Experimental Cell Research 175 (1988) 184–191

A Simple Technique for Quantitation of Low Levels of DNA Damage in Individual Cells[1]

NARENDRA P. SINGH,*[2] MICHAEL T. McCOY,* RAYMOND R. TICE,† and EDWARD L. SCHNEIDER*

**Laboratory of Molecular Genetics, Gerontology Research Center, National Institute on Aging, Baltimore, Maryland 21224, and †Medical Department, Brookhaven National Laboratory, Upton, New York 11973*

Human lymphocytes were either exposed to X-irradiation (25 to 200 rads) or treated with H_2O_2 (9.1 to 291 μM) at 4°C and the extent of DNA migration was measured using a single-cell microgel electrophoresis technique under alkaline conditions. Both agents induced a significant increase in DNA migration, beginning at the lowest dose evaluated. Migration patterns were relatively homogeneous among cells exposed to X-rays but heterogeneous among cells treated with H_2O_2. An analysis of repair kinetics following exposure to 200 rads X-rays was conducted with lymphocytes obtained from three individuals. The bulk of the DNA repair occurred within the first 15 min, while all of the repair was essentially complete by 120 min after exposure. However, some cells demonstrated no repair during this incubation period while other cells demonstrated DNA migration patterns indicative of more damage than that induced by the initial irradiation with X-rays. This technique appears to be sensitive and useful for detecting damage and repair in single cells.

Techniques which permit the sensitive detection of DNA damage have been useful in studies of environmental toxicology, carcinogenesis, and aging [1, 2]. Since the effects of environmental toxicants, cancer, and aging are often tissue and cell-type specific [3–5], it is important to develop techniques which can detect DNA damage in individual cells. Rydberg and Johanson [6] were the first to directly quantitate DNA damage in individual cells by lysing cells embedded in agarose on slides under mild alkali conditions to allow the partial unwinding of DNA. After neutralization, the cells are stained with acridine orange and the extent of DNA damage is quantitated by measuring the ratio of green (indicating double-stranded DNA) to red (indicating single-stranded DNA) fluorescence using a photometer. This technique, however, is not widely used as numerous critical steps are involved in the processing.

To improve the sensitivity for detecting DNA damage in isolated cells, the same laboratory [7] developed a microgel electrophoresis technique. In this technique, cells are embedded in agarose gel on microscope slides, lysed by detergents and high salt, and then electrophoresed for a short period under neutral conditions. Cells with increased DNA damage display increased migration of DNA from the nucleus toward the anode. The migrating DNA is quantitat-

[1] The U.S. Government's right to retain a nonexclusive royalty-free license in and to the copyright covering this paper, for governmental purposes, is acknowledged.

[2] To whom reprint requests should be addressed.

0014-4827/88 $03.00

ed by staining with ethidium bromide and by measuring the intensity of fluorescence at two fixed positions within the migration pattern with a microscope photometer. However, while the neutral conditions for lysis and electrophoresis permit the detection of double-stranded DNA breaks, they do not allow for the detection of single-stranded ones. Since many agents induce from 5- to 2000-fold more single-stranded than double-stranded breaks [8], neutral conditions are clearly not as sensitive as alkaline conditions in detecting DNA damage. Alkaline conditions would also result in the degradation of cellular RNA, which otherwise could interfere in the quantitation of the ethidium bromide-stained samples.

We have modified the microgel electrophoresis technique to permit an evaluation of DNA damage in single cells under alkaline conditions. This approach optimizes DNA denaturation and the migration of single-stranded DNA, thus permitting an evaluation of single-stranded DNA breaks and alkali-labile sites. Details of the technique and some of our studies to validate the applicability of the approach for measuring DNA damage and repair in single cells are presented here.

