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Fluorometric Method for Rapid Detection of DNA Strand Breaks in Human White Blood Cells Produced by Low Doses of Radiation

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ABSTRACT

DNA strand breaks can be detected with great sensitivity by exposing crude cell lysates to alkaline solutions and monitoring the rate of strand unwinding. As little as one strand break per chromosome can be detected. Previous methods for measuring strand unwinding have required physical separation of single- from double-stranded molecules. We now describe conditions under which unwinding can be monitored directly using a fluorescent dye, thus greatly simplifying the analysis. Breaks due to irradiation of blood samples by ^{60}Co γ-rays at doses as low as 0.05 to 0.1 gray (5 to 10 rads) were detectable. Rapid rejoining of strand breaks during *in vitro* incubation at 37° could readily be observed following a dose of one gray. Since the procedure is very rapid and cells can be analyzed directly without the requirement for culturing or radiolabeling, the procedure could be useful in cancer chemotherapy if *in vivo* damage is to be monitored or for testing the *in vitro* sensitivity of cells to drugs.

INTRODUCTION

DNA is generally considered to be the most critical cellular target when considering the lethal, carcinogenic, and mutagenic effects of drugs, radiation, and environmental chemicals. These agents may damage DNA by altering bases or disrupting the sugar-phosphate backbone. Although base damage may have serious consequences for a cell, low levels of base damage are difficult to measure by physical or chemical means (12). By contrast, DNA strand breaks can be detected with great sensitivity by methods which utilize the observation that the rate of unwinding of the 2 DNA strands in alkali is related to the covalent length of the strands (1, 7, 8, 13, 15). As little as 1 break per chromosome [equivalent to approximately 0.04 gray (1 gray = 100 rads) of ^{60}Co γ-irradiation] can give a detectable increase in the rate of unwinding (14). We now report a new procedure, FADU[2], which is appreciably simpler to perform than earlier methods. Details of the procedure are described, and an example of its use for detecting initial DNA damage and its subsequent repair in human peripheral WBC following γ-irradiation is presented. Elsewhere, we have reported that FADU is applicable to other DNA-damaging agents and cells from animal organs (9).

PRINCIPLE OF THE METHOD

When double-stranded DNA is exposed to moderately alkaline solutions, hydrogen bonds are broken and the 2 strands unwind. For relatively small DNA molecules (such as DNA from bacterial viruses), the rate of strand unwinding is very rapid, going to completion in less than 30 sec (3). By contrast, the very large DNA molecules present in mammalian cells may require hours of exposure to alkali for complete unwinding to occur (13). It has been observed that the rate of unwinding of large DNA molecules in alkali is increased by prior exposure of cells to ionizing radiation. This is interpreted to mean that radiation-induced strand breaks are responsible for the increased rate of unwinding and, conversely, that an increased rate of DNA unwinding can be used as a sensitive measure of strand breaks (1, 7, 8, 13, 15).

Earlier methods for detecting DNA unwinding in alkali have required physical separation of single-stranded from double-stranded DNA using a hydroxyapatite column, specific nuclease digestion and precipitation, or filter binding (1, 7, 8, 13, 15). In addition, radiolabeling of cells was required for detection of the small amounts of DNA involved. In cases where radiolabeling was not feasible or was to be avoided, sensitive fluorometric methods were substituted to permit detection and quantitation of DNA after column or filter separation (2, 4–6). We now describe the use of a fluorescent dye for monitoring DNA unwinding in a different way. In our method, the dye is used as a direct probe of DNA structure, obviating the need for physical separation of single-stranded from double-stranded DNA; the final procedure is consequently simpler and more rapid than were earlier methods. The principle of the procedure is as follows. Morgan and Pulleyblank (10) have reported that the fluorescent dye, ethidium bromide, will bind selectively to double-stranded DNA in the presence of single-stranded DNA when short duplex regions in "single-stranded" DNA molecules are destabilized by alkali. We used this observation to develop conditions applicable to crude extracts of mammalian cells under which the dye would show fluorescent enhancement preferentially with double-stranded DNA with little interference by RNA other cell components, or single-stranded DNA. A cell suspension is divided equally among 3 sets of tubes. The contribution to fluorescence by components other than double-stranded DNA (including free dye) is estimated from a blank sample (*B*) in which the cell extract is first sonicated lightly and then treated with alkali under conditions which cause complete unwinding of low-molecular-weight double-stranded DNA. A second sample is used for estimating total fluorescence (*T*), *i.e.*, fluorescence due to the presence of double-stranded DNA plus contaminants. The difference ($T - B$) provides an estimate of the amount of double-stranded DNA in the extracts. A third sample (*P*) is used to estimate the unwinding rate of the DNA. The crude cell extract is exposed to alkaline conditions sufficient to permit partial unwinding of the DNA, the degree of unwinding being related to the size of the DNA. The fluorescence of this sample less the fluorescence of the blank ($P - B$) provides an estimate of the amount of

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[2] The abbreviations used are: FADU, fluorometric analysis of DNA unwinding; Percent D, percent of double-stranded DNA remaining after partial alkali treatment.

