

# Measurement of Radiation-induced Damage in Human Glioma Cells with Flow Cytometry<sup>1</sup>

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## ABSTRACT

Using flow cytometry, we studied DNA supercoiling changes in human glioma cell line SF-126 after irradiation. To release nucleoids (dehistonized DNA in a supercoiled form attached to the nuclear matrix), cells were lysed in a high-salt buffer. Radiation-induced changes in nucleoids were measured by flow cytometry as changes in forward light scatter. Propidium iodide titration curves showed that rewinding of DNA supercoils in irradiated cells was inhibited. To optimize the experimental conditions, we analyzed the effect of lysis time and nucleoid size distribution within the sample. Under optimal conditions, changes in nucleoids were detected after radiation doses as low as 0.5 Gy. The repair of radiation-induced damage in nucleoids followed biphasic kinetics; 50% of the damage was repaired within about 5 min, and the remainder within about 30 min. Interestingly, irradiated S-phase cells showed less damage, as measured by this assay, than irradiated G<sub>1</sub>- or G<sub>2</sub>-phase cells, which is consistent with the relative radioresistance of S-phase cells as measured with cell survival assays. Our findings show that flow cytometric measurement of supercoiling changes is a sensitive and relatively rapid method for quantitating radiation-induced damage in individual cells.

## INTRODUCTION

The nucleoid assay measures changes in the state of DNA supercoils and integrity of the attachment of DNA loops to nuclear matrix in individual eukaryotic cells (1-3). This assay uses a DNA intercalating fluorescent dye, such as PI.<sup>3</sup> The size of the nucleoid increases with an increase in PI concentration up to the maximum point where the DNA supercoils are fully unwound. An additional increase in PI concentration causes rewinding of DNA supercoils and produces a decreased nucleoid size. The rewinding process is dependent on topological constraint of DNA loops and integrity of the attachment of DNA loops to nuclear matrix. Ionizing radiation produces several types of DNA damage, including single- and double-strand breaks, base and sugar damage, and macromolecular cross-links (4). Some of these lesions alter the topological constraints on DNA loops, which inhibits the rewinding of DNA supercoils (2, 3, 5) and results in an increase in nucleoid size.

The nucleoid assay usually has been done with a fluorescence microscope and an image analysis system (1-3, 6). The major disadvantages are that it requires a sophisticated image analysis system and it is time consuming to measure large numbers of nucleoids. In contrast, commercial flow cytometers have become relatively user-friendly and can analyze thousands of nucleoids rapidly (7-9). Flow cytometry of nucleoids stained with fluorescent dye can measure both scattered light and fluorescence intensity; light scattered in the forward direction of the integrating laser beam proportionally represents the cross-sectional area of the measured nucleoid. However, the fragility of nucleoids has made it technically difficult to perform this

assay using flow cytometry. In this study, we optimized the nucleoid assay for measuring radiation-induced damage with flow cytometry in human brain tumor cells.

## MATERIALS AND METHODS

**Cell Culture and Irradiation.** *Mycoplasma*-free SF-126 human brain tumor cells were grown as monolayers in EMEM supplemented with 10% FCS and nonessential amino acids (10). Confluent cells were used for all experiments except those conducted to analyze cell cycle effects, which used log-phase cells. Cells were removed from flasks with 0.05% trypsin, 0.02% EDTA, 0.1% glucose, and 0.058% NaHCO<sub>3</sub>. Single-cell suspensions were washed with cold PBS (Mg<sup>2+</sup> and Ca<sup>2+</sup> free), resuspended in PBS at a concentration of 4 × 10<sup>5</sup> cells/ml, and irradiated on ice with a Phillips X-ray machine (Ridge Inc., Tucker, GA) at a dose rate of 1.26 Gy/min. Cells were centrifuged and resuspended in PBS at a concentration of about 4 × 10<sup>6</sup> cells/ml.

**Preparation of Nucleoids.** Immediately after irradiation, or after a desired repair time at 37°C, nucleoids were prepared by mixing one-tenth volumes of cell suspension (4 × 10<sup>6</sup> cells/ml) with lysis solution [1 M NaCl, 10 mM EDTA, 10 mM Tris (pH 8.0), and 0.2% Triton X-100] containing the desired PI concentration (10 to 80 μg/ml) in a small sample tube; the final nucleoid concentration was 4 × 10<sup>5</sup> nucleoids/ml. Samples were placed on ice in the dark for the desired lysis time and then analyzed by flow cytometry.

