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DNA fragments in Friend erythroleukemia cells induced by dimethyl sulfoxide

(differentiation/DNA degradation)

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ABSTRACT **The idea of the correlation between DNA damage caused by dimethyl sulfoxide (Me_2SO) and the induction of erythrodifferentiation in murine erythroleukemia (MEL) cells needs reevaluation as a result of the hydroxyapatite chromatographic analysis of DNA from Me_2SO-treated and untreated cells as a function of the time in culture. Because Me_2SO causes an arrest in G_1 phase and consequent delay in DNA synthesis, the valid comparison would be of the DNA samples taken from control and induced cells at the same stage of growth rather than at the same time in culture. At the same stage of growth, there is no evidence of the induction of DNA damage by Me_2SO. Presumably, so-called degradation reported in the past stemmed from the fragments of DNA replication.**

Friend *et al.* (1) observed that dimethyl sulfoxide (Me_2SO) stimulated a murine Friend virus-induced erythroleukemia (MEL) cell line to differentiate along the erythroid pathway. Numerous investigations have sought to explain the role of Me_2SO in this apparently simple model of differentiation. A sizeable list of additional organic compounds and even UV radiation have been found to induce hemoglobin synthesis in these cells. These inducers fall into two classifications, those that are known to interact directly with DNA, such as UV irradiation, mitomycin C, actinomycin D, and cytosine arabinonucleoside, and those that probably do not interact directly with DNA, such as Me_2SO, butyric acid, and other simple organic compounds (for review see refs. 2 and 3). Compounds in this second class have been shown to alter transport systems in the plasma membrane (4–6) in addition to affecting chromatin (7–9).

When studied by alkaline sucrose gradient analysis, induction of MEL cells with Me_2SO seems to cause a continuous degradation of cellular DNA (7, 8). In previous studies two peaks were observed in the gradients, sedimenting either at 300 S and 120 S (8) or at 140 S and 17 S (7). The minor (slow sedimenting) fraction rose in Me_2SO-treated cells relative to untreated control cultures. In one study (8) the cellular DNA was totally converted to the smaller component after 64-hr exposure to Me_2SO. In another study (7), the ratio of DNA in the minor peak of Me_2SO-treated cells relative to control cells rose to 2.85 by 12 hr and then slowly decreased, approaching 1 after 50 hr. The large increase in low molecular weight DNA was not seen in neutral-pH sucrose gradient sedimentation; therefore, the fragments must be generated by single-strand breaks.

If Me_2SO causes single-strand breaks in DNA, it is possible that both classes of inducers work by similar mechanisms to activate chromatin by DNA cleavage. On the other hand, if the analytical procedure for determining the presence of single-strand breaks were subject to an unrecognized artifact, DNA cleavage might not be involved in MEL cell differentiation. Because this information is central to further studies of gene activation in this system, we reinvestigated this question.

The measurement of DNA nicking by alkaline sucrose gradient sedimentation can be attended by serious artifacts (10), especially when applied to high molecular weight mammalian DNA, which may not completely separate into single strands. We undertook to examine the accumulation of single-strand breaks by an alternative procedure, alkaline hydroxyapatite column chromatography, which has been successfully used by others (11, 12) on similar problems. Our results have led us to an alternative explanation of the apparent Me_2SO-induced degradation of DNA in MEL cells.

MATERIALS AND METHODS

Treatment of Cells. The MEL cell line DS-19, derived from 745, was originally obtained from R. A. Rifkind. All cultures were maintained in Ham's F-12 medium supplemented with 10% heat-inactivated fetal calf serum at 37°C in humidified atmosphere containing 5% CO_2.

MEL cells were incubated with [2-^{14}C]thymidine (54.6 mCi/mmol) or [*methyl*-^{14}C]thymidine (53.4 mCi/mmol) (New England Nuclear) at a final concentration of 0.1 or 0.2 μCi/ml (1 Ci = 3.7×10^{10} Bq). Me_2SO was obtained from Fisher and used directly from the container without prior treatment or sterilization, at a concentration of 1.5% (vol/vol) (192 mM) or 1.9% (243 mM).

An alkaline separation technique combined with hydroxyapatite chromatography (10, 11) has been used to detect DNA breaks induced by Me_2SO. Between 1 and 2×10^5 cells were removed, centrifuged, washed, and suspended in 50 μl of saline/EDTA (150 mM NaCl/1 mM EDTA, pH 7.5) solution. To this, 1 ml of alkali (30 mM NaOH/10 mM Na_2HPO_4/300 mM NaCl, pH 12.6) was added, and the mixture was kept at 20°C for 25 min in the dark, neutralized with addition of 10 mM NaH_2PO_4 containing phenol red as pH indicator, transferred into an ice-water bath, and sonicated for 15 sec. After sonication, sodium dodecyl sulfate was added to a final concentration of 0.25%.

