberger, C. Greubel, A. Hauptner and P. Reichart, A pulsed proton microbeam. *Nucl. Instrum. Methods B*, in press.
5. J. F. Ziegler and J. P. Biersack, The stopping and range of ions in matter (SRIM2003). Available online at http://www.srim.org
6. C. Greubel, V. Hable, G. A. Drexler, A. Hauptner, S. Dietzel, H. Strickfaden, I. Baur, R. Krücken, T. Cremer and G. Dollinger, Quantitative analysis of DNA-damage response factors after sequential ion microirradiation. *Radiat. Environ Biophys.* **47**, 415–422 (2008).
7. G. C. Jagetia and R. Aruna, Correlation of micronuclei-induction with the cell survival in HeLa cells treated with a base analogue, azidothymidine (AZT) before exposure to different doses of gamma-radiation. *Toxicol. Lett.* **139**, 33–43 (2003).
8. E. Schmid, H. Braselmann and U. Nahrstedt, Comparison of gamma-ray induced dicentric yields in human lymphocytes measured by conventional analysis and FISH. *Mutat. Res.* **348**, 125–130 (1995).
9. ICRP, *Relative Biological Effectiveness (RBE), Quality Factor (Q), and Radiation Weighting Factor ($w_R$)*. Publication 92, International Commission on Radiological Protection, Pergamon Press, Oxford, 2003.

*10.* M. R. Raju, Proton radiobiology, radiosurgery and radiotherapy. *Int. J. Radiat. Biol*. **67**, 237–259 (1995).

*11.* T. C. Yang, Proton radiobiology and uncertainties. *Radiat Meas*. **30**, 383–392 (1999).

*12.* D. Bettega, M. Bombana, T. Pelucchi, A. Poli, L. Tallone Lombardi and A. M. Fuhrman Conti, Multinucleate cells and micronucleus formation in cultured human cells exposed to 12 MeV protons and γ-rays. *Int. J. Radiat. Biol*. **37**, 1–9 (1980).

*13.* D. Bettega, S. Dubini, A. M. Fuhrman Conti, T. Pelucchi and L. Tallone Lombardi, Chromosome aberrations induced by protons up to 31 MeV in cultured human cells. *Radiat. Environ. Biophys*. **19**, 91–100 (1981).

*14.* E. Schmid, H. Roos, G. Rimpl and M. Bauchinger, Chromosome aberration frequencies in human lymphocytes irradiated in a multi-layer array by protons with different LET. *Int. J. Radiat. Biol*. **72**, 661–665 (1997).

*15.* National Research Council, Committee to Assess Health Risks from Exposure to Low Levels of Ionizing Radiation, *Health Risks from Exposure to Low Levels of Ionizing Radiation (BEIR VII)*. National Academies Press, Washington, DC, 2006.

*16.* D. Regulla, E. Schmid, W. Friedland, W. Panzer, U. Heinzmann and D. Harder, Enhanced values of the RBE and H ratio for cytogenetic effects induced by secondary electrons from an X-irradiated gold surface. *Radiat. Res*. **158**, 505–515 (2002).

*17.* M. Fenech and A. A. Morley, Solutions to the kinetic problem in the micronucleus assay. *Cytobios* **43**, 233–246 (1985).

*18.* D. A. Eastmond and J. D. Tucker, Identification of aneuploidy-inducing agents using cytokinesis-blocked human lymphocytes and an antikinetochore antibody. *Environ. Mol. Mutagen*. **13**, 34–43 (1989).

*19.* E. J. Thomson and P. E. Perry, The identification of micronucleated chromosomes: a possible assay for aneuploidy. *Mutagenesis* **3**, 415–441 (1988).

*20.* L. G. Littlefield, A. M. Sayer and E. L. Frome, Comparisons of dose response parameters for radiation induced acentric fragments and micronuclei observed in cytokinesis arrested lymphocytes. *Mutagenesis* **4**, 265–270 (1989).

*21.* A. H. W. Nias, A. J. Swallow, J. P. Keene and B. W. Hodgson, Survival of HeLa cells from 10 nanosecond pulses of electrons. *Int. J. Radiat. Biol*. **17**, 595–598 (1970).

*22.* E. R. Epp, H. Weiss, B. Djordjevic and A. Santomasso, The radiosensitivity of cultured mammalian cells exposed to single high intensity pulses of electrons in various concentrations of oxygen. *Radiat. Res*. **52**, 324–332 (1972).

*23.* L. E. Gerweck, E. R. Epp, H. B. Michaels, C. C. Ling and E. C. Peterson, Repair of sublethal damage in mammalian cells irradiated at ultrahigh dose rates. *Radiat. Res*. **77**, 156–169 (1979).

*24.* S. Antoku, RBE of pulsed radiations by lethality in mice: Cultured mammalian cells and bacteria. *Radiat. Res*. **51**, 696–704 (1972).

*25.* T. Prempree, A. Michelsen and T. Merz, The repair time of chromosome breaks induced by pulsed X-rays of ultra-high dose rate. *Int. J. Radiat. Biol*. **15**, 571–574 (1969).

*26.* R. J. Purrott, E. J. Reeder and S. Lovell, Chromosome aberration yields in human lymphocytes by 15 MeV electrons given at a conventional dose-rate and in microsecond pulses. *Int. J. Radiat. Res*. **31**, 251–256 (1977).

*27.* C. Tillman, G. Grafström, A. C. Jönnon, I. Mercer, S. Mattsson, S. E. Strand and S. Svanberg, Survival of mammalian cells exposed to ultrahigh dose rates from a laser-produced plasma X-ray source. *Radiology* **213**, 860–865 (1999).

*28.* K. Shinohara, H. Nakano, N. Miyazaki, M. Tago and R. Kodama, Effects of single pulse (≤ 1 ps) X-rays from laser-produced plasmas on mammalian cells. *J. Radiat. Res*. **45**, 509–514 (2004).