Alkaline Elution Analysis, a New Approach to the Study of DNA Single-Strand Interruptions in Cells

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SUMMARY

A procedure is described whereby shortened DNA single strands can be selectively eluted from filters upon which cells have been lysed. This provides a sensitive measure of DNA single-strand breakage that may be applicable to the detection of cell damage resulting from certain types of chemotherapeutic agents.

The procedure was applied to the following processes in L1210 cells: (a) production of single-strand breaks in DNA by X-irradiation of cells; (b) repair of these breaks by subsequent incubation of the cells; (c) transient occurrence of newly replicated DNA as relatively short chains.

X-irradiation converts cell DNA from a slowly eluting to a rapidly eluting form. The conversion is first order with respect to X-ray dose and has a D_{37} dose of not more than 400 rads. The DNA single-strand units affected by this first-order process have a minimum size of 1.5 x 10^8 daltons (1500 μm). Repair after an X-ray dose of 1000 rads was essentially complete within 30 min. Newly replicated DNA is in a rapidly eluting form which is slowly converted to the slowly eluting form.

INTRODUCTION

Because of the great variability in the drug sensitivities of different malignant cell lines, successful chemotherapy may ultimately require an in vitro sensitivity test to select a suitable drug regimen. One possibility would be to test for the production of single-strand breaks in DNA at some time after exposure of cells to drug. Although not all antitumor agents produce DNA breaks as their primary action, it seems likely that DNA breakage will in many cases occur secondarily as a result of activation of nucleases at some stage in the evolution of cell damage. In order to investigate this possibility, a convenient quantitative assay for DNA breakage is desirable.

DNA single-strand breakage is commonly studied by sedimentation in alkaline sucrose gradients (1–3, 7, 9–11, 13, 15, 19). Ideally, this technique measures the rate at which individual DNA strands in random coil configuration move in a centrifugal field, from which it is possible to estimate the molecular weight of the strands. In practice, the results are difficult to quantitate and are subject to artifacts due to the tendency of long DNA strands to interact or to form aggregates and due to the dependence of sedimentation rate on the magnitude of the centrifugal field (1–3, 8, 9). We have explored an alternative approach using filters instead of centrifuges. We report here our first studies using this technique, in which we examined the production and repair of DNA breaks by X-rays and the formation of short DNA chains at early stages of replication in untreated cells. The new procedure yielded quantitatively reproducible data and was found to be adaptable to large numbers of determinations. Since a different physical principle is involved, we feel that, in addition to having methodological advantages, the new procedure offers the prospect of new information not attainable by centrifugation techniques.

MATERIALS AND METHODS

Cell Growth and Labeling. Mouse leukemia L1210 cells (12) were grown in suspension culture in Roswell Park Memorial Institute 1630 medium supplemented with 20% fetal bovine serum, penicillin, and streptomycin. Cells growing with a doubling time of 12 hr were labeled for 20 hr with thymidine-2-¹⁴C (51.8 mCi/mmole; 0.02 μCi/ml; Schwarz/Mann, Orangeburg, N. Y.) or thymidine-methyl-³H (49.2 Ci/mmole; 0.2 μCi/ml; New England Nuclear, Boston, Mass.; diluted with unlabeled thymidine to give a concentration of 10⁻⁶ M in the culture). For labeling of newly synthesized DNA, thymidine-methyl-³H (0.27 μCi/ml) was used without dilution with unlabeled thymidine. After labeling, cells were diluted with 3 to 10 volumes of PBS¹ at 0° and then washed and resuspended in PBS.

Filtration and Elution. One million cells were filtered through a stack of four 25-mm-diameter, 1.2-μm-pore-size filters (cellulose triacetate; Gelman Instrument Co., Ann Arbor, Mich.) and washed with cold PBS. The filters were then washed with approximately 10 ml of HSD (14) at room temperature (23–25°) at a flow rate of approximately 3 ml/min. The HSD solution was removed by washing with about 5 ml of 10⁻³ M trisodium EDTA. Up to the abbreviations used are: PBS, phosphate-buffered saline; HSD, high-salt detergent (2 M NaCl-0.02 M trisodium EDTA-0.2% Sarkosyl-0.08% deoxycholate, pH 9).

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to this point there was little or no loss of cell DNA from
the filters. Control experiments in which cells were diluted
and chilled immediately after addition of labeled thymi-
dine, or in which 2.5 mM hydroxyurea was added 5 min
prior to incubation with labeled thymidine, gave no sig-
nificant labeling of filters processed up to this point, thus
showing that all nonincorporated radioactivity was re-
moved and all incorporated radioactivity was in DNA.

