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ACTINOMYCIN D-ASSOCIATED LESIONS MIMICKING DNA–DNA INTERSTRAND CROSSLINKS DETECTED BY ALKALINE ELUTION IN CULTURED MAMMALIAN CELLS*

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Abstract—Cultured human fibroblasts were exposed to various concentrations of actinomycin D, a DNA intercalating agent, and studied by various alkaline elution techniques for the presence of DNA lesions. DNA–protein crosslinks increased proportionately with increasing actinomycin D, with 1.53 crosslinks/10^6 nucleotides after 5 μg/ml exposure. However, in the single-strand DNA break assay, elution of DNA initially increased as expected with increasing actinomycin D but thereafter decreased, with only 0.09 breaks/10^6 nucleotides detected after 25 μg/ml exposure, suggesting the presence of DNA crosslinking. A standard alkaline elution assay for DNA–DNA crosslinking was performed, and lesions which mimicked such crosslinks were detected, with a relative crosslink frequency of 2.30 after 5 μg/ml exposure. These actinomycin D-associated lesions disappeared when the alkaline elution procedure was modified to include additional proteinase digestion and use of the detergent sodium dodecyl sulfate (SDS) in the elution buffer, suggesting that they represented undigested DNA–protein crosslinks or nonspecific protein on the filters inhibiting DNA elution. Greater than ten times as many DNA–protein crosslinks were detected in fibroblasts than the number of single-strand DNA breaks after cellular exposure to actinomycin, even after determining breaks using the modified methodology for decreasing cellular protein interference. The data suggest that the actinomycin–DNA complex is associated with the formation of DNA–protein crosslinks which represent lesions other than endonuclease-associated DNA strand scission.

Actinomycin D, a widely used cancer chemotherapeutic agent, is an antibiotic which structurally has a phenoxazone ring system to which identical cyclic polypeptides are attached [1]. The phenoxazone chromophore appears to intercalate between the bases of double helical DNA, with the 2-amino group of guanine playing an important role in the formation of stable hydrogen binding between the drug and DNA [2]. Intercalation of actinomycin D disrupts the tertiary structure of DNA, leading to local uncoiling and subsequent inhibition of DNA and RNA synthesis.

Ross and associates [3, 4] have studied the qualitative and quantitative aspects of intercalator–DNA interaction. Compounds such as doxorubicin and ellipticine were found to be associated with large numbers of single-strand DNA breaks as well as with the formation of DNA–protein crosslinks, with a similar frequency of breaks and crosslinks [3]. It was hypothesized that the strand breaks are induced by a DNA enzyme, such as a topoisomerase, which becomes bound to one end of the DNA at the break, forming a DNA–protein crosslink, as part of the process of repairing the intercalator-induced distortion of the DNA helix [4]. As an intercalator, actinomycin D was shown to lead to the formation of both single-strand DNA breaks and DNA–protein crosslinks, but the precise quantitative association between these two lesions after actinomycin exposure was not determined [4]. Actinomycin D is not thought to bond covalently with DNA because it does not undergo strong electrophilic reactions which would be necessary for the formation of such bonds to nucleic acids. However, Muller and Crothers [2] have stated that actinomycin "increases the tendency of parts of the DNA molecule to interact with each other, leading to a kind of 'crosslinking' of the long randomly coiling chain, possibly through the generation of free radicals".

We have investigated the various DNA lesions associated with mammalian cellular exposure to actinomycin D, using the alkaline elution techniques originally designed by Kohn and associates [5–7]. These methods utilize the biophysical properties of DNA eluting from filters in high pH under various conditions. For example, the elution of DNA is enhanced with increasing DNA strand scission, while the presence of DNA crosslinking, either DNA–protein or DNA–DNA interstrand crosslinks, retards the elution of DNA. The use of a proteinase digestion step allows one to remove DNA–protein crosslinks so that there is no interference with the detection of the other two lesions. Our data suggest that actinomycin D is associated with the formation of large numbers of DNA–protein crosslinks after

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exposure of cultured mammalian cells to this antibiotic, and that this DNA lesion is found 10-fold more frequently than single-strand DNA breaks. Furthermore, using a standard proteinase digestion step in the alkaline elution assay, lesions were found which mimicked DNA–DNA interstrand crosslinks which could be removed by modifying the assay to include an additional proteinase digestion step associated with use of detergent in the elution buffer.

