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REPAIR OF DEOXYRIBONUCLEIC ACID LESIONS CAUSED BY ADRIAMYCIN AND ELLIPTICINE*

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Abstract—The intercalating agents adriamycin and ellipticine caused DNA single and double strand breaks and DNA–protein crosslinks in mammalian cells. Ellipticine caused a much higher frequency of each of these lesions than adriamycin, at equitoxic doses. However, adriamycin-induced DNA effects were slowly and incompletely repaired over 24 hr after drug removal while the effects of ellipticine were virtually completely repaired within 30 min. Repair of ellipticine-induced lesions was observed only if cells were diluted (~100-fold) after drug treatment, suggesting that significant amounts of drug may have been sequestered in the cellular membranes. Cell dilution did not alter the effects of adriamycin. Removal of the DNA lesions was temperature dependent. Finally, the similarity in the rates of repair for DNA single and double strand breaks and DNA–protein crosslinks supports the hypothesis that they are functionally related.

A variety of drugs which bind to DNA by intercalation between base pairs have been examined for antitumor activity. While major differences in cytotoxicity exist, it is not clear what pharmacological properties are responsible for these differences. One such property of potential interest is the disruption of the structural integrity of DNA when cells are exposed to these drugs. A number of investigators have shown that intercalating agents cause DNA single strand breaks in mammalian cells [1–3]. Ross *et al.* [3, 4] have presented evidence that these strand breaks occur in association with another lesion, DNA–protein crosslinks. In addition, we have reported recently that DNA double strand breaks result when cells are treated with intercalating agents [5]. Double strand breaks are generally considered to be a highly lethal form of DNA damage. Unfortunately, direct evidence relating intercalator-induced DNA damage to cytotoxicity has not uniformly supported a cause–effect relationship. For example, when the weakly cytotoxic ellipticine was directly compared with the more potent adriamycin and actinomycin D, the less potent drug generated a much greater frequency of DNA strand breaks than the more toxic agents [6]. Ellipticine also causes a higher frequency of DNA double strand breaks in L1210 cells than either adriamycin or actinomycin D at equitoxic doses [5].

In our current work, we have examined the repair of various forms of DNA damage observed in L1210 cells following exposure to adriamycin and ellipticine. In addition to noticing major differences in repair rates, we report a methodologic problem which may be relevant to future studies using this group of drugs.

MATERIALS AND METHODS

Drugs were obtained from the Developmental Therapeutics Program of the Division of Cancer Treatment, NCI.

Mouse leukemia L1210 cells were grown in suspension in RPMI 1630 medium with 10% horse serum. Details of tissue culture technique and the labeling of cells with thymidine have been published previously [7].

Cells were resuspended in fresh warm medium at $5–7 \times 10^5$ cells/ml, 1 hr prior to drug exposure. Cells were treated with drug for 1 hr after which they were centrifuged and resuspended in fresh medium. Because our experimental results were highly dependent on the method of drug removal, this is described in greater detail in Results.

Cytotoxicity was determined by the decrease in colony formation in soft agar, as described by Chu and Fisher [8]. Following drug removal, cells were serially diluted into tubes containing medium and 1% agar. Cell densities ranged from 10^2 to 10^5 cells per 4-ml tube. Colony-forming efficiency of untreated control cells was 50–70%.

The DNA alkaline elution technique was used to assay DNA single and double strand breaks as well as DNA–protein crosslinks. The methods for each of these assays have been described in detail elsewhere [5, 9]. For the single strand break assay, cells containing ^{14}C-labeled DNA are layered onto a membrane filter and lysed in the presence of proteinase K, and the DNA is eluted from the filter using tetrapropylammonium hydroxide at pH 12.1. The rate of elution from the filter is inversely related to strand size and, therefore, is directly related to the frequency of single strand breaks. The DNA double strand break assay is similar except that the eluting

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buffer is at a non-denaturing pH, namely 9.6 [5]. The DNA–protein crosslink assay is based on the fact that, under standard elution conditions, protein adsorbs to the filter [10].

By administering a high dose of gamma radiation (3000 rads) to the cells immediately prior to elution and by lysing the cells in the absence of proteinase K, the DNA which elutes from the filter is separated into fast and slow components. The former represents DNA reduced in size by radiation but not bound to protein while the latter consists of DNA bound to the filter by virtue of proteins which have become crosslinked to it. Thus, the retention of DNA on the filter increases with the frequency of DNA–protein crosslinks.

RESULTS

The relative cytotoxicity of adriamycin and ellipticine to L1210 cells is shown in Fig. 1. Following 1 hr of exposure to adriamycin over a concentration range of 0.5 to 1.5 μg/ml, a marked loss of clonogenic potential was observed. In contrast, ellipticine was far less cytotoxic, even over a concentration range of 1.25 to 15 μg/ml.