**Flow Cytometric Analysis.** Flow cytometry was performed on a FACScan (Becton Dickinson, San Jose, CA) bench top flow cytometer equipped with an air-cooled argon-ion laser at 488 nm. Nucleoid suspensions were run at a flow rate of 12 μl/min; the sample tube was clad with an external ice water jacket tube to reduce effects of temperature change. All samples were collected with a standard sample line, with the tip positioned at the bottom of the sample tube. Isoton II, a balanced electrolyte solution (Coulter, Hialeah, FL), was used as sheath fluid. All experiments were done in a darkened room to minimize photobleaching. Nucleoid size was monitored by FSC, fluorescent light from PI-bound DNA was measured at 575-626 nm (*FL2-H*, fluorescence pulse height), and doublets were discriminated by fluorescence pulse width (*FL2-W*, fluorescence pulse width) and area (*FL2-A*, fluorescence pulse area). Normally, about 20 to 35% of the particles in unirradiated and irradiated samples was discriminated as doublets, clumps, and debris. Sample collection time was recorded at 1- or 2-s intervals, depending on sample volume. All data were acquired within the 1024-channel dynamic range of the photomultiplier detectors. The acquisition threshold was set on fluorescent-positive events above channels 36-68, depending on the PI concentration; all event gating was done after acquisition.

The data were analyzed with the Lysis II (Becton Dickinson) program. After gating the data to exclude doublets, clumps, and debris, the mean channel number of FSC was taken as a measure of nucleoid size. Cells in different phases of the cycle were determined from histograms of DNA content (fluorescence intensity of PI-stained DNA) from nucleoids. This is based on the fact that G<sub>1</sub>-phase cells contain one-half as much DNA as G<sub>2</sub>-M-phase cells and that the DNA content of S-phase cells is between that of G<sub>1</sub> and G<sub>2</sub>-M-phase cells. When nucleoids were prepared in this study, the G<sub>2</sub>-M peak contained only G<sub>2</sub>-phase nucleoids; M-phase nucleoids were seen as individual chromosomes.

**Statistical Analysis.** Six experiments were used to evaluate the effect of radiation on cells in S, G<sub>1</sub>, or G<sub>2</sub> phase. For each experiment, three differences, S - G<sub>1</sub>, S - G<sub>2</sub>, and G<sub>1</sub> - G<sub>2</sub>, were calculated. All subsequent analyses were based on these differences. To test for differences at baseline (0 Gy), we used the Wilcoxon signed rank test with the difference from each experiment as the observation. To test for a differential dose effect, the difference, e.g., S - G<sub>1</sub>, was considered as a dependent variable in an analysis of covariance, with dose

Received 8/11/95; accepted 10/27/95.

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<sup>1</sup> This study was supported by NIH Grants CA-09215 and CA-13525.

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<sup>3</sup> The abbreviations used are: PI, propidium iodide; FSC, forward light scatter.

and experiment in the model. Statistically significant coefficients for the dose effects would indicate that the size of the difference changed with dose, implying a differential dose effect. Because it is possible that at the higher doses the damage (expressed as FSC) in the S phase might have reached a plateau, the analysis excluded the 2-Gy dose. Because of the small sample size, no adjustment was made for multiple testing.

**RESULTS**

**Effects of Lysis Time and Nucleoid Size Distribution.** The effect of lysis time on FSC is shown in Fig. 1. In both irradiated and unirradiated nucleoids, FSC increased with increasing lysis time. FSC was greater in irradiated nucleoids than in unirradiated nucleoids at all lysis times longer than 10 min. When all nucleoids in the sample tube were collected, we found that the smaller ones tended to stay at the bottom of the tube and, therefore, were collected before the larger nucleoids, which were mostly found at the top of the tube (Fig. 2). The uneven size distribution was more obvious in large samples (0.5 ml) than in small samples (0.1 to 0.15 ml). In general, nucleoids from control samples could be collected in less time than nucleoids from irradiated samples of equal volume, perhaps because irradiated cells had larger halos, which increased the viscosity of the solution.

When all nucleoids in the sample were collected, radiation-induced changes in FSC were similar after 15 or 30 min of lysis (Fig. 3). Therefore, we selected 15 min as our standard lysis time, and we collected all of the nucleoids in small sample volumes (0.1–0.15 ml).