MATERIALS AND METHODS

Cell strains, labeling, and actinomycin D exposure. Normal, non-transformed human fibroblasts (cell strain HF-26) were initiated from a newborn foreskin sample [8]. Fibroblasts were maintained in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum, L-glutamine and sodium pyruvate, 100 I.U./ml penicillin, 100 μg/ml streptomycin, 10 μg/ml gentamicin, and 2.5 μg/ml amphotericin, and split every 7–14 days. All studies were performed on strains in passages 7–13.

Fibroblasts ($3–5 \times 10^4$) were plated on 60×15 mm tissue culture dishes with [2-^{14}C]thymidine, 0.02 μCi/ml (58.0 Ci/mole) (New England Nuclear). The radioactive medium was replaced with non-radioactive medium after 3 days, and the cells were allowed to incubate another day until the experiments were performed, at which time the cells were in exponential growth.

HF-26 cells were exposed to actinomycin D at concentrations from 0 to 25 μg/ml (20 μM) for 1 hr at 37° in tissue culture medium as described above. Care was taken to protect both the drug and the cells from direct light during experiments. After 1-hr exposure to actinomycin D, cells were washed three times with cold phosphate-buffered saline (PBS: 0.15 M NaCl, 0.7 mM KH_2PO_4, 4.3 mM K_2HPO_4) at 4°. Cells were then scraped off plates after the addition of Tris-glucose, 0.5 mM Na_2EDTA buffer and resuspended in 5 ml of PBS at 4° before being packed on ice.

DNA-protein crosslink assay. The method used to analyze DNA–protein crosslinks was a modification of the DNA alkaline elution technique described by Kohn and Ewig [7] in which the rate of DNA elution through a filter is retarded by increasing numbers of DNA–protein crosslinks. Mouse leukemia L1210 cells, grown in suspension for use as internal controls, were maintained at an approximate density of 1×10^6 cells/ml in RPMI 1640 (Gibco) with 10% horse serum, 100 I.U./ml penicillin and 100 μg/ml streptomycin in 25 cm^2 tissue culture flasks (Falcon No. 3013). Exponentially growing L1210 cells were labeled with [6-^{3}H]thymidine (0.1 μCi/ml medium, 21.5 Ci/mmole; New England Nuclear) for 18–72 hr prior to the day of experiment. These cells were then washed three times by centrifugation at 1500 rpm for 5 min followed by resuspension in 10 ml cold PBS. Following the washes, a cell count was done, and aliquots containing approximately 5×10^5 cells were diluted up to 10 ml with cold PBS in Falcon tubes and kept on ice continuously through irradiation with 150 rad by a ^{137}Cs gamma radiation source (JL Shepherd, Mark I) at a rate of 150 rad/min until used in experiments.

For each assay of DNA–protein crosslinking, approximately 5×10^5 [2-^{14}C]thymidine-labeled human fibroblasts were prepared for elution after treatment with actinomycin D and then irradiated on ice with 3000 rad. The actinomycin D-treated human cells and L1210 internal standard cells were then collected in PBS onto a polyvinyl chloride filter, 2.0 μm pore size (Millipore Corp., Bedford, MA), prewetted with 0.02 M Na_2EDTA. The cells were then lysed with 5 ml of a solution containing 2% sodium dodecyl sulfate (SDS), 0.02 M Na_2EDTA and 0.04 M glycine, pH 10, followed by 3 ml of 0.02 M Na_2EDTA, pH 10, with both solutions allowed to flow through the filter by gravity. The filter apparatus was then connected to a constant speed, multi-channel, peristaltic pump (Gilson Minipuls 2) and the DNA eluted at 1.9 ml/hr with a solution consisting of 0.02 M H_4EDTA and a sufficient quantity of filtered tetrapropyl ammonium hydroxide to give a final pH of 12.1 ± 0.02. Ten 90-min fractions of elution solution were collected (2.9 ml/fraction). The filter was then removed, incubated in 0.4 ml of 1 N HCl at 60° for 1 hr to release any DNA remaining on the filter, and neutralized with 2.5 ml of 0.4 N NaOH for 1 hr. The filter funnel was washed with 10 ml of 0.4 N NaOH, and an aliquot was saved for scintillation counting. The ^{14}C and ^{3}H radioactivity of all samples was determined after the addition of Aquasol II scintillation fluid (New England Nuclear) containing 0.78% (v/v) glacial acetic acid. The percent of [^{14}C]actinomycin D-treated fibroblasts retained on the filters was then determined as a function of percent of ^{3}H-labeled L1210 cells retained on the filters for each fraction, with the HF-26 cells analyzed in comparison to the standard elution pattern of the L1210 DNA, which is semilogarithmic with time [5, 6], to correct for slight differences between filters and thereby improve quantitation, as well as to allow for accurate comparison of elution profiles from different experiments.