MATERIALS AND METHODS

Low-melting-temperature agarose was purchased from BRL (Gathersburg, MD); Triton X-100 from Bio-Rad laboratories (Richmond, CA); sodium sarcosinate, ethylenediaminetetraacetic acid, disodium salt (Na_2-EDTA), Tris base, and ethidium bromide from Sigma Chemical Company (St. Louis, MO); hydrogen peroxide (H_2O_2), 30% solution, from Fisher Scientific (Fair Lawn, NJ); phosphate-buffered saline (PBS), without calcium and magnesium, and RPMI 1640 medium from GIBCO (Grand Island, NY); lymphocyte separation medium from Litton Bionetics, Inc. (Charleston, SC); and TRI X 135, ASA 400, black and white film from Eastman Kodak (Rochester, NY).

Lymphocytes were separated from whole blood utilizing Ficoll–Hypaque lymphocyte separation medium, washed in RPMI 1640, and suspended in PBS at a concentration of 30 million cells/ml. The blood used in these DNA damage studies was obtained from the same adult male donor. From 1000 to 500,000 cells were mixed with 25 μl of 0.5% low melting temperature agarose at 37°C and then placed on a precleaned microscope slide (Curtin Matheson Scientific Inc., Houston, TX, USA, Cat No. 267-0960) which were already covered with thin layer of 0.5% normal melting agarose to promote even and firm attachment of second layer. The cell suspension was immediately covered with a No. 1 coverglass, and the slides were then kept at 4°C for 5 min to allow solidification of the agarose. After gently removing the coverglass, the slides were covered with a third layer of low melting agarose by using a coverglass and then placed horizontally in a steel tray and returned to 4°C. The cells embedded in the agarose on the slides were exposed to X-rays or to H_2O_2 within 20 min of their preparation.

For X-irradiation, a Phillips Model MG 300 X-ray machine (Ridge Instrument Company, Inc., Tucker, GA) was used at a dose rate of 200 rads/min. Slides were treated with various concentrations of H_2O_2 in cold PBS. In the initial damage studies, both X-ray and H_2O_2 treatments were kept at 4°C to avoid repair of damage induced by these agents. To assess the kinetics of DNA repair, lymphocytes isolated from blood obtained from three adult male individuals were exposed to 200 rads and then incubated for various times in RPMI 1640 (supplemented with 10% fetal bovine serum) at 37°C in a 5% CO_2:95% air incubator. Cells were centrifuged at 4°C, resuspended in a small volume of PBS, and mixed with agarose and slides were prepared as described before. After the solidification of the agarose covering, the slides were immersed in a lysing solution (1% sodium sarcosinate, 2.5 *M* NaCl, 100 m*M* Na^2-EDTA, 10 m*M* Tris, pH 10, and 1% Triton X-100, added fresh) for 1 h to lyse the cells and to permit DNA unfolding. The slides were then removed from the lysing solution and placed on a horizontal gel electrophoresis unit. The unit was filled with fresh electrophoretic buffer (1 m*M* Na_2-EDTA and 300 m*M* NaOH) to a level 0.25 cm above the slides. The slides were allowed to set in this high-pH buffer for 20 min to allow unwinding of DNA before electrophoresis. Electrophoresis was conducted for the next 20 min at 25 V using an electrophoresis compact power supply (International Biotechnologies, Inc., New Haven, CT).

All of the steps described above were conducted under yellow light or in the dark to prevent

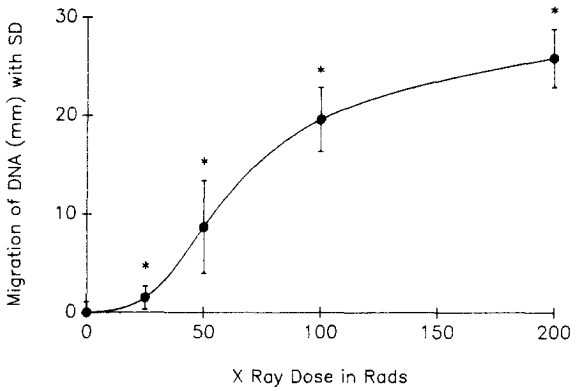

Fig. 1. Length of DNA migration as a function of X-ray dose. The average nuclear size of unexposed cells has been subtracted from each exposed group to obtain DNA migration. Each point represents the mean of 20 cells. The range bars indicate standard deviations. * indicates significantly different from control data at $P<0.0125$ (α of 0.05 Bonferroni corrected for four pairwise comparisons), based on Student t test using separate variances.