Hydroxyapatite Column Chromatography. A water-jacketed glass column (1 × 25 cm) was packed with 250 mg of hydroxyapatite (DNA grade Bio-Gel HTP) and maintained at 60°C. Elution buffers used were 0.01 M sodium phosphate (pH 6.8) for washing and 0.13 and 0.25 M potassium phosphate (pH 6.8) for single-stranded (ss) and double-stranded (ds) DNA, respectively. Fractions were dialyzed overnight and aliquots were removed for fluorometric and radioactivity measurements. Sam-

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Abbreviations: MEL, murine erythroleukemia (Friend virus-induced); Me_2SO, dimethyl sulfoxide; ds DNA, double-stranded DNA; ss DNA, single-stranded DNA.

ples were precipitated with 10% trichloroacetic acid after an addition of a carrier DNA, and radioactivity in precipitates was measured with a scintillation counter (Beckman LS-230).

Fluorometric Procedure for DNA Assay. See ref. 13. Diaminobenzoic acid (Eastman Kodak) at 1.1 M was shaken with activated charcoal (Sigma) at 50 mg/ml to reduce the background fluorescence and filtered through a 0.2-μm pore size disposable disc filter. *Escherichia coli* DNA (Worthington) was used as a standard. Fluorescence measurements were made with a Perkin–Elmer fluorimeter, with excitation at 397 nm and emission scanned from 480 to 520 nm.

RESULTS AND DISCUSSION

The growth and [^{14}C]thymidine incorporation of MEL cells, clone DS19, are practically identical for induced and uninduced cells (Fig. 1), except that Me_2SO-treated cells lag behind untreated cells. Although the lag extends for 2 hr in 0.19 M Me_2SO and for 6 hr in 0.24 M Me_2SO, the cultures reach the same cell density after 2.5 days. After 3–4 days of culture, DNA degradation occurs as the cells age.

Analyses of DNA samples from Me_2SO-treated and control cells by hydroxyapatite chromatography are shown in Fig. 2, where the percentage of the ds DNA is plotted as a function of time in culture. The differences observed between the fluorometric and isotope incorporation methods are within experimental error. The ds DNA percentage decreases for about a day, slowly increases to a plateau, remains at this level for about 1 day, and finally begins to decline.

In the hydroxyapatite method the DNA unwinds in alkaline solution from single- or double-stranded breaks until the solution is neutralized and the DNA is sonicated. The amount of DNA that is rendered single-stranded and passes through the hydroxyapatite column depends upon the duration of the alkaline incubation; however, the *relative changes* in the measured percent ds DNA during the culture period are not affected by length of exposure to base (Fig. 3).

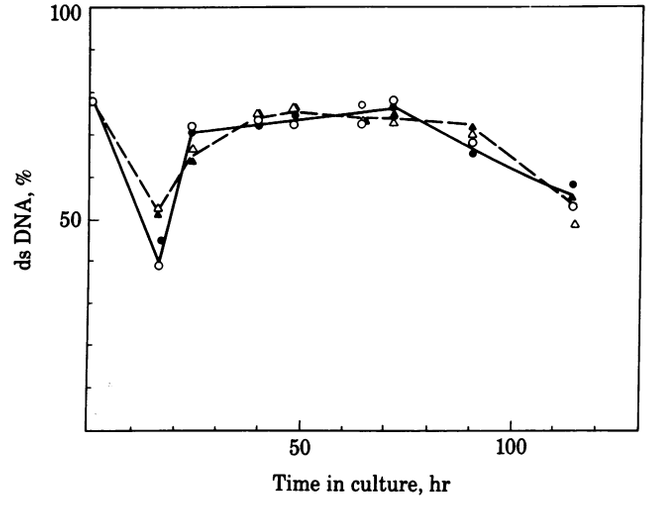

FIG. 2. Hydroxyapatite chromatographic analysis of DNA samples during culture of MEL cells with Me_2SO. DS19 cells were cultured in the presence (192 mM) or absence of Me_2SO and in the presence of [^{14}C]thymidine at 0.1 or 0.2 μCi/ml. ○, Control, fluorometric method; ●, control, radioactivity measurement; △, Me_2SO-treated, fluorometric method; ▲, Me_2SO-treated, radioactivity measurement.