Single-strand DNA break and DNA–DNA crosslink assays. Single-strand DNA breaks and DNA–DNA interstrand crosslinks were analyzed using modifications [9] of the DNA alkaline elution technique described by Kohn *et al.* [5, 6]. Actinomycin D-treated [^{14}C]thymidine-labeled human fibroblasts and [^{3}H]thymidine L1210 internal standard cells were collected onto polyvinyl chloride filters in PBS. Cells were then exposed to 1.5 ml of a solution containing 2% SDS, 0.02 M Na_2EDTA, 0.04 M glycine, and proteinase K (E. Merck) 0.5 mg/ml, pH 9.6, for 45 min. The DNA was then eluted with the proteinase K/lysis solution pumped ahead of the elution solution. For analyzing single-strand breaks, cells were treated with actinomycin D and placed directly on filters; increasing drug-induced breaks leads to increased DNA elution. For analyzing DNA–DNA crosslinks, cells were treated with actinomycin D, washed, packed in ice as above, and irradiated with 300 rad to introduce a known number of DNA breaks [6] before being placed on filters; increasing crosslinks leads to decreased DNA elution.

After initial data from experiments described below were obtained, the above procedures were

amended to include an additional proteinase K step. Specifically, cells were treated for a minimum of 1 hr with the proteinase K/SDS lysis solution. The solution was allowed to flow through the filter by gravity, and a second proteinase K/SDS lysis solution identical to above was re-layered on the filters, with the lysed cells exposed for an additional 45 min before this solution was pumped ahead of the elution solution, which was identical to that described except for the addition of 0.1% SDS. The ^{14}C and ^{3}H radioactivity of the filter, funnel wash, and each of the ten elution fractions was determined as described above.

Determination of protein binding to elution filters. To determine the contribution of multiple proteinase steps and addition of 0.1% SDS in the elution buffer to protein elution, fibroblasts were labeled with L-[^{14}C-U]leucine, 0.1 μCi/ml (335 Ci/mole; New England Nuclear), for 3 days, exposed to actinomycin D, and studied by the various elution methods as described above, including the use of irradiated ^{3}H-labeled L1210 internal standard cells.

RESULTS

Utilizing the alkaline elution technique without the proteinase K digestion step, DNA–protein crosslinks were examined in HF-26 cells after a 1-hr exposure of actinomycin D at concentrations between 0 and 25 μg/ml (0–20 μM). DNA–protein crosslinks were quantitated using the "randomly distributed lesions" model as described by Ross *et al.* [4] in which the frequency of drug-induced DNA–protein crosslinks, P_d, equals $[(1 - r)^{-1/2} - (1 - r_0)^{-1/2}] \times p_b$, where r_0 represents the extrapolated percentage of DNA retained on the filter in the slow component of the DNA elution profile in cells not exposed to drug, r represents the same factor for DNA from cells exposed to various concentrations of actinomycin D, and P_b is the frequency of gamma radiation induced single-strand breaks. As can be seen in Fig. 1, there is an orderly increase in DNA–protein crosslinks detected with exposure of HF-26 cells to increasing concentrations of actinomycin D. The relationship between DNA–protein crosslinks and actinomycin D appears linear through all drug concentrations between 0 and 5 μg/ml (correlation coefficient $r = 0.93$, with a slope of 0.306 and a Y intercept of 0.020). DNA–protein crosslinks determined after 25 μg/ml exposure were relatively less due to limitations in the ability to detect more than the maximum amount of DNA filter retention after cellular exposure to this concentration. Specifically, on the average 1.53 DNA–protein crosslinks/10^6 DNA nucleotides were detected after 5 μg/ml exposure to actinomycin D and 3.00 crosslinks/10^6 nucleotides after exposure to 25 μg/ml.

