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INDUCTION OF DNA SINGLE- AND DOUBLE-STRAND BREAKS BY DIETHYLSTILBESTROL IN MURINE L5178Y LYMPHOBLASTS *IN VITRO**

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Abstract—The mechanism of action of the synthetic estrogen diethylstilbestrol (DES) was investigated in murine L5178Y lymphoblasts. The dose–survival curve of cells treated with DES in serum-free medium for 1 hr was characterized by a prominent shoulder followed by a simple exponential decline; the D_o, the dose of DES reducing cell survival to 1/*e*, was 1.52 nmoles/ml. DNA single-strand breaks, as measured by the alkaline elution method, were observed in DES-treated cells, and these followed a dose–response relationship after an apparent threshold of 10 μM DES was exceeded. Protein-associated strand breaks, which represent the increment in single-strand breaks that occurs by exposing drug-treated cells to proteinase K, were also noted. DNA double-strand breaks as measured by filter elution technology at pH 9.6 were observed and increased markedly to reach a level of approximately 9000 rad equivalents at a DES concentration of 20 μM. The measured ratio (mean ± S.E.) of single- to double-strand breaks induced by DES in L5178Y limphoblasts was 0.09 ± 0.035. A comparison of the ratio of single- to double-strand breaks induced by DES to that observed following radiation suggested that all of the single-strand breaks produced by DES could be attributed to double-strand breaks. The close correspondence of the dose–response curve for cytocidal activity of DES with that obtained for induction of DNA double-strand breaks suggested that such breaks may play an important role in the mechanism of cell kill by DES.

The synthetic estrogen, diethylstilbestrol (DES), is known to be carcinogenic in experimental animals [1, 2] and in man [3]. Furthermore, DES has proven useful clinically as an antitumor agent in the therapy of carcinoma of the breast [4] and carcinoma of the prostate [5]. However, the mechanism whereby DES exerts either its carcinogenic or antitumor activity is unkonwn. Indeed there are many conflicting reports in the literature on the biological effects induced by DES.

DES is inactive in many short-term tests used to detect carcinogens; DES does not produce mutations in several bacterial [6, 7] and mammalian [8, 9] cell assays. However, DES has been reported to be mutagenic in the presence of oxidizing agents in other systems including yeast [10], the L5178Y/TK mutagenic assay [11] and the rat alkaline elution-hepatocyte assay [12]. Conflicting reports have also appeared on the effect of DES on unscheduled DNA synthesis, which indirectly detects DNA damage by measuring DNA repair [13–15], and on the induction of chromosomal aberrations [15–19].

Alkylating agents and ionizing radiation, like DES, are both carcinogenic and have clear-cut antitumor activity. Alkylating agents and radiation both induce changes in DNA; with alkylators the predominant reaction is cross-linking [20, 21], whereas with radiation DNA strand breakage occurs [20, 22]. DES has been shown to bind covalently to DNA by oxidative and photochemical linkage [23] and after incubation with a liver microsomal preparation [24]. In this report, evidence is presented that DES treatment of murine L5178Y lymphoblasts *in vitro* is associated with the inducation of DNA single- and double-strand breaks and that these changes appear to correlate with cytocidal activity.

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MATERIALS AND METHODS

Drugs and chemicals. [^{14}C]Thymidine (sp. act. 50 mCi/mmole) and [^{3}H]thymidine (sp. act. 50–80 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA, and DES was purchased from the Sigma Chemical Co., St. Louis, MO. Proteinase K was from E. Merck, Darmstadt, Germany, and tetrapropylammonium hydroxide was from the Eastman Kodak Co., Rochester, NY.

Determination of cytocidal activity of DES. Murine L5178Y lymphoblasts were grown in Fischer's medium (Grand Island Biological Co., Grand Island, NY) supplemented with 10% horse serum as described previously [25]. Exponential phase cells at a concentration of 2–3 × 10^5 cells/ml were treated with DES in serum-free medium for 1 hr at 37°. The treated cells were washed twice to remove drug and resuspended in whole medium; a dose–survival curve of the DES-treated cells was determined by the cloning method of Chu and Fischer [26].