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double-stranded DNA remaining. Percent D is given by the relationship

$$\text{Percent D} = (P - B) \div (T - B) \times 100$$

MATERIALS AND METHODS

Irradiation of Blood. Samples from human volunteers were collected in 5-ml B-D Vacutainer tubes containing EDTA (3.6 mM) as an anticoagulant. In some experiments, samples from 3 to 4 donors were pooled. For irradiation, 3 ml of blood at 0° was exposed to ^{60}Co γ-rays at a dose rate of 0.04 to 0.16 gray/min using a GammaBeam 150 source (Atomic Energy of Canada Limited). For the experiment involving *in vitro* repair of DNA strand breaks, the blood sample was diluted 1:1 before irradiation with Roswell Park Memorial Institute Tissue Culture Medium 1640 (Grand Island Biological Co., Grand Island, N. Y.).

Buffer Solutions. Composition of the solutions used was as follows: Solution A, 0.87% NH_4Cl-10 mM Tris-HCl (pH 7.2); Solution B, 0.25 M meso-inositol-10 mM sodium phosphate-1 mM $MgCl_2$ (pH 7.2); Solution C, 9 M urea-10 mM NaOH-2.5 mM cyclohexanediaminetetraacetate-0.1% sodium dodecyl sulfate; Solution D, 0.45 volume Solution C in 0.20 N NaOH; Solution E, 0.40 volume Solution C in 0.20 N NaOH; Solution F, 1 M glucose-14 mM mercaptoethanol; Solution G, ethidium bromide, 6.7 μg/ml-13.3 mM NaOH. Cyclohexanediaminetetraacetate (diaminocyclohexanetetraacetic acid) was purchased from Aldrich Chemical Co., Milwaukee, Wis., and urea was purchased from BDH Chemicals.

Fluorometric Analysis of DNA Unwinding. A typical analysis of WBC from 3 ml of blood is carried out as follows. The blood is mixed with 9 ml of Solution A and held at 0° for 20 to 30 min until lysis of RBC is complete. The suspension is centrifuged (0°, 20 min, 400 × *g*), the pellet is suspended in 3 ml of Solution A, and cells are again centrifuged for 10 min. This pellet is suspended in 2.7 ml of Solution B to give a total WBC concentration of 5 to 10 × 10^6/ml. Aliquots of this suspension (0.2 ml) are distributed to 12 disposable glass tubes, designated T, P, or B in groups of 4. To each is added 0.2 ml of Solution C, and incubation at 0° is allowed to proceed for 10 min. During this time, cell lysis and chromatin disruption occurs. To the P and B tubes, 0.1 ml of Solution D and 0.1 ml of Solution E are very gently added without mixing. During the subsequent incubation at 0° for 30 min, the alkali diffuses into the viscous lysate to give a final pH of approximately 12.8 (measured at 23°). The contents of the B tubes are sonicated for 1–2 sec to ensure rapid denaturation of the DNA in the alkaline solution. P and B tubes are incubated at 15° for 60 min (or other times as indicated in the charts). Denaturation is stopped by chilling to 0° and addition of 0.4 ml of Solution F with mixing. This lowers the pH to about 11.0 (measured at 23°). The lysates are sonicated briefly to render them homogeneous and diluted with 1.5 ml of Solution G, and their fluorescence is read at room temperature in a Farrand Mark I spectrofluorometer (excitation, 520 nm; analyzer, 590 nm). The T tubes differ from P tubes in that the neutralizing solution, Solution F, is added before the alkaline Solutions D and E so that the DNA is never exposed to a denaturing pH. The extent of DNA unwinding after a given time of exposure of cell extracts to alkali is calculated from the fluorescent values of the T, P, and B samples. Percent D is given by $(P - B) \div (T - B) \times 100$; the use of 12 tubes permits estimation of Percent D in quadruplicate. As discussed above, the B samples are used as a blank, *i.e.*, an estimate of the fluorescence due to all cell components other than double-stranded DNA; sonication before alkali treatment ensures that the DNA is broken sufficiently to become completely single stranded during the exposure to alkali. The reagents as described were designed to give a final pH value which is high enough to destabilize single-stranded DNA and RNA (to give low *B* fluorescence values), yet still below the denaturing pH for double-stranded DNA (high *T* values). Typically, the *T* values are 2 to 2.5 times the *B* values.