Single-strand DNA breaks in cultured human fibroblasts were then examined after actinomycin exposure using the standard alkaline elution technique, with a 45-min proteinase K digestion step [6]. Break frequency was determined using the relative retention of DNA on filters compared to cells irradiated with known doses of gamma radiation, with a linear relationship between log of relative retention and number of single-strand DNA breaks induced [5]. The amount of DNA elution increased initially with increasing actinomycin D dose, with 0.144 break equivalents/10^6 nucleotides noted after 2.5 μg/ml actinomycin D exposure. However, with increasing actinomycin D concentrations, the elution of DNA appeared to be retarded, with only 0.091 single-strand DNA break equivalents/10^6 nucleotides

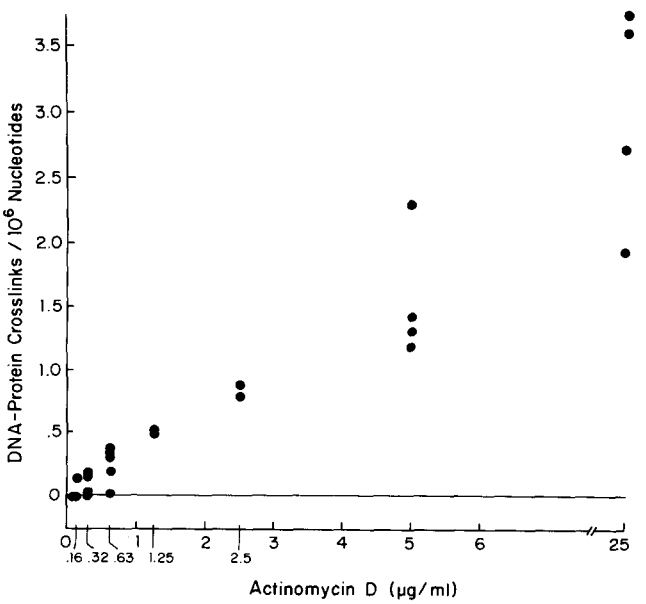

Fig. 1. DNA–protein crosslinks as a function of actinomycin D dose in cultured human fibroblasts. HF-26 cells were exposed to various concentrations of actinomycin D for 1 hr, and the number of DNA–protein crosslinks was determined using the alkaline elution assay [7].

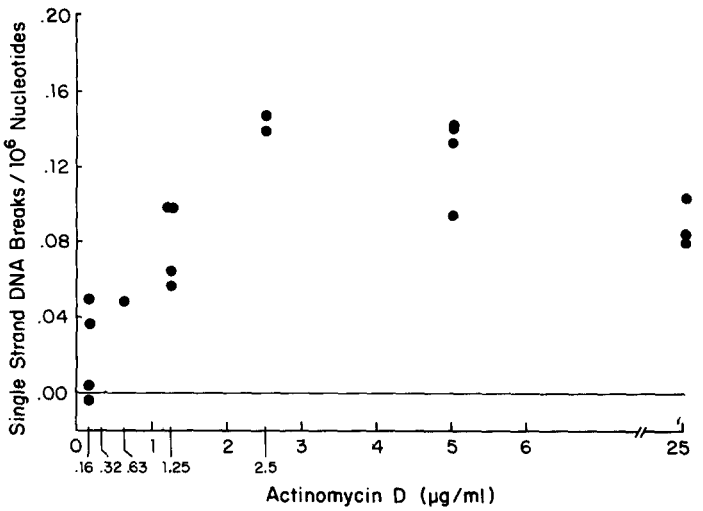

Fig. 2. Single-strand DNA break equivalents as a function of actinomycin D dose in cultured human fibroblasts. HF-26 cells were exposed to various concentrations of actinomycin D for 1 hr, and the number of single-strand DNA breaks was determined using a standard alkaline elution assay [5, 6].

detected after exposure to 25 μg/ml actinomycin D (Fig. 2).

To examine the possibility that the decrease in single-strand break equivalents noted after exposure to large concentrations of actinomycin was due to interference by DNA–DNA crosslinks, relative retention of DNA on filters was examined in HF-26 cells after actinomycin D exposure, using the standard alkaline elution technique [6]. Relative retention of DNA, determined as $\log r_0/\log r$ (where r_0 represents the relative retention of DNA after no drug exposure and r represents the relative retention of DNA after drug exposure), is plotted versus actinomycin D concentration in Fig. 3 (closed circles). The results clearly show an increase in relative retention with increasing actinomycin D exposure in these cultured human cells, with a relative retention of 1.64 ± 0.51 (S.E.M.) after 2.5 μg/ml exposure of actinomycin D, 2.30 ± 0.35 after 5 μg/ml exposure, and 3.55 ± 0.59 after 25 μg/ml exposure.