To examine the relationship between cytotoxicity and DNA damage, 10 ml of 5–7 × 10^5 cells/ml was treated with adriamycin (1.0 μg/ml) or ellipticine (3.75 μg/ml) for 1 hr, twice washed and resuspended in equal volumes of fresh warm medium, and examined at various times thereafter by the alkaline elution technique. The single strand break frequencies

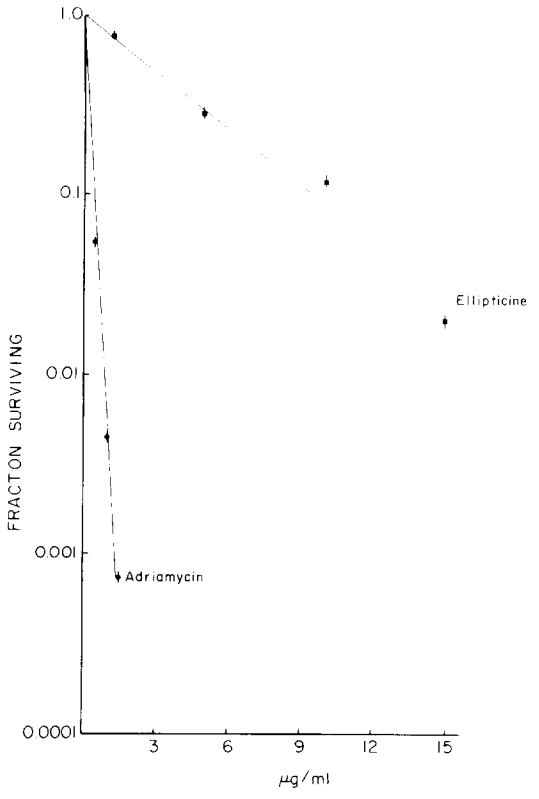

Fig. 1. Colony forming ability of L1210 cells following a 1-hr treatment with adriamycin or ellipticine at various doses. Each point is the mean of at least three experiments ± S.E.M.

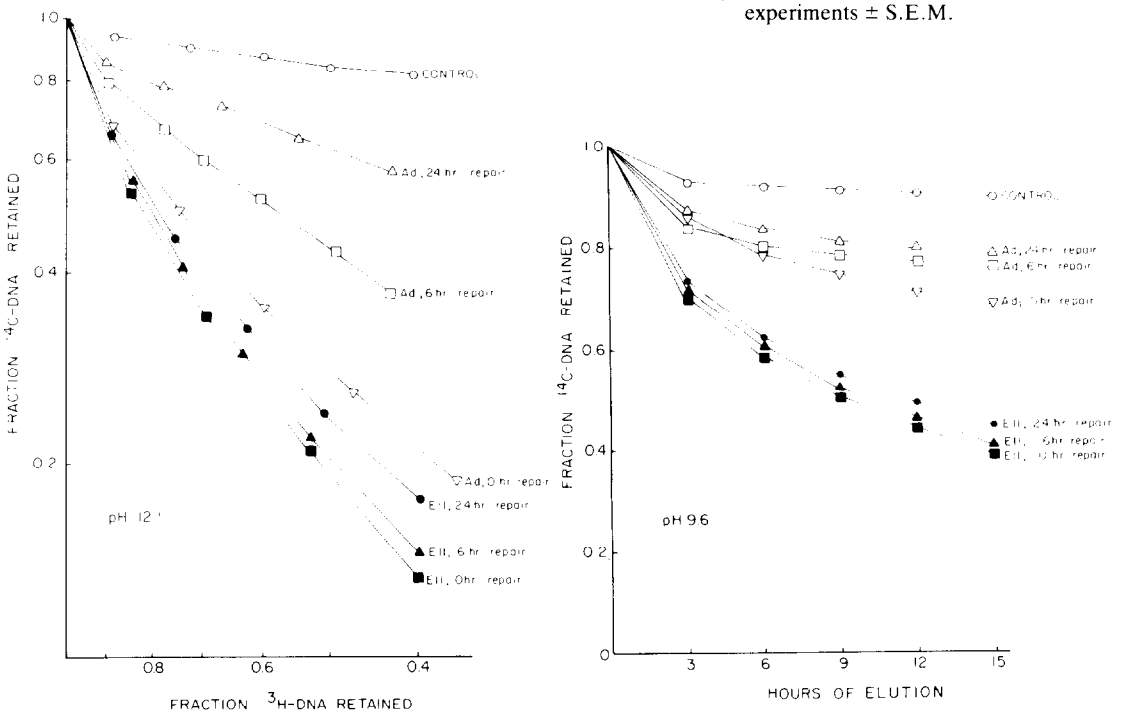

Fig. 2. Repair of DNA single and double strand breaks in L1210 cells following treatment with adriamycin and ellipticine. Cells containing ^{14}C-labeled DNA were exposed to ellipticine (3.75 μg/ml) or adriamycin (1.0 μg/ml) for 1 hr and then resuspended twice in equal volumes of fresh warm medium (37°). Single strand break frequency (left) was determined by alkaline elution at a denaturing pH 12.1. Experimental [^{14}C]DNA is plotted against simultaneously eluted internal standard [^{3}H]DNA. Double strand breaks were determined by alkaline elution at a non-denaturing pH 9.6 (right).