additional DNA damage. After electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining, by placing them horizontally and flooding them slowly with 0.4 *M* Tris, pH 7.5. After 5 min, the slides were stained by placing 25 μl of 20 μg/ml ethidium bromide in distilled water solution on each slide, and then covering the slide with a coverglass. Observations were made using an Axiomat microscope (Zeiss, RG), equipped with an excitation filter of 515–560 nm and a barrier filter of 590 nm. Photomicrographs of single cells were taken at 400× magnification using TRI X 135 black and white film, ASA 400. DNA migration was determined on a negative photomicrograph by measuring the nuclear DNA and the migrating DNA in 20 randomly selected cells in each exposure group.

The effect of dose on the length of the DNA migration was analyzed using a one-tailed trend test, with the α level set at 0.05. For a determination of the regression coefficient, multiple linear regression analysis was used. To determine the lowest dose at which a significant increase in the length of migration occurred, multiple pairwise comparisons were conducted between the control data and each dose using Student t test, with the α level appropriately Bonferroni corrected for the number of comparisons made.

RESULTS AND DISCUSSION

A significant increase (one-tailed trend $P<0.001$) in the length of DNA migration was observed in human lymphocytes exposed to ionizing radiation over a dose range of 25 to 200 rads (Fig. 1). Photomicrographs of typical lymphocytes in control samples or exposed to X-rays are presented in Fig. 2. Under the electrophoretic conditions used, no migration of DNA occurred among the majority of the control cells and an approximately linear increase in the length of DNA migration was observed for doses between 25 and 100 rads (correlation coefficient $r=0.92$). By 200 rads, the length of migration appeared to plateau, while the extent of DNA damage in cells exposed to greater doses was too great to permit an accurate measurement of the migration pattern. At each dose of radiation, a relatively homogeneous response in the extent of DNA migration among cells was observed (Fig. 3).

In human lymphocytes exposed to H_2O_2, a significant increase in the migration of DNA occurred at concentrations between 9.1 and 291 μ*M* ($P<0.001$) (Fig. 4).

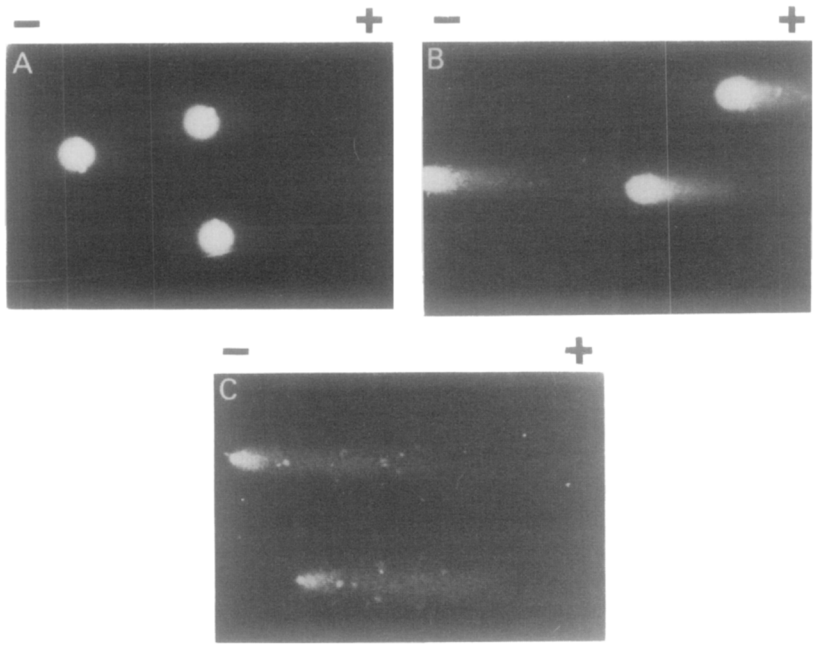

Fig. 2. Photomicrograph negatives of typical DNA migration patterns in (*A*) untreated human lymphocytes; (*B*) human lymphocytes exposed to 50 rads of X-rays; (*C*) human lymphocytes exposed to 100 rads of X-rays. Pictures were taken with TRI X 135, ASA 400, black and white film.