The initial decrease in the percentage of ds DNA observed in Fig. 2 coincides with the early logarithmic phase of cell growth (Fig. 1) and it, probably, results from the burst of DNA synthesis that occurs whenever the cells are transferred into a fresh medium at a low concentration, as shown in Fig. 4. As the cells enter the late logarithmic phase of growth, the percentage of cells undergoing division decreases, and this corresponds to the slow increase portion of the curve in Fig. 2.

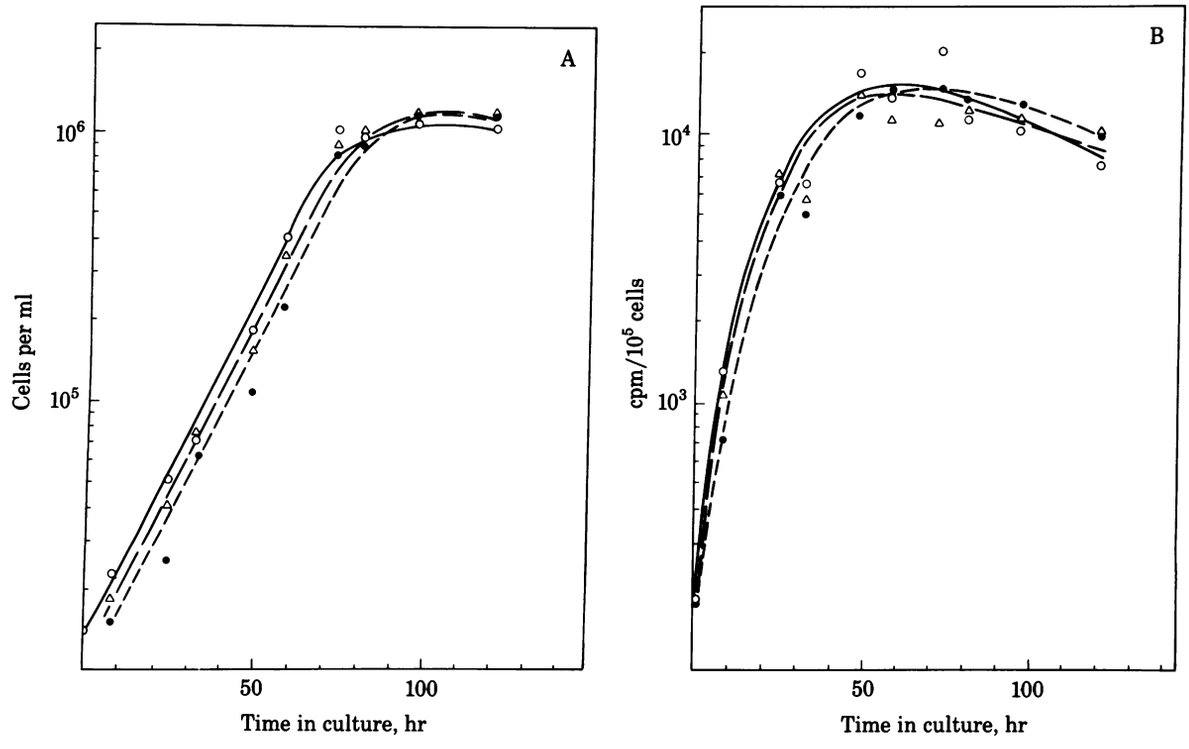

FIG. 1. Cell growth and DNA synthesis. MEL cells, clone DS19, were cultured in the presence (1.5% = 192 mM and 1.9% = 243 mM) and the absence of Me_2SO. At times indicated, the cells were removed for determination of cell number (*A*) and DNA synthesis (*B*) by measuring [^{14}C]-thymidine incorporation into trichloroacetic acid-precipitable material. ○, Control; △, 192 mM; ●, 243 mM.

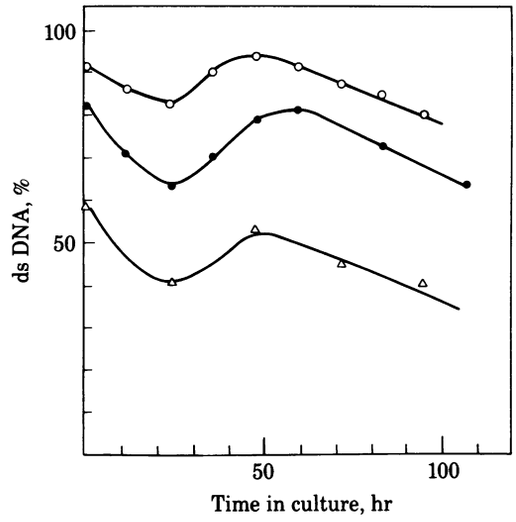

FIG. 3. Effect of the length of alkali treatment on the hydroxyapatite chromatographic profile. DS19 cells were cultured in the presence of 192 mM Me_2SO and samples were taken at certain time intervals and subjected to lysis by alkali for various lengths of time. ○, 10 min at 0°C; ●, 25 min at 20°C; △, 40 min at 20°C.