The data presented in Fig. 3 suggested the presence of DNA–DNA crosslinks in cultured HF-26

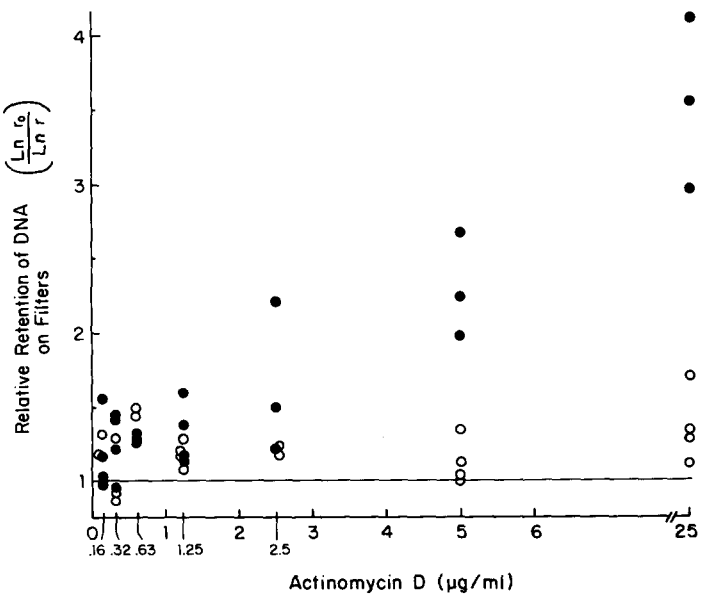

Fig. 3. Relative retention of DNA on filters as a function of actinomycin D in cultured human fibroblasts. HF-26 cells were exposed to various concentrations of actinomycin D for 1 hr, and the relative DNA–DNA crosslinking was determined using the alkaline elution assay [6]. Symbols: (●) crosslinking determined using standard alkaline elution method; and (○) crosslinking determined by the alkaline elution assay modified to include an additional proteinase K digestion step as well as 0.1% SDS in the elution buffer.

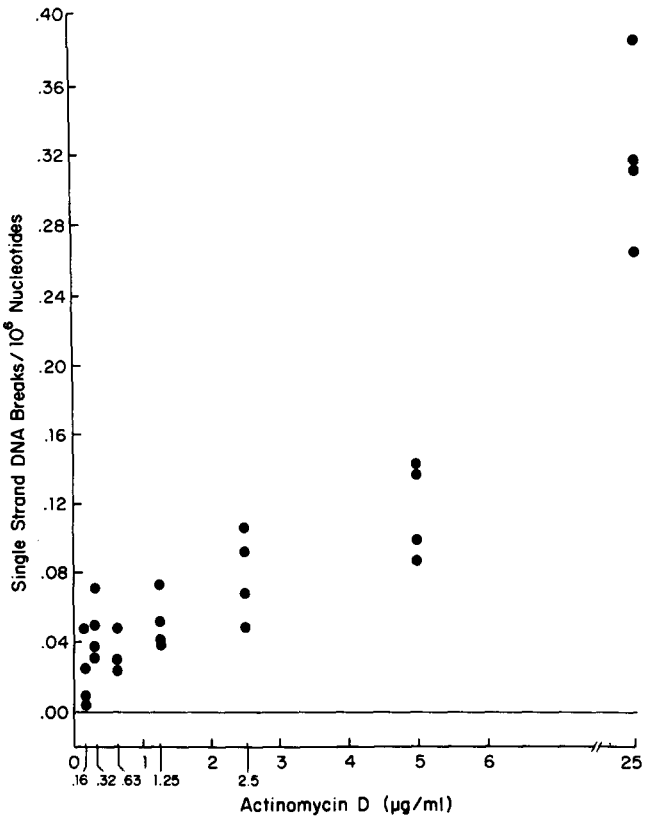

Fig. 4. Single-strand DNA break equivalents as a function of actinomycin D dose in cultured human fibroblasts determined by the alkaline elution assay modified to include an additional proteinase digestion step as well as detergent in the elution buffer. Cells were studied as in Fig. 1, except that the procedure was modified as described in Materials and Methods.

cells after actinomycin D exposure. Because actinomycin D is not felt to form covalent bonds leading to crosslinking of nucleic acids, the elution methodology was modified to include an additional proteinase K step, in which the lysed cells on filters were re-exposed to an additional incubation with 0.5 mg/ml proteinase K before DNA elution in a buffer containing 0.1% SDS (see Materials and Methods above). Relative retention of DNA on filters, $\log r_0/\log r$, was redetermined for HF-26 cells exposed to various concentrations of actinomycin and treated twice with proteinase K (Fig. 3; open circles). No DNA–DNA crosslinks could now be detected, even after cellular exposure to high concentrations of the antibiotic.