which resulted at the drug concentrations used were roughly equivalent immediately after drug removal (Fig. 2, left), but over the ensuing 24 hr much of the damage caused by adriamycin was repaired while there was little, if any, repair of ellipticine-induced single strand breaks. As previously reported [5], DNA double strand breaks were observed following treatment of L1210 cells with adriamycin and ellipticine. The rate of repair of these double strand breaks (Fig. 2, right) appeared to be similar to that of the single strand breaks.

In comparing the techniques for determining drug-induced cytotoxicity and DNA repair, it is important to note that, in the former, the cells were serially diluted prior to seeding in soft agar whereas in the DNA repair studies cells are generally maintained at densities of 5–7 × 10^5 cells/ml. Because the difference in cell density may have an important influence on drug efflux for a lipophilic drug such as ellipticine, the resuspension procedure was modified in the following way. After exposure to ellipticine, the cells were twice centrifuged and resuspended in 10 ml of fresh medium; an aliquot of 10^6 cells was then diluted in 100 ml of fresh medium and incubated at 37° for various times. As seen in Fig. 3, the cells, which were diluted prior to post-treatment incubation, exhibited rapid repair of both single and double strand breaks. In fact, the repair was virtually complete by 30 min. Interestingly, if the cells were held at 0° during this incubation period, no repair was seen. Similar dilutions of adriamycin-treated cells were performed, but there was no effect on repair of DNA strand breaks (data not shown).

The results shown in Fig. 3 suggest that, despite resuspension of ellipticine-treated cells in drug-free medium, most of the drug remained in the cells and effluxed only if the intracellular:extracellular concentration gradient were enhanced by increasing the relative extracellular volume. To test the hypothesis that the cells could act as a drug sink, cells containing unlabeled DNA were treated with ellipticine for 1 hr and resuspended in fresh warm medium at a cell density of 7 × 10^5/ml. Cells containing ^{3}H-labeled DNA were then added to the drug-treated cells, allowed to incubate for 1 hr, and then examined by alkaline elution. No cell lysis occurred during the cell admixture period. Nearly the same frequency of single strand breaks was observed in the ^{3}H-labelled DNA as would be expected if the cells had been directly treated with ellipticine (data not shown). In a separate control tube, medium containing drug but no cells was centrifuged, decanted, and replaced with medium containing ^{3}H-labeled cells but no drug. No DNA strand breaks were observed in these cells. Thus, following removal of ellipticine-containing medium, remaining drug derived primarily from the previously exposed cells rather than from adsorption to plastic or precipitation of the drug itself.

We have shown previously that treating L1210 cells with a variety of intercalating agents causes DNA–protein crosslinks in addition to strand breaks [3, 4]. Because the breaks and crosslinks are spatially and quantitatively related, we have hypothesized that these proteins may represent enzymes which nick DNA in response to intercalation of drug. It was thus of interest to determine if the DNA–protein crosslinks were removed at the same rate as the