The onset of the decline in the percentage of ds DNA, after 3 or 4 days of culture in both induced and control cells, coincides with the decrease observed in the [^{14}C]thymidine incorporated per 10^5 cells as shown in Fig. 1; therefore it stems from the DNA degradation due to cell aging and, in Me_2SO-treated cells, probably to differentiation. Induced MEL cells appear to progress to specialized erythroid cells resembling orthochromatic erythroblasts, without undergoing the final stage of differentiation to become reticulocytes. However, at the stage of late normoblast, the mitotic apparatus shrinks to a blackish-brown mass of irregularly shaped chromatin and begins to break down (1, 14). The extrusion of the nucleus has not been observed in this system (1).

By this comparative analysis of Figs. 1 and 2, we conclude that the initial decrease in the percentage of ds DNA observed by the hydroxyapatite chromatography is related to an intense DNA replication occurring at the early logarithmic phase of growth of the cells. Probably these ss DNA fragments are replicating intermediates and equivalent to nicks in providing sites for alkali-induced unwinding.

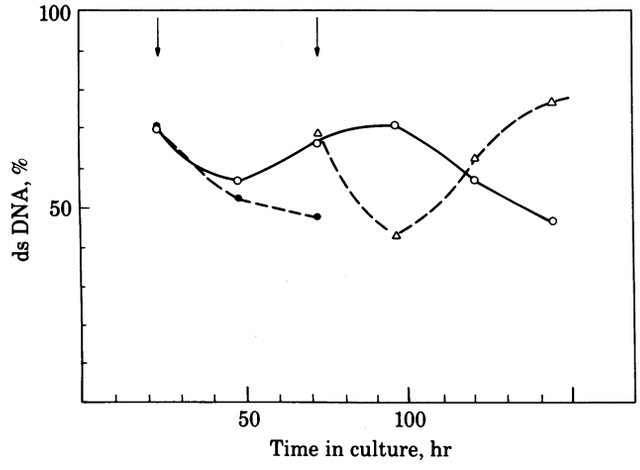

FIG. 4. Hydroxyapatite chromatographic pattern of DNA from stationary-phase cells transferred to a fresh medium at a low concentration. DS19 cells were cultured in the absence of Me_2SO and, after reaching stationary phase, a fraction was transferred, as indicated by arrow, to a fresh medium at 5×10^4 cells per ml (○, the first transfer; △, the second transfer), and the rest were left in the original culture. ●, Original culture after the first transfer; ○, original culture after the second transfer.

The Me_2SO-treated cells lag behind the untreated cells in both the initial drop and in the subsequent rise in ds DNA. At subsequent times the Me_2SO-induced lag in DNA replication produces a *higher* fraction of ss DNA in Me_2SO-treated cells, a result that could be interpreted as the induction of DNA strand breaks if the delaying effect on replication were not considered.

The replication intermediate-like fragments can also be observed by alkaline sucrose gradient centrifugation (Fig. 5). As the rate of cell growth slows the fraction of small fragments declines (Fig. 4 *B* and *C*), as was observed by using the hydroxyapatite method. The high molecular weight peak is composed primarily of ds DNA, whereas the low molecular weight peaks consist of unwound single-stranded fragments (8). The extent of unwinding depends upon the duration of the incubation in alkali before concentration (8) and probably upon the duration of centrifugation as well. Because unwinding can occur during centrifugation, this method seems to provide less reliable estimates of ss DNA content.

Using the alkaline gradient technique, others have observed the appearance of small ss DNA fragments in rapidly dividing MEL cells cultured in the absence of Me_2SO (7–9). Because of differences in experimental procedures and strains used, we cannot directly compare these sets of data with our own; however, they are not inconsistent with the proposal that the fragments are, probably, predominantly related to replication intermediates.