Estimation of DNA single-strand breaks by alkaline elution. DNA single-strand breaks were measured by the alkaline elution technique of Kohn as described

previously [20, 21]. The DNA of L5178Y cells was labeled with [^{14}C]thymidine by growing the cells overnight in Fischer's medium containing 10% dialyzed horse serum and [^{14}C]thymidine (0.01 to 0.02 μCi/ml) at a concentration of 1 μM. Cells were washed twice to remove exogenous label, resuspended in Fischer's medium without serum at a concentration of approximately 2×10^5 cells/ml, and treated with DES for 1 hr at 37°. Approximately 5×10^5 drug-treated cells were applied to an elution filter and washed twice with cold medium to remove DES. An internal standard was routinely used to determine flow rate through the filter and this consisted of an equal number of L5178Y lymphoblasts labeled with [^{3}H]thymidine. Cells were labeled by overnight culture in Fischer's medium with 10% dialyzed horse serum containing [^{3}H]thymidine (0.05 to 0.1 μCi/ml) at a concentration of 1 μM. The [^{3}H] labeled cells were washed and irradiated with 150 rads on ice using a radioactive cobalt-60 source at a dose rate of 95 rads/min. The combined cell suspension was analyzed for DNA single-strand breaks by a modification of the method of Kohn *et al.* [20], using 2 μm polyvinylchloride filters (Millipore Corp., Bedford, MA) fitted on a polyethylene filter holder (Swinnex, Millipore Corp., Bedford, MA) modified to hold a volume of 20 ml as described previously [21]. Protein-associated single-strand breaks were detected by a modification of the procedure of Zwelling *et al.* [27, 28] in which 0.8 μm polycarbonate filters (Nucleopore Corp., Pleasanton, CA) were used and the cells were exposed to a lysing solution containing 0.5 mg/ml proteinase K on the filter for 1 hr prior to elution. In both methods, DNA was eluted with a buffer solution consisting of tetrapropylammonium hydroxide at pH 12.1 to 12.2.

Estimation of DNA double-strand breaks by neutral elution. To determine DNA double-strand breaks, non-irradiated drug-treated cells labeled with [^{14}C]thymidine were combined with an internal standard, consisting of cells labeled with [^{3}H]thymidine and irradiated with 3000 rads. The combined cell suspension was placed on a 2.0 μm polycarbonate filter (Nucleopore Corp.) and lysed for 1 hr with a solution containing 0.5 mg/ml proteinase K. DNA double-strand breaks were detected by the neutral elution technique using a buffer consisting of 0.02 M ethylenediaminetetraacetic acid and sufficient quantity of 10% tetrapropylammonium hydroxide to give a final pH of 9.6 as described previously [22].

The level of DNA single- and double-strand breaks was calculated from the elution profiles and was expressed as rad equivalents (dose of radiation inducing an equivalent number of breaks) as determined from calibration curves [20, 27–29].

RESULTS

Dose–survival curve of L5178Y lymphoblasts treated with DES. A dose–survival curve of L5178Y lymphoblasts treated with DES for 1 hr in serum-free medium is shown in Fig. 1. A prominent shoulder is noted followed by a simple exponential decline. The presence of a shoulder suggests that, as with ionizing radiation, the cell must accumulate multiple lesions before death ensues and/or that the cell is capable of repairing sub-lethal damage [30, 31]. The segment of exponential decline suggests that in this region cell kill follows first-order kinetics; the D_o, the dose of DES reducing cell survival to $1/e$, was 1.52 nmoles/ml.