Up to the point at which Solution D is added, all steps are carried out under ordinary room illumination; after this step, manipulations are under subdued light and incubations are in a covered bath. All solutions are kept at 0° except Solutions C and G; these are kept at room temperature.

RESULTS AND DISCUSSION

In this report, we describe a procedure, FADU, which allows the rate of DNA unwinding to be measured after transfer of unlabeled cells to alkali. This procedure was applied to human peripheral WBC, and the rate of unwinding of DNA from unirradiated and irradiated (1 gray of ^{60}Co γ-rays) cells was compared (Chart 1). This dose of γ-irradiation produced a readily observable effect upon unwinding rate. The *dotted lines* represent unwinding which occurs at 0° during the 30-min period of equilibration with alkali. Although the rate for irradiated cells is much faster than that for control cells during this initial exposure to alkali, the greatest absolute difference between the 2 curves (Δ*D*) requires subsequent incubation in alkali at 15°. A maximum Δ*D* is observed after 60 min at 15°, following which the 2 curves remain approximately parallel for at least 120 min (data not shown). It is interesting to note that the kinetics of unwinding as monitored by FADU is very similar to

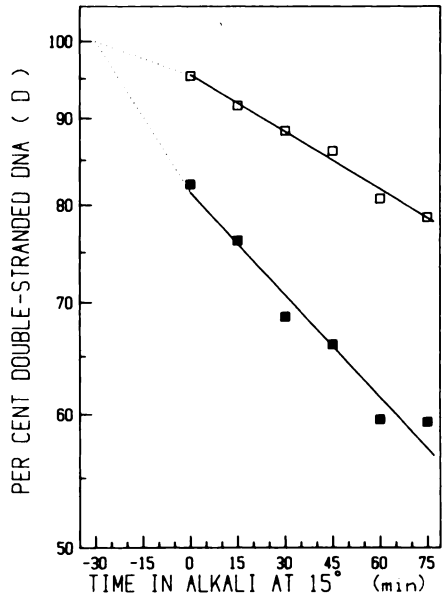

Chart 1. Kinetics of unwinding of control (□) and γ-irradiated (1 gray) (■) human WBC DNA. The fluorometric procedure as described in "Materials and Methods" was used to monitor unwinding. After addition of alkali, extracts were maintained at 0° for 30 min prior to transfer to a 15° bath (*dotted lines*).

unwinding as determined by hydroxyapatite chromatography, although the alkaline solutions used were very different (13). When tetrapropylammonium hydroxide was substituted for NaOH, very similar results were also obtained (data not shown).

The use of a Percent D value measured after 60 min of alkali treatment at 15° has proven to be the most reliable procedure for assessing unwinding rate. When measured in quadruplicate as described in "Materials and Methods," replicate analyses in a single experiment give Percent D values which are within 3%. Percent D values measured on blood samples from different donors over a period of several months have ranged from 76 to 84%. An indication of the sensitivity of the method is given by the dose-response curve for irradiated whole blood shown in Chart 2. ΔD represents the difference between the Percent D of unirradiated cells (84.1%) and cells irradiated with the indicated dose. The effect of a dose as low as 0.05 gray was detectable. This represents DNA damage equivalent to approximately 1 strand break per average human chromosome and approaches the sensitivity hitherto attainable only by use of a double-label radioisotopic technique (14). The dose-response curve is slightly nonlinear below 0.02 gray and above 1 gray, the reasons for which have not yet been established. In this experiment, the dose rate of different samples varied from 0.04 to 0.16 gray/min but, since irradiation was carried out at 0°, no differences due to dose rate would be expected. Conditions for decreasing the sensitivity to allow more precise measurements at higher doses (>2 to 3 grays) have not been fully explored. See "Addendum."