Experiments utilizing [^{14}C-U]leucine-labelled cells were performed to examine protein binding to elution filters under various conditions. After cellular exposure to 25 μg/ml actinomycin, no proteinase digestion, and no detergent in the elution buffer, 5.4% of the original protein remained on the filter at 40% retention of the internal standard DNA. Increasing protein elution was seen with one proteinase step (3.8% filter retention), but even after two proteinase treatments 2.4% of the original protein remained on the filter without use of SDS in the elution buffer. Addition of detergent increased the protein elution (1.9% filter retention). Precise quantitation of results was impeded by the low total [^{14}C-U]leucine counts remaining on the filter after elution such that background radioactivity became significant.

The single-strand DNA break frequency in cells exposed to increasing concentrations of actinomycin was redetermined using the additional proteinase K step with SDS in the elution buffer (Fig. 4). Although no increase in single-strand break frequency was detected in cells exposed to low concentrations of actinomycin (less than 5 μg/ml) and studied by the modified procedure, a large difference was apparent at higher doses: 0.28 single-strand breaks/10^6 nucleotides were detected versus 0.08 breaks/10^6 nucleotides found using the more standard assay, a difference of 3.5-fold. Small differences detected in single-strand break frequency utilizing the two procedures at lower concentrations of the intercalator can be attributed to experimental error in the methodologies used (actual points from separate experiments are plotted on Figs. 2 and 4). Using the modified procedure, a positive relationship between single-strand breaks and actinomycin D concentration was apparent, giving a line with a slope of 0.011, a Y intercept of 0.04, and a correlation coefficient $r = 0.97$ (Fig. 4).

Using the modified procedure to determine single-strand DNA breaks less influenced by protein, the relationship between DNA–protein crosslink formation and single-strand DNA break formation in

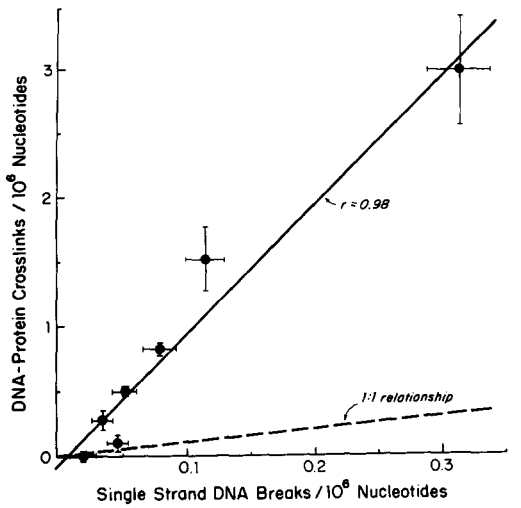

Fig. 5. Relationship between DNA–protein crosslinks and single-strand DNA breaks in actinomycin D-treated human fibroblasts. Points are plotted ± S.E.M. The dashed line represents a one-to-one relationship between DNA–protein crosslinking and single-strand DNA breaks.

HF-26 cells exposed to various concentrations of actinomycin D was then explored (Fig. 5). Increasing concentrations of actinomycin D led to increases in DNA–protein crosslinks and single-strand breaks, with a linear relationship noted. However, contrary to the one-to-one relationship found between single-strand breaks and DNA–protein crosslinks for intercalating agents, as described by Ross *et al.* [4], considerably more DNA–protein crosslinks were detected compared to single-strand DNA breaks in HF-26 cells, with a slope of 10.3, a correlation coefficient $r = 0.98$, and a Y intercept of -0.09 DNA–protein crosslinks/10^6 nucleotides.

DISCUSSION

DNA intercalating agents, such as the anthracyclines daunomycin and doxorubicin, and actinomycin D, have been important because many exhibit antitumor activity. Although the cytotoxicity of these agents is felt to be related to their intercalating abilities, little is known at the molecular level about how a cell is initially affected by or subsequently responds to this type of DNA damage. It has been suggested that, for the anthracyclines, ellipticine, and 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (m-AMSA), DNA intercalation initially leads to the formation of DNA single-strand breaks that are intimately associated with the formation of covalent bonding between DNA and protein, hypothesized to be at the scission site [4, 10–12]. Endogenous endonucleases such as topoisomerases may be responsible for the DNA breaks when they covalently bind to DNA in an attempt to repair the tertiary structural abnormalities which have occurred [10, 13, 14].