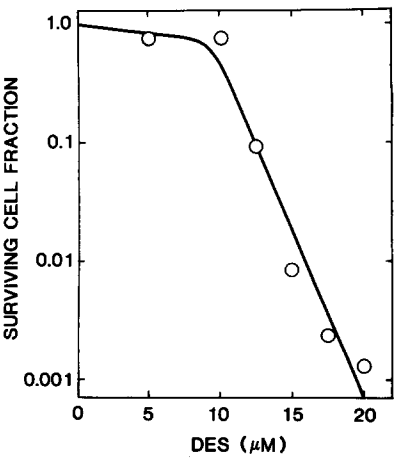

Fig. 1. Dose–survival curve of murine L5178Y lymphoblasts treated *in vitro* with DES for 1 hr and cloned in soft agar by the method of Chu and Fischer [26]. The regression equation for the linear portion of the dose–survival curve was $\log_e y = -0.656x + 5.82$ with a correlation coefficient of −0.98. Each point represents the mean ± S.E. of four determinations; the confidence intervals were too small to be illustrated.

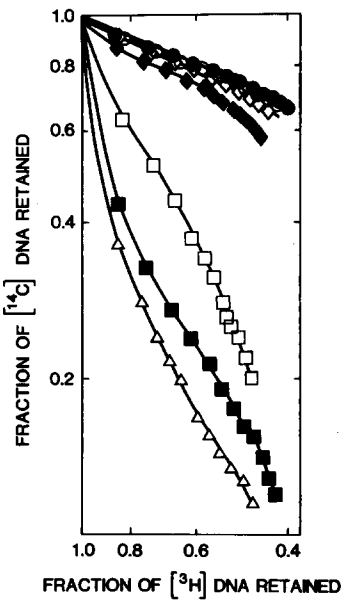

Fig. 2. Determination of DNA single-strand breaks in L5178Y lymphoblasts treated with DES for 1 hr in serum-free Fischer's medium using the alkaline elution method without proteinase K as described in the text and in the literature [20, 21, 27, 28]. Elution of [^{14}C]DNA from DES-treated cells is plotted against the simultaneous elution of [^{3}H]DNA from the internal standard cells as described in the text and previously [20, 21, 27, 28]. Cells were treated with DES at concentrations of 0 μM (○), 5 μM (●), 10 μM (◇), 15 μM (◆), 20 μM (□), 30 μM (■) and 40 μM (△).

Induction of DNA single-strand breaks in L5178Y lymphoblasts treated with DES. DNA single-strand breaks induced in L5178Y lymphoblasts by treatment with DES in serum-free medium for 1 hr were determined by the alkaline elution procedure of Kohn *et al.* [20, 21] as illustrated in Fig. 2. The rate of elution of DNA, which is a function of the number of DNA single-strand breaks, increased proportionately as the dose of DES was increased from 5 to 40 μM.

From a standard calibration curve obtained by treating L5178Y lymphoblasts with a dose range of ionizing radiation, the number of DNA single-strand breaks induced by any agent may be expressed as the dose of radiation in rads producing an equivalent number of breaks. DNA single-strand breaks induced by DES and measured in the absence and presence of proteinase K were plotted as a function of DES concentration (Fig. 3). The number of single-strand breaks increased with increasing drug concentration after a threshold dose of approximately 10 μM DES was exceeded. The increment in single-strand breaks that occurs by exposing DES-treated cells to proteinase K prior to elution represents protein-associated strand breaks [27, 28]. The number of single-strand breaks produced in cells treated with 30 μM DES for 1 hr was approximately 650 rad equivalents in the absence of proteinase K, and that in the presence of proteinase K was approximately 970 rad equivalents (Fig. 3); the difference of 320 rad equivalents between these two determinations is attributable to protein-associated strand breaks.