The filters were then eluted with 0.1 N NaOH-0.01 M
trisodium EDTA at a rate of 0.5 ml/min by means of a
peristaltic pump. (The funnel and tubing distal to the fil-
ters had a dead space of 1.5 ml for which no correction was
made.) The elutions were carried out at room temperature
(23–25°).

In some experiments, cells were diluted into PBS con-
taining 0.2% Triton X-100-1 mM spermidine and kept in
ice for 20 min. This step disrupted the cells but kept the
nuclei intact. The suspension was then applied to the fil-
ters and washed with PBS-Triton-spermidine prior to
lysis with HSD solution. The results were the same as when
whole cells were filtered.

Radioactivity of 14C and 3H in the eluted fractions was
determined by scintillation counting in 3.3 volumes of
Aqua sol (New England Nuclear) containing 0.3% acetic
acid. Radioactivity remaining on the filters was determined
as follows. Filters were placed in a scintillation vial and
heated for 30 min at 70° in the presence of 0.4 ml of 1 N
HCl; 2.5 ml of 0.4 N NaOH were then added; 30 min
later, the solution was mixed with Aquasol as above.

RESULTS

The HSD solution used for cell lysis is essentially the
same as that used by Ormerod and Lehman (14). These
workers had found that lysis of cells in this medium pre-
erved the DNA in a rapidly sedimenting form, from which
it could be released by X-irradiation of the cells.

The effect of HSD solution on L1210 cells was observed
microscopically using Nomarski interference optics (Carl
Zeiss). When HSD solution was passed under the cover-
slip, the nuclear and cytoplasmic structures gradually
vanished, leaving only occasional bodies resembling nu-
cleoli. All other structures, including membranes ap-
peared to be completely dissolved.

In our earliest experiments, cells were mixed with HSD
solution by inversion in a test tube and then filtered. Under
these conditions, most of the DNA passed through the
filters. The fraction of the DNA retained on the filters de-
creased from 15 to 1% as the time and vigor of mixing was
increased. The DNA retained on the filters was consistently
enriched 3- to 12-fold in newly synthesized DNA (10-min
thymidine-3H label) relative to 20-hr labeled DNA-14C.
If the filters were then washed with 0.1 N NaOH-0.01 M
EDTA, all of the remaining DNA was promptly washed
out.

It was apparent that the DNA in the lysate was in a
highly extended state and easily broken by mild shear.
Therefore, in the experiments to be described, intact cells
were first filtered and then lysed on the filters with HSD
solution. In this way shearing was minimized and at least
95% of the DNA (including newly replicated DNA) was
retained on the filters. When filters were washed with alkali,
newly replicated DNA was now found to be washed out
more rapidly than bulk DNA. This served as the basis
of a new measure of DNA single-strand interruptions.

Single-Strand Breaks Produced by X-ray. The DNA of
L1210 cells was labeled for 20 hr with thymidine-14C or
thymidine-3H. After being washed with cold PBS, cells
labeled with one isotope were X-rayed and then mixed with
control cells labeled with the other isotope. Alkaline elu-
tion was carried out as described under "Materials and
Methods." Typical results are shown in Chart 1. Control
cells gave reproducible elution patterns, independent of
whether they were labeled with 14C or 3H. (The 20% of
control DNA remaining on the filter at the end of the elu-
tion in Chart 1 was in other experiments found to elute
after longer times and probably does not constitute a
separate component.) An X-ray dose as low as 250 rads
markedly increased the rate of elution from the filters.

The dose dependence of the increased elution rate was
investigated by eluting with NaOH for 2 min and then

Chart 1. Alkaline elution patterns of DNA from L1210 cells on
filters. Effects of X-ray. •, A, control cells labeled with 14C or 3H,
respectively, and mixed before filtration. O, A, mixture of 14C-labeled
cells irradiated with 250 rad (O) and 3H-labeled cells irradiated with
500 rad (A). X-irradiation conditions: 200 kV (1 mm Cu), 139 rads/min,
0-4°.
With increasing elution time, the dose dependence of filter-retained DNA remained approximately 1st order, but the $D_{37}$ dose decreased as shown in Chart 3. The 1st point on the left is derived from the data for DNA-$^{14}C$ in Chart 2. The fraction of DNA retained on the filter in the case of control cells decreased with NaOH elution time, as expected from Chart 4a; this fraction ranged from 0.93 to 0.70.