Little work has been done to specifically examine DNA lesions associated with actinomycin D intercalation in mammalian cells. Although actinomycin leads to the formation of single-strand breaks and DNA–protein crosslinks [4], as do the other intercalating agents, the exact relationship of these lesions has not before been characterized. In the experiments reported herein, the single-strand DNA break frequency in cultured cells exposed to actinomycin, as detected by the standard alkaline elution method, initially increased with increasing concentrations of drug as anticipated but then decreased (Fig. 2). However, with a more aggressive treatment of cells to remove protein after actinomycin exposure, increased numbers of single-strand breaks were identified at all concentrations of drug studied (Fig. 4). The standard proteinase digestion step during the alkaline elution procedure removes about 99% of cellular protein in L1210 cells [6], but the quantity of histone and non-histone protein, or chromatin, associated with DNA which is removed is unknown. Sequential proteinase steps coupled with the addition of a detergent in the elution buffer removed more total cellular protein in the human fibroblasts studied herein (98.1% vs 96.2% for the standard proteinase treatment). Exact quantitation of nuclear protein removed was not determined. The modified procedure allowed a more accurate quantitative determination of the number of single-strand breaks formed after intercalator exposure (Fig. 4).

Despite detecting a linear relationship between single-strand DNA breaks and actinomycin D concentration with increased protein removal during alkaline elution, the DNA–protein crosslink frequency appeared greater than 10-fold in excess of the DNA break frequency (Fig. 5). Breaks and crosslinks were studied at multiple concentrations of actinomycin D, with a linear relationship seen up to the limits of the methodologies used. DNA–protein crosslinks were determined using a model assuming that the crosslinks are randomly distributed rather than associated 1:1 with single-strand DNA segments [4]; if one recalculates the frequency of drug-induced DNA–protein crosslinks using a model assuming one crosslink per single-strand DNA segment [4], the ratio was even greater (data not shown). Furthermore, the method used to study DNA–protein crosslinks, with 2% SDS in the lysis solution, may somewhat inhibit DNA–protein crosslink absorption to filters compared with use of other detergents such as Sarkosyl [7], so that the quantity of DNA–protein crosslinks may have been underestimated. These data suggest that actinomycin D leads to the formation of DNA–protein crosslinks which are not associated with single-strand DNA breaks. If the model of intercalator-induced DNA strand scission applies to actinomycin-induced cellular damage, other forms of DNA–protein crosslinks must also be present. Alternatively, none of the single-strand breaks and DNA–protein crosslinks found in cells after actinomycin exposure may be related to endonuclease action. Regardless, cells exposed to actinomycin D appear to exhibit DNA–protein crosslinks by a mechanism which to date is unexplained. Future experiments could be performed utilizing topoisomerase inhibitors, such as berenil [15], to determine if any of the DNA–protein crosslinks and single-strand breaks formed after actinomycin D exposure are associated with this endonuclease.

The formation and disappearance of protein-associated DNA scission may be important in determining the cytotoxocity of intercalating agents. For example, ellipticine, a weakly cytotoxic agent, induces a high frequency of these lesions which disappear within an hour [15]. On the other hand, doxorubicin, a potent chemotherapeutic agent, leads to less protein-associated strand scission, even at equally toxic doses, which are not totally removed even 24 hr after drug exposure [15]. Future studies examining the formation and disappearance of these lesions with time after cellular exposure to actinomycin may shed light on the role of endonucleases in responding to the induced alterations in DNA helical structure.