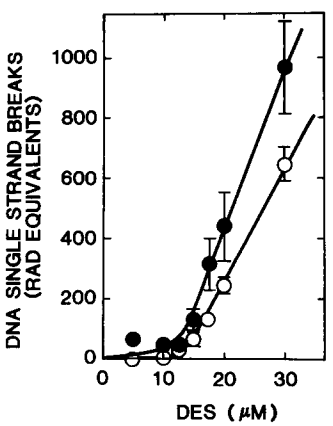

Fig. 3. Dose–response plot of DNA single-strand breaks in L5178Y lymphoblasts treated with DES for 1 hr as described in the text and the legend of Fig. 2. DNA single-strand breaks were determined in the presence (●) and absence (○) of proteinase K by the method of alkaline elution as described in the text and previously [20, 21, 27, 28]. The number of breaks, expressed as rad equivalents (dose of radiation producing an equivalent number of breaks), is plotted against the concentration of DES. Each point represents the mean ± S.E. of at least three determinations. The lines were determined by linear regression analysis. The linear regression equation for single-strand breaks measured in the presence of proteinase K was $y = 53.5x - 635$, with a correlation coefficient of 0.99, and that for breaks determined in the absence of proteinase K was $y = 36.7x - 471$, with a correlation coefficient of 0.99.

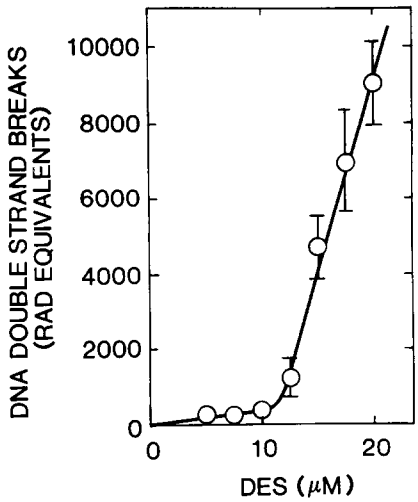

Fig. 4. Dose–response plot of DNA double-strand breaks in L5178Y lymphoblasts treated with DES for 1 hr using the neutral elution method as described in the text and previously [20, 22, 27, 28]. The number of double-strand breaks, expressed as rad equivalents, is plotted against DES concentration. Each point represents the mean ± S.E. of two to five determinations. The linear regression equation for double-strand breaks was $y = 1030x - 11{,}260$ with a correlation coefficient of 0.99.

Induction of DNA double-strand breaks in L5178Y lymphoblasts treated with DES. The generation of DNA double-strand breaks in L5178Y lymphoblasts exposed to DES was measured by the neutral elution technique as described in the literature [20, 22]. DNA double-strand breaks expressed as rad equivalents were plotted as a function of DES concentration (Fig. 4). Once again, as with single-strand breaks, few double-strand breaks appeared below an apparent threshold dose of approximately 10 μM DES. Thereafter, double-strand breaks increased sharply, reaching a level of approximately 9000 rad equivalents at a DES concentration of 20 μM.

Relative production of DNA double-strand breaks and single-strand breaks in L5178Y lymphoblasts treated with DES. To determine the true frequency of single- and double-strand breaks induced by DES a formula was used, which compares the observed values obtained with DES to the ratio of actual single-strand breaks and double-strand breaks induced by X-ray [27, 28]. The formula takes into account that single-strand break frequency measured by alkaline elution includes both single-strand breaks arising from double-strand breaks (each double-strand break gives rise to two single-strand breaks) as well as true single-strand breaks. The formula for calculating the ratio of true single-strand breaks, s, to actual double-strand breaks, d, produced by DES is:

$$\frac{s}{d} = \left(\frac{k_{RS}}{k_{RD}}\right)\left(\frac{[SSB]}{[DSB]}\right) - 2 \qquad (1)$$

where k_{RS} is the single-strand break frequency produced per unit of X-ray dose, k_{RD} is the double-strand break frequency per unit of X-ray dose, and