Following an irradiation insult, most cultured cells have the ability to rejoin rapidly the DNA strand breaks which are produced (11). That FADU may be used to follow this process in human peripheral WBC is indicated by the experiment depicted in Chart 3. A sample of blood was diluted with an equal volume

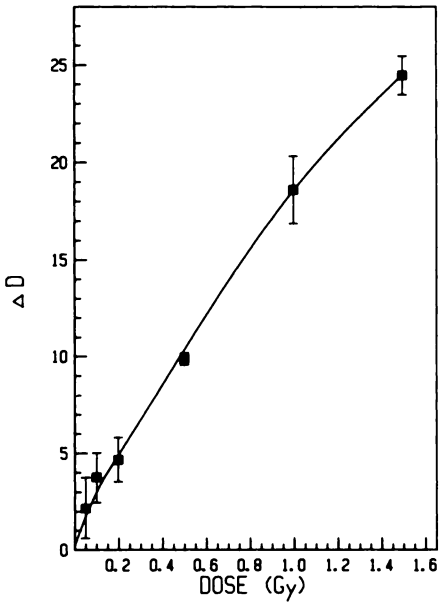

Chart 2. Dose-response curve of DNA strand breaks in human WBC irradiated with ^{60}Co γ-rays. Irradiation of 3-ml aliquots of whole blood was carried out at 0° at a dose rate of 0.04 to 0.16 gray/min. Percent D was determined as in "Materials and Methods" at 60 min following transfer of samples to a 15° bath. Each estimate of Percent D was carried out in quadruplicate. ΔD, the difference between Percent D values of unirradiated and irradiated cells. ΔD values were determined in 4 experiments for doses ≤0.2 gray and in 2 experiments for doses >0.2 gray. *Points*, averages. *Bars*, S.D., (≤0.2 gray) or ranges (>0.2 gray).

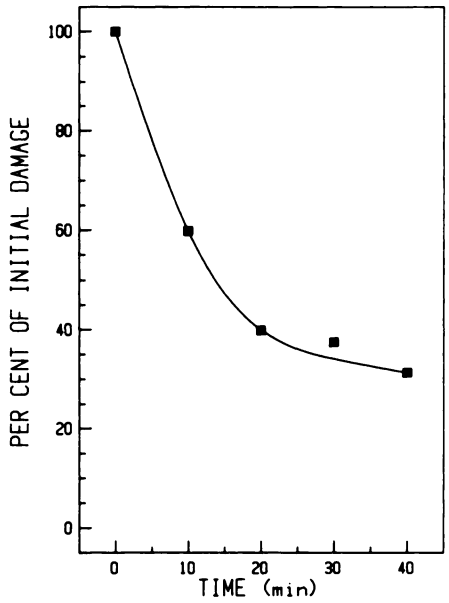

Chart 3. *In vitro* repair of DNA strand breaks following γ-irradiation of human blood at a dose of 1 gray. Volumes (3 ml) of human blood were diluted 1:1 with Roswell Park Memorial Institute Tissue Culture Medium 1640 and irradiated at 0°. Samples were analyzed immediately or after a period of incubation at 37° to allow repair of strand breaks to occur. ΔD values were calculated with reference to unirradiated blood samples treated in a similar manner (Percent D = 81.8%). The initial ΔD value (reflecting the initial damage) was 17.9; decreasing ΔD values which indicate repair of strand breaks during incubation at 37° are shown as a percentage of this value.

of Roswell Park Memorial Institute Tissue Culture Medium 1640 and irradiated at 0° with a dose of 1 gray. Aliquots were incubated at 37° for the times indicated, and repair of DNA strand breaks was monitored by FADU. A dose of 1 gray produced an initial ΔD of 17.9%, a value similar to that seen in Chart 2. The rapid decrease in DNA damage as indicated by decreasing ΔD values provides preliminary evidence that nondividing cells can repair DNA strand breaks rapidly. A more detailed study of strand rejoining in purified lymphocytes will be presented elsewhere.[3]

Because FADU is simple, rapid, and sensitive, it may have application in several areas related to human health. Basically, DNA strand breaks can be monitored in any mammalian cell type that can be prepared as a homogeneous suspension. For example, if an agent which is known to produce strand breaks is to be used in chemotherapy, then the *in vivo* or *in vitro* response of cells from a particular individual to that agent could be monitored. Information about cell sensitivity could be helpful in selecting the most appropriate drug and its dosage. Another application is for the development of short-term test systems which could detect DNA-damaging environmental agents. One such system using FADU has been described, and strand breakage in human peripheral WBC DNA produced by some metal ions has been demonstrated (9). Further applications are under investigation.

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[3] R. S. McWilliams, W. G. Cross, J. G. Kaplan, and H. C. Birnboim, manuscript in preparation.

ADDENDUM

We have recently shown that a preferred method for plotting dose-effect curves, such as in Chart 2 is to plot dose versus log (percent $D_{control}$)-log (percent $D_{radiated}$). This gives a linear relationship up to a dose of at least 7 gray, whereas a plot of dose versus ΔD, which is (percent $D_{control}$)-(percent $D_{radiated}$), is curvilinear.

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