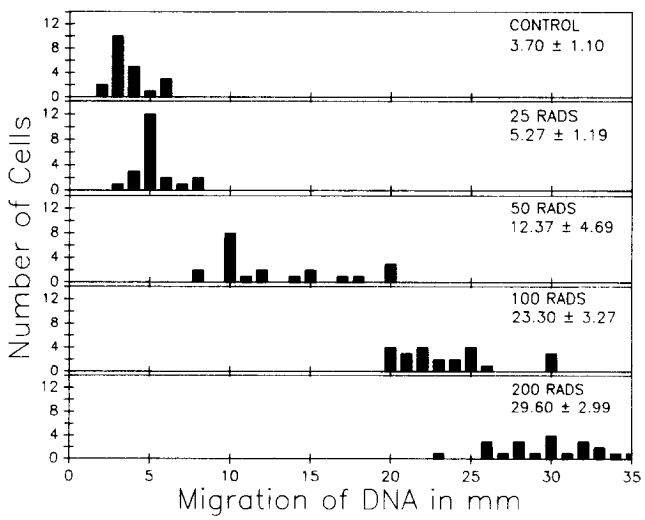

Fig. 3. Histogram of the distribution of the length of migration observed among 20 cells as a function of X-ray dose. The mean and standard deviation of each distribution are provided. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells includes the size of the nucleus. The width of each bar represents 1 mm.

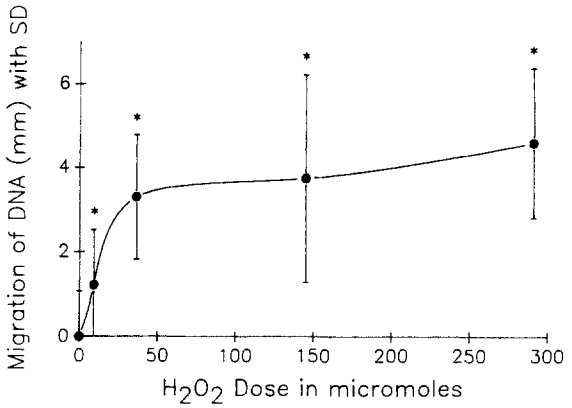

Fig. 4. Length of DNA migration as a function of H_2O_2 concentration. The average nuclear size of unexposed cells has been subtracted from each exposed group to obtain DNA migration. Each point represents the mean of 20 cells. The range bars indicate standard deviations. * indicates significantly different from control data at $P<0.0125$ (α of 0.05 Bonferroni corrected for four pairwise comparisons), based on Student t test using separate variances.

The extent of migration plateaued at H_2O_2 concentrations above 36.4 μM. However, in contrast to the relatively homogeneous DNA migration patterns observed for lymphocytes exposed to X-rays, extensive differences in the length of DNA migration, and thus in the extent of DNA damage, were observed among cells exposed to H_2O_2 (Fig. 5). There are several possible explanations for the differential response observed for these two agents. Individual cells may vary in their permeability to H_2O_2, their radical scavenging capabilities, the access of H_2O_2 or its metabolites to DNA, and other mechanisms which either enhance or