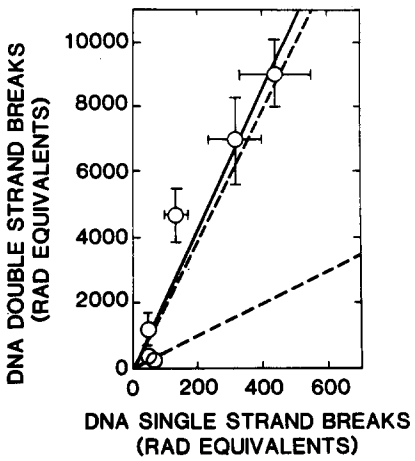

Fig. 5. Relationship between DNA double-strand breaks and DNA single-strand breaks in L5178Y lymphoblasts treated with DES for 1 hr. Double-strand breaks were measured by filter elution at pH 9.6 as described in the text and previously [20, 22, 27, 28]; single-strand breaks were determined by the method of alkaline elution as described in the text and previously [20, 21, 27, 28] and strand-break frequency was expressed in rad equivalents (the dose of radiation producing an equivalent number of strand breaks). The points represent the mean ± S.E. of two to five determinations. The solid line was obtained by linear regression analysis of the measured single- and double-strand breaks produced by DES; the regression equation was $y = 21.75x - 66.98$ with a correlation coefficient of 0.96. The broken lines represent the values of single- and double-strand breaks obtained for cells treated with X-radiation assuming that all single-strand breaks arose from double-strand breaks (i.e. $s/d = 0$ in equation 1) for k_{RS}/k_{RD} ratio of 10 (lower broken line) or 40 (upper broken line) as described in the text.

$[SSB]$ and $[DSB]$ are the measured frequencies in rad equivalents of single- and double-strand breaks in the presence of proteinase K induced by DES. Values for the ration k_{RS}/k_{RD} of actual single-strand breaks (excluding those arising from double-strand breaks) to double-strand breaks induced by X-radiation range from 10 to 40 in the literature [27, 28]. Thus, from equation 1, if DES produced double-strand breaks exclusively, s would equal 0, and the measured values for $[SSB]/[DSB]$ for DES would fall between 0.05 and 0.2 for k_{RS}/k_{RD} values of 40 and 10, respectively, as illustrated by the broken lines in Fig. 5. In this study almost all the experimental determinations of single- and double-strand breaks induced by DES fell within this range and indeed the linear regression line of these data was almost identical to the theoretical plot obtained assuming a k_{RS}/k_{RD} for X-ray of 40 (Fig. 5).

The measured ratio (mean ± S.E.) of $[SSB]/[DSB]$ in L5178Y lymphoblasts treated with DES over a concentration range of 5 to 20 μM for 1 hr was 0.09 ± 0.035. From equation (1) the true ratio of single- to double-strand breaks (s/d) produced by DES was calculated between 0 and 2, for a k_{RS}/k_{RD} of 10 and 40 respectively [27, 28].

Correlation of cell cytotoxicity with DNA damage induced by DES. Cell cytotoxicity as measured by the clonogenic assay of Chu and Fischer [26] was plotted on a long scale against DNA damage induced by DES on a linear scale and measured by the alkaline [20, 21, 27, 28] and neutral [20, 22, 27, 28] elution assays (Fig. 6). The number of DNA single-strand breaks, measured in the absence and presence of proteinase K, and the number of double-strand breaks appeared to correlate with the level of cell kill.