**Radiation-induced Damage and Repair in SF-126 Cells.** When cells were stained with increasing PI concentrations (10 to 80 µg/ml), rewinding of DNA supercoils was inhibited by 1 Gy of radiation (Fig. 4). At a high PI concentration (40 µg/ml), FSC increased with increasing radiation doses from 0.5 to 5 Gy and then reached a plateau. (Fig. 5).

The radiation dose-response curves of cells in different phases of the cell cycles are shown in Fig. 6. FSC from unirradiated cells was

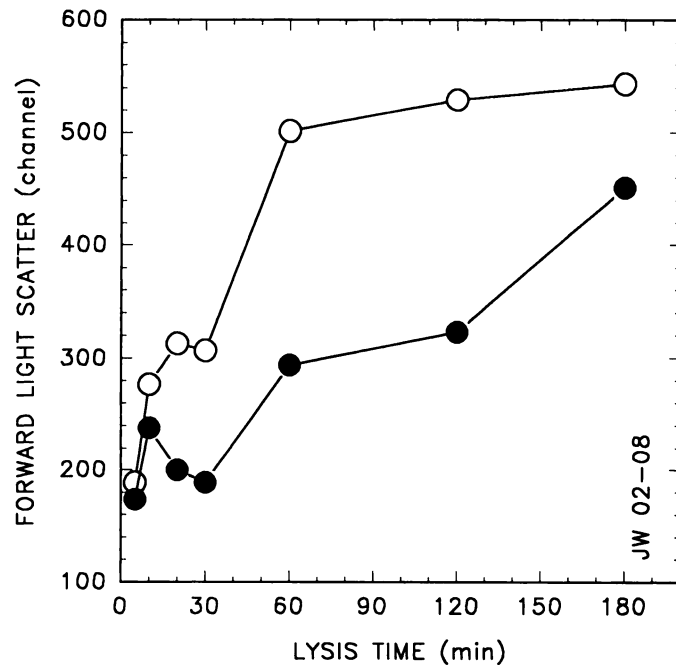


Fig. 1. Effect of lysis time on measurement of FSC from irradiated (5 Gy) nucleoids (○) and unirradiated nucleoids (●). Cells were placed in 0.2 ml of lysis buffer containing PI (40 µg/ml) on ice and left undisturbed until the samples were collected. For each data point, 5000 nucleoids were collected over 1–2 min. The mean FSC from G<sub>1</sub> cells was plotted as a function of lysis time.

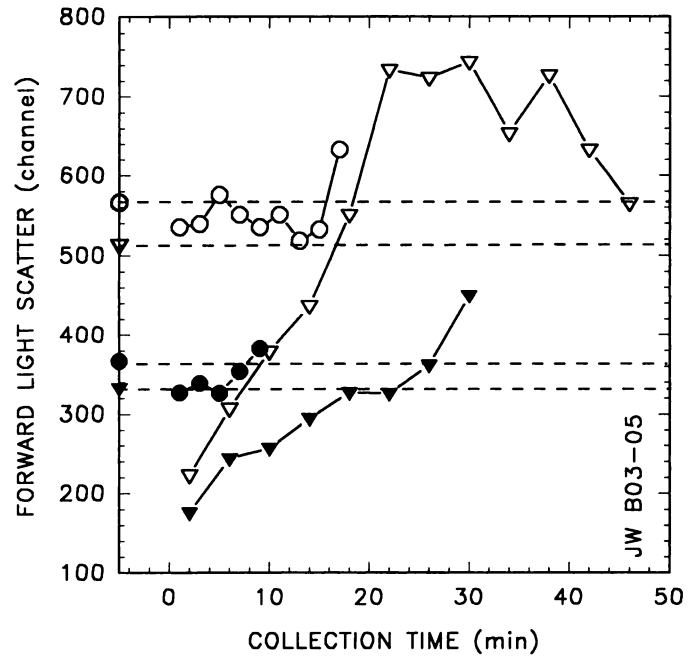


Fig. 2. Distribution of FSC from nucleoids in the sampling tube. Cells were placed in 0.5 ml (▽, ▼) or 0.15 ml (○, ●) lysis buffer containing PI (40 µg/ml) and kept on ice for 15 min. All nucleoids in the sample were collected. The mean FSC was calculated every 2 min for 0.15 ml samples or every 4 min for 0.5 ml samples. Cells were either irradiated (5 Gy, ▽, ○) or unirradiated (▼, ●). — — —, the mean FSC of all the nucleoids collected for each sample.