These results lead to the following explanation for the larger amount of small DNA fragments in Me_2SO-treated cells: Me_2SO

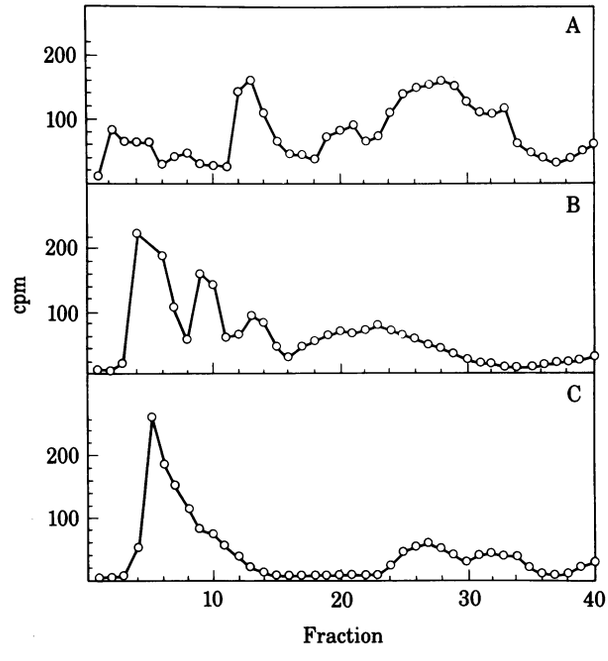

FIG. 5. Alkaline sucrose gradient analysis of MEL cells at different stages of growth. DS19 cells were cultured in the presence of [2-^{14}C]thymidine (54.6 mCi/mmol) at a concentration of 0.1 or 0.2 μCi/ml, in the absence of Me_2SO. Analysis was performed according to Terada *et al.* (8), except using the Beckman SW 60 rotor at 30,000 rpm for 50 min. Direction of sedimentation is from right to left. Between 1 and 2×10^4 cells were used for each run. (*A*) Early logarithmic phase (24 hr); (*B*) middle logarithmic phase (48 hr); (*C*) late logarithmic phase (72 hr).

may slow the entrance of MEL cells into S phase; meanwhile, control cells have passed through S phase and have incorporated the replication intermediates into full-length DNA. In a nonsynchronized culture this delay will lead to an excess of replication intermediates in Me_2SO-treated cells at any time after the control cells have passed through their first S phase.

Although these results do not eliminate the possibility that Me_2SO induces globin gene expression by causing single-strand nicks in DNA, they do indicate that nicks cannot be unambiguously demonstrated in a replicating system in which DNA fragments normally are formed. Nicks are particularly difficult to demonstrate by a method that relies upon labeling DNA with radioactive precursors, which, as Scher and Friend have noted (7) cause radiolytic cleavage of DNA.

Other approaches can be undertaken to assess the extent of DNA nicking in Me_2SO-treated cells. In other systems DNA-nicking activates ADP-ribosyltransferase, a nuclear enzyme that catalyzes the formation of poly(ADP-ribose)-modified nuclear proteins from NAD^+ (15, 16). If Me_2SO produced DNA nicking, it would be expected that the activity of this enzyme would be increased. Our experiments (not shown here) indicate that although the enzyme activity appeared to depend on the state of cell growth, there was no difference in ADP-ribosyltransferase activity between Me_2SO-treated and untreated MEL cells. Conversely, an inhibitor of this enzyme would be expected to increase the rate of nicking in Me_2SO-treated cells and to potentiate hemoglobin induction. However, the potent inhibitors (17) 3-aminobenzamide (3 mM) and 3-methoxybenzamide (1.5 mM) did not potentiate the induction of hemoglobin at the concentrations used in the absence of Me_2SO.

Although Me_2SO-mediated DNA nicking is difficult to demonstrate in dividing cells, other effects of the inducer have been observed. Me_2SO stimulates the uptake of Ca^{2+} by MEL cells (18) and the blocking of Ca^{2+} uptake by amiloride, an inhibitor of passive Na^+ transport, prevents induction by Me_2SO. Moreover, reversal of cellular Ca^{2+} uptake inhibition by Ca^{2+} ionophore A23187 stimulated Me_2SO-treated MEL cells to resume differentiating. This finding suggests involvement of the Na^+/Ca^{2+} antiport system in MEL cell differentiation (18, 19).

Nomura and Oishi established that at least two processes are involved in MEL cell differentiation (20). They found that any of several types of cell—e.g., BHK (baby hamster kidney)—irradiated with UV light and fused to MEL cells that had been briefly tested with Me_2SO would induce globin synthesis. The brief Me_2SO treatment was insufficient to induce globin synthesis without the *trans*-acting substance formed in the UV-irradiated cells. One interpretation of these results is that DNA damage promotes the accumulation of a substance that acts in concert with another Me_2SO-promoted function to induce globin synthesis. A further inference from these data is that prolonged Me_2SO treatment that is sufficient to induce globin synthesis may also cause generalized DNA damage. This conclusion is premature, because other pathways to produce the inducer substances may also exist. Other approaches are needed to assess the involvement of DNA damage in the differentiation of MEL cells.

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