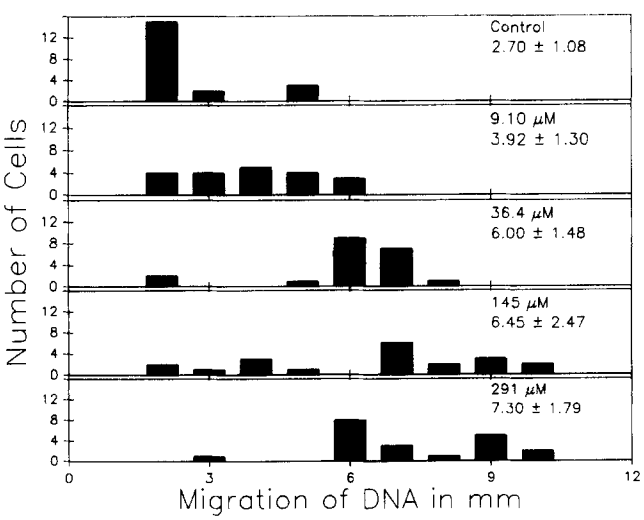

Fig. 5. Histogram of the distribution of the length of migration observed among 20 cells as a function of H_2O_2 concentration. The mean and standard deviation of each distribution are provided. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells included the size of the nucleus. The width of ear bar represents 1 mm.

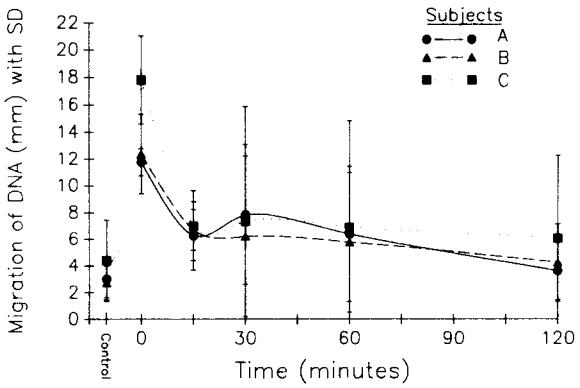

Fig. 6. Length of DNA migration in human lymphocytes exposed to 200 rads of X-rays as a function of postexposure incubation time. Each point represent the mean migration length, including nuclear size, for 20 cells, while the error bars indicate the standard deviation.

diminish the effects of H_2O_2. Whatever may be the mechanism for this differential response to these two agents, our data demonstrate the usefulness of this technique for examining DNA in individual cells.

To further examine the potential of this technique, human peripheral blood lymphocytes obtained from three donors were exposed to 200 rads of X-irradiation and incubated at 37°C in complete medium for from 15 to 120 min to assess the kinetics of DNA repair. In the lymphocytes from all three individuals, the bulk of the repair occcurred within the first 15 min, with a second, slower component that was essentially complete by the end of the 120-min incubation period (Fig. 6). However, there was considerable variability among cells in their ability to repair X-ray-induced DNA damage (Fig. 7). Even at 120 min after

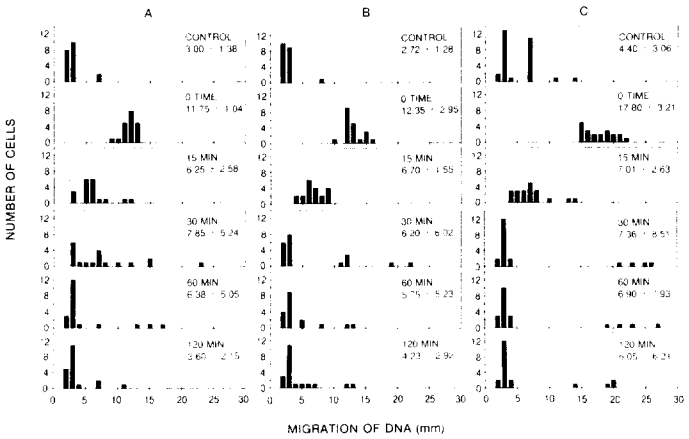

Fig. 7. Histogram of the distribution of the length of migration observed among 20 cells in three subjects as a function of postexposure incubation time after treatment with 200 rads of X-rays. The control data represent the mean nuclear size since no migration of DNA occurred. The length of migration among exposed cells includes the size of the nucleus. The mean and standard deviation of each distribution are provided. The width of each bar represents 1 mm.

treatment, the extent of DNA migration in some cells in each individual suggested a complete lack of repair capacity. Furthermore, some cells exhibited DNA migration patterns in length greater than that which occurred immediately after the X-ray treatment, which may be indicative of cell death.