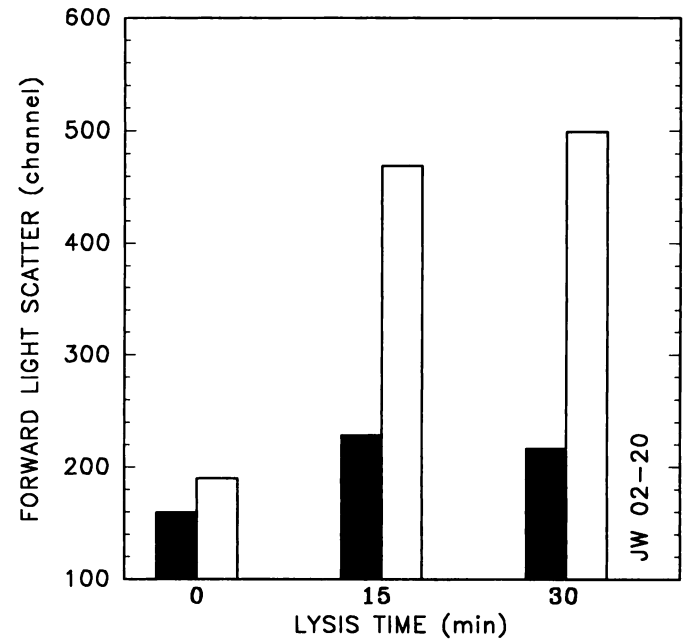


Fig. 3. Effect of lysis time on FSC. Cells were placed in 0.15 ml of lysis buffer containing PI (40 µg/ml) and kept on ice for the desired time before all nucleoids in the sample were collected; collection time was approximately 10 min. The mean FSC of the whole sample was calculated. Cells were either irradiated (5 Gy, □) or unirradiated (■).

dependent on cell cycle phase with S-phase cells > G<sub>2</sub> phase cells > G<sub>1</sub> phase cells (*P* = 0.03). Evaluation of dose effects showed no difference between G<sub>1</sub> and G<sub>2</sub> (*P* = 0.19). The differences in FSC between S and G<sub>1</sub> or S and G<sub>2</sub> decreased with increasing dose (*P* < 0.001). This represents a differential dose response, with S-phase cells less affected by radiation.

The repair of radiation-induced damage expressed in nucleoids is

shown in Fig. 7. The curve followed biphasic kinetics. Radiation-induced damage in nucleoids was repaired very quickly; ~50% of damage was repaired within 5 min, and all damage was repaired within 30 min.

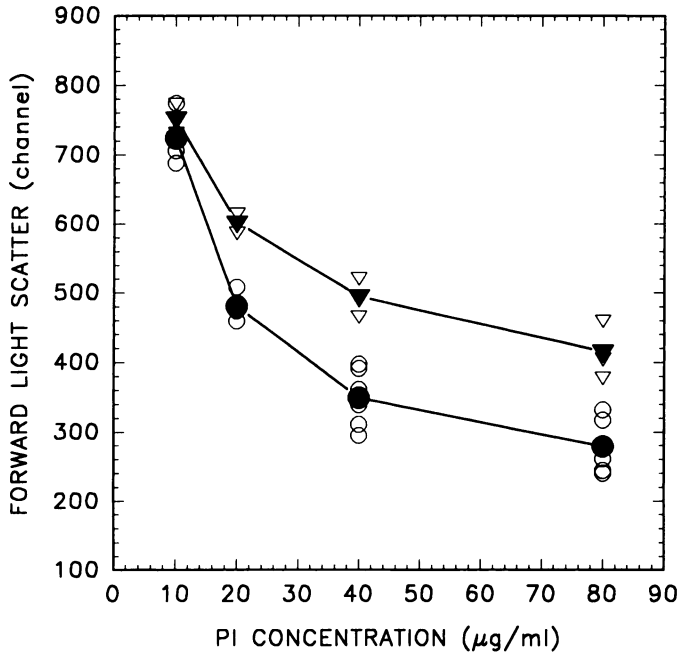


Fig. 4. Change in FSC as a function of PI concentration in nucleoids from unirradiated and irradiated cells. Cells were placed in 0.1 ml of lysis buffer containing the desired PI concentration and kept on ice for 15 min. All nucleoids in the sample were collected. The mean FSC was plotted as a function of PI concentration. Cells were either irradiated (1 Gy, ▽, ▼) or unirradiated (○, ●). ▽ and ○, data from three to five experiments; ▼ and ●, the average FSC at each PI concentration.