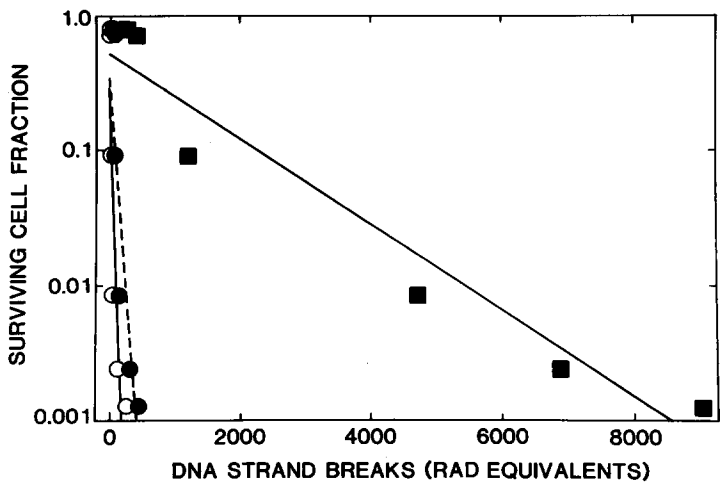

Fig. 6. Correlation of cytotoxicity of L5178Y lymphoblasts exposed to DES for 1 hr with the appearance of DNA single-strand breaks measured by alkaline elution in the absence (○) or presence of proteinase K (●), and of DNA double-strand breaks (■) measured by neutral elution as described in the text and previously [20, 22, 27, 28]. Cytotoxicity was determined by the clonogenic assay of Chu and Fischer [26] as described in the text and in the legend of Fig. 1, and was plotted as surviving cell fraction on the ordinate against number of DNA single- or double-strand breaks expressed as rad equivalents on the abscissa. The linear regression equation for DNA single-strand breaks in the absence of proteinase K was $\log_e y = -0.0265x - 1.24$ with a correlation coefficient of −0.89, that for single-strand breaks in the presence of proteinase K was $\log_e y = -0.0151x - 0.728$ with a correlation coefficient of −0.88, and that for DNA double-strand breaks was $\log_e y = -0.00074x - 0.619$ with a correlation coefficient of −0.97.

DISCUSSION

In a previous study, the sensitivity of ER-positive and -negative human breast cancer cells to the cytocidal activity of DES was identical [32]. That observation together with the inability of equimolar concentrations of estradiol to protect cells from the cytocidal activity of DES suggested that the mechanism of cell kill might be independent of ER status. In this study, the D_0 for L5178Y cells treated with DES for 1 hr in serum-free medium was 1.52 nmoles/ml. Thus, L5178Y lymphoblasts are 1.5- to 1.8-fold more sensitive to DES than either ER-positive or -negative human breast cancer cells [32]. This finding lends further support to the notion that the mechanism of cell kill by DES may be ER-independent.

An alternative to the interpretation that the mechanism of cell kill by DES is ER-indpendent is the possibility that the observed cytotoxicity is "nonspecific" and of questionable biological relevance. Although the data do not provide a definitive choice between these two alternatives, we favor the first interpretation. In evaluating the pharmacological relevance of dose–survival studies such as this, the product of drug concentration x treatment time is particularly pertinent. If one extrapolates and extends the treatment time from 1 to 24 hr, then the "corrected" D_0 for DES treatment of L5178Y lymphoblasts would be 0.063 nmole/ml or 63 nM. The plasma DES levels achievable in cancer patients receiving 15–20 mg drug daily would be expected to range from 3.7 to 149 nM [32]. Thus, it would be reasonable to assume that the effective dose of DES used in this study would be clinically attainable and therefore of possible biological significance.

Treatment of L5178Y lymphoblasts with DES resulted in the appearance of DNA single-strand breaks as determined by the alkaline elution technique (Fig. 3). The induction of single-strand breaks followed a dose–response relationship varying directly with DES concentration after a threshold dose of 10 μM DES was exceeded. Furthermore, there appeared to be a correlation between the cytocidal activity of DES as measured by the clonogenic assay and the formation of single-strand breaks (Fig. 6). However, the single-strand breaks induced by DES may be attributed entirely to those arising from double-strand breaks (Fig. 5). The measured ratio of $[SSB]/[DSB]$ produced by DES was 0.09 ± 0.035, which was identical to that reported for ellipticine in L1210 cells [28] and approximately one-half that reported for adriamycin [27]. The true ratio of single- to double-strand breaks (s/d) produced by DES was between 0 and 2, which is lower than that recorded for adriamycin [27] or radiation [27, 28, 33]. Thus, with respect to DNA damage, DES resembles the intercalating agent ellipticine in apparently producing double-strand breaks exclusively.