In the experiments above, lesions were found in human fibroblasts exposed to actinomycin D which mimicked DNA–DNA interstrand crosslinks as detected by the alkaline elution assay. These lesions disappeared when the assay was modified to include a second proteinase digestion step with SDS in the elution buffer, thereby suggesting that DNA–protein crosslinks and/or nonspecific protein in the filters were interfering with the DNA–DNA crosslink assay by inhibiting DNA elution. The actual mechanisms involved in the reduction of this interference in the modified assay are unknown. From experiments performed above, use of proteinase for two 45-min cellular exposures reduced the amount of total cellular protein adhering to elution filters compared to use of a single proteinase step. Use of detergent in the elution buffer further contributed to increased protein elution. One other variable was introduced in the procedure change: in the modified assay, the first SDS-proteinase K lysis buffer was allowed to drip through the filter by gravity and the second proteinase solution was pumped ahead of the elution solution, similar to the procedure in the original assay. Gravity dripping could conceivably remove more protein than pumping or conversely could increase DNA breaks, with either mechanism leading to the appearance of less DNA retention on the filters, a sign of less DNA crosslinking. Increased DNA breaks induced by the modified procedure can be ruled out, because elution patterns of control, gamma-irradiated fibroblasts not exposed to actinomycin and included in each single and double proteinase assay did not change, regardless of the method employed. However, preliminary experiments examining gravity dripping versus pumping for protein removal suggest an advantage for the former technique. Future experiments will examine this methodological aspect more closely. Unfortunately, quantitation of total cellular protein removed by a modified elution procedure does not necessarily reflect effects of such treatment on protein associated with DNA. Therefore, no firm conclusions can be made as to the contribution of various aspects of the modified procedure on elimination of interfering DNA–protein crosslinks.

No evidence was hence found for true covalent bonding between DNA strands after actinomycin D exposure. Fibroblast exposure to this intercalating agent is, however, associated with the production of large numbers of DNA–protein crosslinks, with over 3 crosslinks found/10^6 nucleotides after a 1-hr exposure to concentrations of 25 μg/ml. Preliminary experiments not shown suggest that the level of DNA–protein crosslinks may be somewhat determined by the cell strain studied, as mouse L1210 leukemia cells did not exhibit the degree of crosslinking seen in the normal human fibroblast cell strain used in the experiments above. However, because certain pharmacologic agents may lead to the formation of a sufficient quantity of DNA–protein crosslinks to both mask single-strand breaks as well as mimic DNA–DNA crosslinks using standard alkaline elution assays, we suggest that these procedures be modified to include an additional proteinase step with gravity dripping and detergent in the elution buffer to ensure minimal protein interference.

In summary, actinomycin D induced large numbers of DNA–protein crosslinks that mimicked DNA–DNA interstrand crosslinks in cultured mammalian fibroblasts as detected by a standard alkaline elution assay. Furthermore, the frequency of DNA–protein crosslinks formed in cells exposed to this intercalator exceeded by 10-fold the frequency of single-strand DNA breaks, suggesting that the cellular response to actinomycin D–DNA complexes involves more than endonuclease-associated DNA strand scission.

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REFERENCES

1. E. F. Gale, E. Cundliffe, P. E. Reynolds, M. H. Richmond and M. J. Waring, *The Molecular Basis of Antibiotic Action*, pp. 258–401. John Wiley, London (1981).
2. W. Muller and D. M. Crothers, *J. molec. Biol.* **35**, 251 (1968).
3. W. E. Ross, D. L. Glaubiger and K. W. Kohn, *Biochim. biophys. Acta* **519**, 23 (1978).
4. W. E. Ross, D. Glaubiger and K. W. Kohn, *Biochim. biophys. Acta* **562**, 41 (1979).
5. K. W. Kohn, L. C. Erickson, R. A. G. Ewig and C. A. Friedman, *Biochemistry* **15**, 4629 (1976).
6. K. W. Kohn, *Meth. Cancer Res.* **16**, 291 (1979).
7. K. W. Kohn and R. A. G. Ewig, *Biochim. biophys. Acta* **562**, 32 (1979).
8. W. G. Woods, *Biochim. biophys. Acta* **655**, 342 (1981).
9. M. E. Steiner and W. G. Woods, *Mutation Res.* **95**, 515 (1982).
10. Y. Pommier, D. Kerrigan, R. Schwartz and L. A. Zwelling, *Biochem. biophys. Res. Commun.* **107**, 576 (1982).
11. L. A. Zwelling, D. Kerrigan and S. Michaels, *Cancer Res.* **42**, 2687 (1982).
12. L. A. Zwelling, S. Michaels, D. Kerrigan, Y. Pommier and K. W. Kohn, *Biochem. Pharmac.* **31**, 3261 (1982).
13. W. Ross, C. Ross and M. Smith, *Proc. Am. Ass. Cancer Res.* **22**, 243 (1981).
14. L. A. Zwelling, S. Michaels, L. C. Erickson, R. S. Ungerleider, M. Nichols and K. W. Kohn, *Biochemistry* **20**, 6553 (1981).
15. W. E. Ross and M. C. Smith, *Biochem. Pharmac.* **31**, 1931 (1982).