**Repair of Breaks.** Previous studies using the alkaline sucrose gradient technique have demonstrated that mammalian cells are capable of repairing the single-strand DNA breaks produced by X-ray (2, 3, 7, 11, 15). In Chart 4, this phenomenon is demonstrated for L1210 cells by means of alkaline elution from filters. The elution patterns of control cells, labeled for 20 hr with thymidine-$^{3}H$, are shown in Chart 4a. In order to show the details of the elution kinetics, the elution rate (fraction of total radioactivity eluted per min) is plotted on a logarithmic scale; aside from this difference in mode of plotting, the result is similar to that of the control curves in Chart 1. The rate of elution rises during the 1st 20 min of elution and then falls exponentially over the next 2 hr. This pattern was reproduced in several independent experiments and was the same for the 2 radioisotopes.

Labeled cells were irradiated with 1000 rads (640 rads/min) in their normal culture medium at about 30°. Part of each sample was then chilled in ice, and part was incubated further at 36°. For each filtration and elution run, $5 \times 10^6$ tritium-labeled cells were mixed with an equal number of $^{14}C$-labeled cells, so that in each run there is an internal comparison between the 2 labels. The 2 cell measuring the fraction of the DNA radioactivity remaining on the filters (Chart 2). Cells were labeled for 20 hr with thymidine-$^{14}C$ and 10 min with thymidine-$^{3}H$.

Chart 2 shows how the fraction of the DNA that remains on the filter after 2 min of NaOH elution decreases with increasing X-ray dose. In the case of the 20-hr labeled DNA-$^{14}C$, the decrease was 1st order, with a $D_{37}$ dose of 2050 rads. This would suggest that a single X-ray event can cause the change that allows a DNA molecule to be eluted within 2 min. However, a 2-min period allows the collection of only the initial part of the rapid elution peak of Chart 1. (The 2-min elution corresponds to approximately 5 min on the time axis of Chart 1, because of the solution initially presents in the dead space of the funnel and pump tubing; see "Materials and Methods").

The 10-min-labeled DNA-$^{3}H$ in Chart 2 shows markedly increased elution, even without X-ray. This will be considered later in connection with Chart 5. The part of the DNA-$^{3}H$ that does not have this high elution capacity, however, had an X-ray sensitivity not very different from normal, since the $^{3}H$ curve in Chart 2, although not linear, has an overall slope comparable to that of the $^{14}C$ line.

![Chart 2. Dose dependence of the effect of X-ray on the rapid rate of elution of DNA from filters. Elution with 15 ml of 0.1 N NaOH-0.01 M trisodium EDTA for 2 min. L1210 cells were labeled with thymidine-$^{14}C$ (○) for 20 hr and with thymidine-$^{3}H$ (□) for 10 min. X-irradiation conditions: 200 kV, 640 rad/min, 0-4°.](chart2)

![Chart 3. Dependence of the apparent X-ray sensitivity ($D_{37}$ dose) on NaOH elution time. Experiments similar to those in Chart 2 but with various NaOH elution times.](chart3)
Chart 4. Repair of single-strand DNA breaks produced by X-ray. Elution rate is the fraction of the total radioactivity eluted per min. Log-phase L1210 cells were labeled for 20 hr with \(^{14}\text{C}\)- or \(^{3}\text{H}\)-labeled thymidine as in Chart 1. Cells were irradiated in culture medium at 25—30\(^\circ\) at a dose rate of 640 rads/min. \(a\), un-irradiated control cells labeled with \(^{14}\text{C}\) (○) or \(^{3}\text{H}\) (Δ); \(b\), Δ, \(^{3}\text{H}\)-labeled cells irradiated with 1000 rads and diluted immediately with chilled PBS; ○, \(^{14}\text{C}\)-labeled cells irradiated with 1000 rads and then incubated for 30 min at 36\(^\circ\). The \(^{14}\text{C}\) and \(^{3}\text{H}\) cells were then mixed and analyzed together. - - - - , control pattern from \(a\). \(c\), Same as \(b\), except that incubation after X-ray was for 2 hr. Duplicate experiments are shown with opposite pairs of isotopes. Solid symbols, \(^{14}\text{C}\); open symbols, \(^{3}\text{H}\); circles, no incubation after X-ray; triangles, incubated for 2 hr.

The accelerated elution of the irradiated unincubated sample is largely reversed in the sample that was incubated after X-ray for 30 min. Similar results are shown in Chart 4c for the case of 120 min incubation after X-ray. Two experiments are shown with opposite combinations of isotopes; the results were identical. The curves are derived from Chart 4b and show the agreement between the 2 sets of data. The agreement between the patterns for 30 versus 120 min incubation after X-ray shows that repair, as measured by this procedure, was completed within 30 min.