The number of DNA single-strand breaks measured by the assay increased if the drug-treated cells were exposed to proteinase K prior to elution (Fig. 3). This increment in single-strand breaks was substantial, amounting to 320 rad equivalents for cells exposed to 30 μM DES for 1 hr. From this study it is not possible to determine if this finding represents protein-associated strand breaks or DNA–protein cross-links. The intercalating agents adriamycin and ellipticine have been reported to produce protein-associated strand breaks [27, 28]. It has been suggested that protein-associated strand breaks may be unrelated to cytotoxicity and may represent a protective mechanism that counteracts the effect of intercalators on DNA torsion [27, 28]. Protein-associated strand breaks may arise from activation of enzymes such as topoisomerases, which produce strand breaks and become covalently bound to DNA at the break site; such enzyme activity would serve to relieve strain caused by intercalation [27]. Conversely, other drugs including alkylating agents [20, 21] and the epipodophyllotoxin VP-16 [34] have been reported to form DNA–protein cross-links; however, cross-linking by DES was not directly examined in this study.

Treatment of L5178Y cells with DES was also associated with the marked appearance of DNA double-strand breaks, as measured by the filter elution assay at pH 9.6 (Fig. 4). Double-strand breaks have been considered to represent the lethal lesion induced by ionizing radiation [33, 35], and some drugs [34]. The finding of a correlation between cytocidal activity and the number of DNA double-strand breaks (Fig. 6) suggests that these breaks may play a central role in the mechanism of cell kill by DES.

The question arises as to whether the induction of DNA double-strand breaks at cytotoxic concentrations of DES is primary, due to direct chemical attack on or interaction with some portion of the macromolecule, or secondary, resulting from nonspecific cell lysis, extensive cell repair or release of intracellular degradative enzymes. The data do not permit a clear choice between these two options. However, the striking similarity in the shape of the dose–response curves for cytotoxicity and induction of DNA double-strand breaks is consistent with a primary effect. Furthermore, evidence that DNA scission is not simply the result of dying cells degrading their own DNA was the finding that over 96% of L5178Y cells exposed to 15 μM DES for 1 hr were viable as measured by trypan blue dye exclusion (data not shown), although such treatment produced 4700 rad equivalents of DNA double-strand breaks (Fig. 4) and reduced surviving cell fraction to 8.6×10^{-3} in the clonogenic assay (Fig. 1). Finally, Birnboim* using a non-radioactive, fluorescent method to measure DNA damage has found changes in the DNA of human leukocytes and mouse thymocytes following DES, that are quantitatively similar to those reported here.

Many of the biological effects attributed to DES imply an interaction with DNA; these include reports on mutagenic activity [10–12], chromosomal changes such as induction of aneuploidy [16] and sister chromatid exchange [17, 19], and stimulation of unscheduled DNA synthesis [14, 15]. Evidence of direct binding of DES to DNA has been reported following oxidative and photochemical linkage [23] and after incubation with a liver microsomal preparation [24]. The finding that DES induces DNA double-strand breaks in L5178Y lymphoblasts *in vitro* might pro-

* Personal communication.

vide a possible mechanism for several of the above biological effects.

This study provides no information as to the mechanism whereby DES might produce DNA double-strand cleavage. Of interest are the recent reports on induction of DNA single- and double-strand breaks in L1210 cells treated with the epipodophyllotoxin VP-16 [34, 36]. Evidence was presented that VP-16 is oxidized by a dehydrogenase with generation of an active intermediate, possibly a phenoxy- or quinone-type radical from the phenol group of the pendant ring, which reacts with DNA causing strand breaks [36]. DES, which contains two phenol groups, might be activated by a similar mechanism to form an active intermediate capable of damaging DNA; clearly further studies are required to test this hypothesis.

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