Using the same microgel electrophoresis technique, but under neutral pH conditions, we were unable to detect any migration of DNA at up to 100 rads of X-rays. Under neutral conditions, DNA remains double-stranded and will migrate most readily in an electrophoretic field in regions of double-stranded breaks. The ratio of double-stranded to single-stranded DNA breaks is 1 : 20 for X-rays and 1 : 2000 for H_2O_2 [8]. Thus, it is clear that alkaline conditions will permit a more sensitive detection of DNA damage, including single- and double-stranded DNA breaks and alkali-labile regions, such as apurinic and apyrimidinic sites [9] and phosphotriesters [10].

Using two agents, X-rays and H_2O_2, at relatively low doses, we have demonstrated the detection of DNA damage in individual human lymphocytes. As few as 1 cell and as many as 500,000 cells can be placed on a single slide, making the technique applicable to microsampling procedures. For best results, the slides should be examined shortly after electrophoresis. The 37°C temperature for the agarose appears to be optimum for cell viability and for the adherence of the cells to the slides. The use of this temperature also seems to aid in the easy removal of the coverglass after the agarose has solidified at 4°C. Twenty minutes appears to be the optimum time for allowing the DNA to unwind under alkaline conditions. In our experiments, whole blood has been used with success and thus eliminates the necessity for lymphocyte isolation. Also, since the length of migration depends upon the percentage of agarose in the gel and upon the duration of electrophoresis, it should be possible by using either higher-percentage agarose gels or shorter electrophoretic times to quantitate greater amounts of DNA damage in single cells. Conversely, increasing the duration of electrophoresis would perhaps permit an evaluation of extremely low levels of DNA damage. Finally, quantitation of fluorescence intensity throughout the DNA migration pattern by the use of a microdensitometric or image-analyzing system should provide a more quantitative assessment of DNA damage.

In conclusion, we have developed a simple approach for the sensitive detection of DNA damage as well as the assessment of DNA repair in individual cells. In the applications described above, we have observed cellular heterogeneity both in the response to DNA damage (with H_2O_2) and in DNA repair. Further application of this approach should facilitate insight into these differences in cellular response to DNA damage.

N.P.S. was supported by the Glenn Foundation for Biomedical Research and by the Donald H. and Charity Yarborough Fund. M.M. is supported by the John D. and Catherine T. MacArthur Foundation Research Program on Successful Aging. R.R.T. is supported at Brookhaven National Laboratory by the Department of Energy under prime contract DE-AC02-76CH00016. This research was supported by the National Institute on Aging. Our sincere thanks to Dr. Ronald W. Hart, director of the National Center for Toxicological Research, Jefferson, Arkansas, Dr Douglas Brash and Kirsten Watson of the National Cancer Institute, Bethesda, Maryland, for their valuable suggestions.

REFERENCES

1. Ames, B. N. (1983) *Science* **221**, 1256–1264.
2. Cerutti, P. A. (1985) *Science* **227**, 367–381.
3. Ono, T., and Okada, S. (1976) *Exp. Gerontol.* **11**, 127–132.
4. Lewensohn, R., Ringborg, U., Baral, E., and Lambert, B. (1982) *J. Cell Sci.* **54**, 69–78.
5. Niedermuller, H., Hofecker, G., and Skalicky, M. (1985) *Mech. Aging Dev.* **29**, 221–238.
6. Rydberg, B., and Johanson, K. J. (1978) *in* DNA Repair Mechanism (Hanawalt, P. C., and Friedberg, E. C., Eds), pp. 465–468, Academic Press, New York.
7. Ostling, O., and Johanson, K. J. (1984) *Biochem. Biophys. Res. Commun.* **123**, 291–298.
8. Bradley, M. O., and K. W. (1979) *Nucleic Acids Res.* **7**, 793–804.
9. Ross, J., and Tang, M. (1985) *Anal. Biochem.* **144**, 212–217.
10. Conrad, J., Muller, N., and Eisenbrand, G. (1986) *Chem. Biol. Interact.* **60**, 57–65.

Received September 18, 1987

13–888333

Printed in Sweden