The exponential phase of the control pattern (linear segment in Chart 4a) was reproduced in several experiments. The pattern is not sensitive to changes in flow rate of the eluting solution but rather depends mainly on the duration of exposure to eluting solution. The elution does not depend on time required for disentanglement of DNA chains from an intercellular aggregate, since the elution patterns did not change when the number of cells loaded onto the filters was varied over a wide range. The elution rate does not reflect an alkali-catalyzed chemical reaction, since the elution patterns showed little change with hydroxide ion concentration between 0.1 and 0.4 M.

Newly Synthesized DNA. Several reports have indicated that newly synthesized DNA chains in mammalian cells are demonstrably shorter than old DNA chains (4, 16—18). Chart 5 compares the behavior of newly synthesized DNA with that of old DNA by alkaline elution from filters. Cells were labeled for 20 hr with thymidine-\(^{14}\text{C}\) and then 3 min or 20 min with thymidine-\(^{3}\text{H}\). In contrast to the old DNA, most of the newly synthesized DNA eluted almost immediately. The 20-min \(^{3}\text{H}\) pattern is shifted towards the old DNA curve, but the deviation from the 3-min curve is relatively small.

**DISCUSSION**

The findings with DNA from X-rayed cells and with pulse-labeled DNA show that the alkaline elution of DNA...
Alkaline Elution of Cell DNA

from filters is markedly increased by single-strand interruptions. The high sensitivity of the effect and the 1st-order kinetics with X-ray dose indicate that a single X-ray event can convert a slowly eluting DNA segment to a rapidly eluting one. This conversion has an X-ray sensitivity corresponding to a 1-hit dose (D37) of 400 rads or less (Chart 3). The size of the DNA segment subject to this 1-hit process can be roughly estimated from the sensitivity of intracellular DNA to a single-strand scission by X-rays. The number of single-strand breaks per cell per rad has been estimated in mouse leukemia L5178Y cells to be 3 for cells irradiated in complete medium (19) and 8 for another line of these cells irradiated in 0.9% NaCl solution (7). Taking the value 8 as more likely to be correct, since our cells were irradiated in 0.9% NaCl solution, and assuming 5 x 10^{12} daltons of DNA per cell, the minimum size of the DNA single-strand units detected in our experiments is (5 x 10^{12} daltons/cell)/(8 breaks/cell/rad x 400 rad) = 1.5 x 10^{12} daltons. This corresponds to a minimum length of 1500 μm.

The sensitivity of the alkaline elution procedure to detect X-ray breaks is comparable to that of the extremely sensitive DNA “complex” studied by Elkind (1–3) using alkaline sucrose gradient sedimentation. The 2 procedures may be measuring the same DNA entity.

The alkaline elution procedure has the advantage of facilitating quantitatively reproducible measurements. A limitation of the procedure however has been the elution of the control uninterrupted DNA (Charts 1, 4, and 5). This prevented an accurate determination of the D37 dose for the conversion of slowly eluting to rapidly eluting DNA (Chart 3), since the long elution times required to account for all of the rapidly eluting DNA also removed substantial amounts of the control slowly eluting DNA.

The reproducibility of the elution pattern of control DNA under the conditions described at first suggested that the pattern may reflect a significant property of the control DNA. More recent work, however, has shown that the control elution pattern depends on the ionic composition of the eluting solution and on the filter material, while the elution of DNA from X-rayed cells or of pulse-labeled DNA is essentially unaffected by these changes. This problem is now under study with the view of possible improvement of the resolution between broken and intact DNA strands.

Newly replicated DNA eluted even more rapidly than the bulk DNA from cells irradiated with 1000 rads. The time required for conversion to slowly eluting DNA was found to be much greater than 20 min (Chart 5). Although the kinetics of this conversion is still under study, it is clear that a single replicating point cannot copy a DNA unit 1500 μm long in 1 S phase [since at a replication rate of 1 μm/min (6) the time required would be about 1500 min, which is much longer than the duration of S phase]. The replication of these units can be pictured from the autoradiograms obtained by Huberman and Riggs (5) which reveal tandem replication points separated by 30 to 40 μm. Each unit must encompass several of these replication points.

Although the present work documents the sensitivity and quantitative reproducibility of the alkaline elution methodology, its potential as a cell sensitivities assay remains to be investigated. The X-ray sensitivity of the L1210 cell culture line has not yet been determined. The sensitivity (Dα dose) of a similar cell line, L5178Y, however, is of the order of 180 rads (19). The single-strand DNA breakage caused by this dose in L1210 cells was detectable by the alkaline elution method. These breaks, however, are subject to rapid repair and may not in themselves be responsible for cell death.

REFERENCES

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