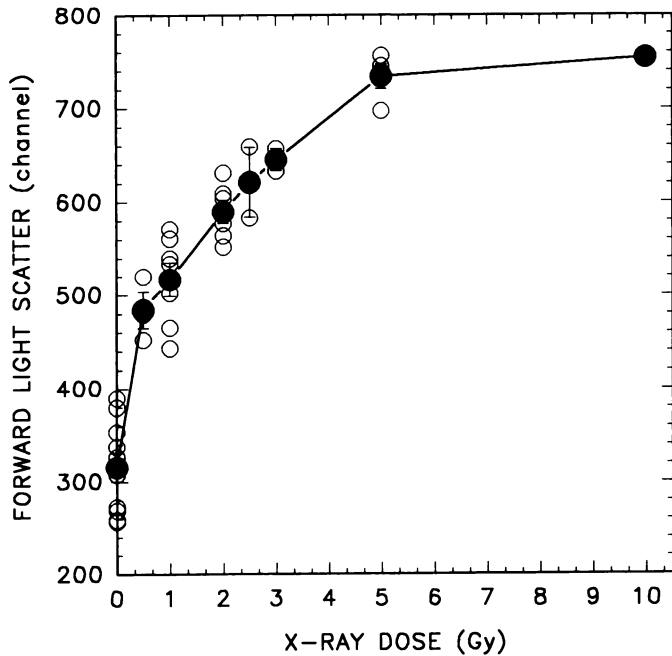


Fig. 5. Radiation-induced changes in FSC from irradiated and unirradiated nucleoids. Cells were placed in 0.1 ml of lysis buffer containing PI (40 µg/ml) and kept on ice for 15 min. All the nucleoids in the sample were collected. The mean FSC was plotted as a function of radiation dose. ○, data from 2 to 10 experiments; ●, the average FSC for each dose. Bars, SEMs from 2 to 10 experiments.

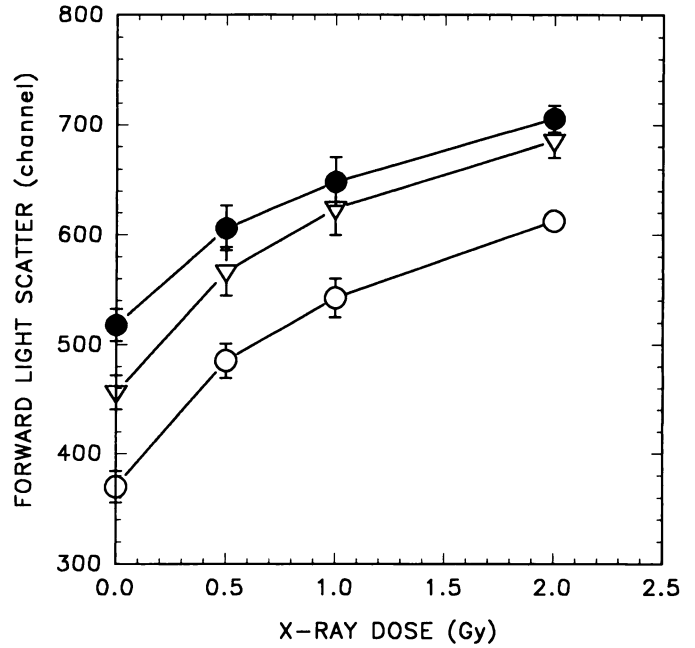


Fig. 6. Radiation-induced changes in FSC of nucleoids from cells in different phases of the cell cycle, as determined from the fluorescence profiles of PI-DNA. Log-phase cells were irradiated with desired doses, and nucleoids were prepared and analyzed as described in Fig. 5. ○, G<sub>1</sub> phase; ●, S phase; ▽, G<sub>2</sub> phase. Bars, SEMs from six experiments.

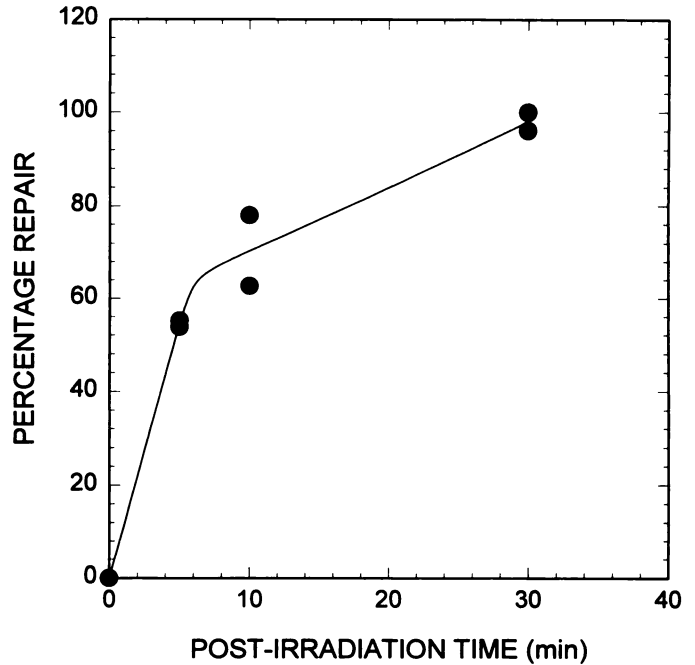


Fig. 7. Repair of radiation-induced (2 Gy) nucleoid damage as a function of postirradiation time. Damage in nucleoids was measured under the conditions described in Fig. 5. ●, data from individual experiments.

## DISCUSSION

This study shows that radiation-induced damage in human brain tumor cells can be measured by the nucleoid assay performed with flow cytometry. However, the nucleoids are fragile, and the conditions of the assay must be optimized, including detergent (Triton X-100) concentration, salt (NaCl) concentration, cell concentration, lysis time, and the number of cells collected. To detect damage produced by a low radiation dose, we found that SF-126 cells must be lysed for

at least 10 min on ice. To obtain a representative measurement in this cell line, we found that all nucleoids from small samples (0.15 ml or less) must be analyzed because the size distribution of nucleoids within samples is uneven. The uneven size distribution may result from different densities of individual nucleoids; larger nucleoids have low density and float on the top of the sample, and smaller nucleoids have higher density and settle toward the bottom of the sample.

Under optimal conditions, this assay is very sensitive for measuring radiation-induced damage in cells. At radiation doses as low as 0.5 Gy, a 52% increase in FSC was observed. The kinetics of repair of radiation-induced nucleoid damage were biphasic. Because nucleoid damage is mostly due to DNA single-strand breaks, it was repaired quickly (11). About 50% of the initial damage was repaired within 5 min, and the remainder of the damage was repaired within 30 min.

The higher FSC in unirradiated G<sub>2</sub>-phase nucleoids compared to G<sub>1</sub>-phase nucleoids is to be expected, considering the larger size of G<sub>2</sub> cells. Unirradiated S-phase nucleoids showed an even higher FSC than G<sub>2</sub>-phase nucleoids. This may result from the loose attachment of DNA loops to nuclear matrix during DNA replication in S phase and/or from the growth forks that act like strand breaks in this assay (1, 12). Our results also showed less radiation-induced nucleoid damage in S-phase cells than in cells in G<sub>1</sub> or G<sub>2</sub> phases. Such differences were not due to the saturation of the assay, because they were statistically significant without including the highest dose (2 Gy). This finding is consistent with unpublished results from colony-forming assays of SF-126 cell survival observed in our laboratory, which have shown increased radioresistance in S-phase cells.<sup>4</sup> The relative radioresistance of S-phase cells is also common to other cell lines (13).

Other investigators have shown less radiation-induced nucleoid damage in radioresistant than in radiosensitive cells using flow cytometric analyses (7, 9, 14) or using a microscope combined with an image analysis system (15–20). These findings suggest that the nucleoid assay may be a potentially useful method for determining the radiosensitivity of human brain tumor cells.

## ACKNOWLEDGMENTS

We thank Dr. Andrew Vaughan (Loyola University, Chicago, IL), Dr. Hiram Basu (University of Wisconsin, Madison, WI), and Dr. Burt Feuerstein (University of California, San Francisco, CA) for helpful discussions. We also

<sup>4</sup> N. Gupta, L. Hu, and D. F. Deen, unpublished data.

thank Cheryl Christensen for manuscript preparation and Stephen Ordway for editorial assistance.

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