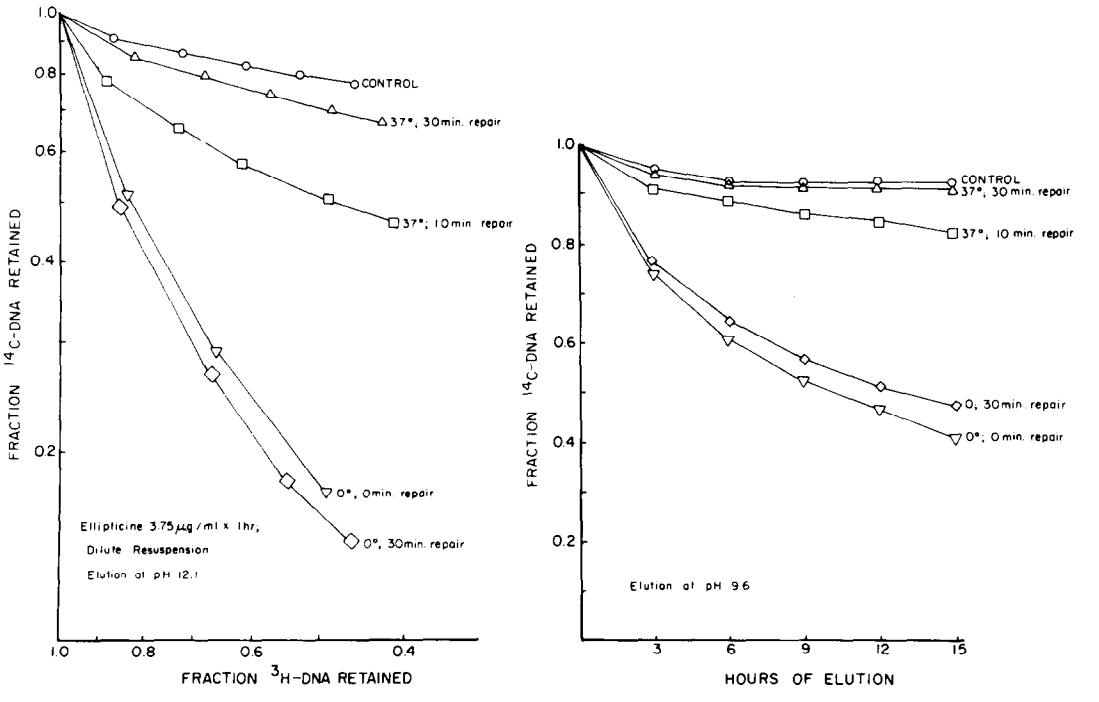

Fig. 3. Effect of cell dilution and temperature on repair of ellipticine-induced DNA strand breaks. These experiments were identical to those in Fig. 2, except that following drug treatment cells were diluted to a cell density of 10^4/ml in fresh medium. Where noted in the graph, the cells were maintained in ice-cold medium for various periods prior to elution. Cells allowed to repair at 37° were resuspended in warm medium following drug treatment.

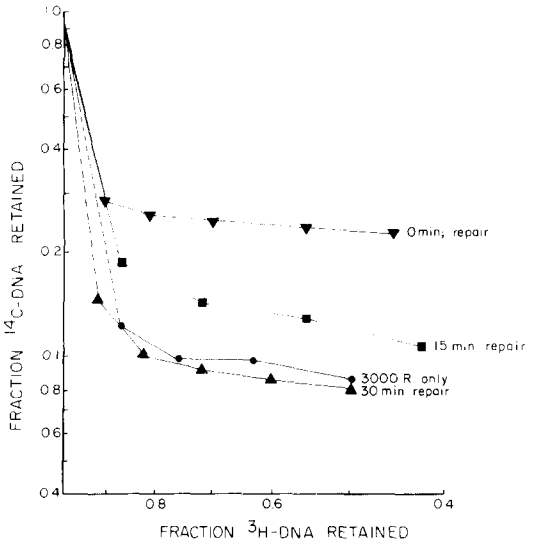

Fig. 4. Repair of DNA–protein crosslinks following a 1-hr treatment with ellipticine, 3.75 μg/ml. Following drug treatment cells were resuspended in fresh medium at a cell density of 10^4 cells/ml. Immediately prior to elution, cells were cooled on ice, irradiated with 3000 rads, and deposited on the elution filter. Cell lysis was performed without proteinase K, and the pH of the elution buffer was 12.1.

strand breaks. Indeed, Figs. 4 and 5 show that repair of DNA–protein crosslinks following a 1-hr exposure to ellipticine and adriamycin occurred over approximately the same period of time as the DNA strand breaks.

DISCUSSION

Previous work from this laboratory has shown that, in the L1210 cell line, adriamycin is more cytotoxic but causes less measurable DNA damage than ellipticine. This result had been difficult to reconcile with the premise that DNA strand breakage was causally related to cytotoxicity as suggested by others [1, 2]. However, our current work indicates that, while the effects of ellipticine on DNA are greater initially than those of adriamycin, repair of the former is much more rapid. The simplest explanation for these observations is that delayed repair of the adriamycin-induced DNA lesions reflects persistence of intracellular drug and that cytotoxicity is, therefore, largely dependent on the continued presence of drug. A similar conclusion was reached by Zwelling *et al.* [11] who studied the effect of the intercalating agent 4′-(9-acridinylamino)-methanesulfon-*m*-ansidide (*m*-AMSA). They found that this drug effluxed rapidly from L1210 cells after resuspension in drug-free medium and that the DNA strand breaks were repaired within 30 min. They also noted slow repair of adriamycin-induced DNA single strand breaks but did not examine repair of double strand breaks or DNA–protein crosslinks. The role of intracellular retention of adriamycin in determining cytotoxicity has also been noted in regard to acquired resistance. Inaba *et al.* [12] found that P-388 cells, made resistant to the adriamycin analogue, daunomycin, exhibited a higher drug efflux rate due to an energy-dependent transport mechanism. In this case, decreased drug retention caused decreased cytotoxicity.

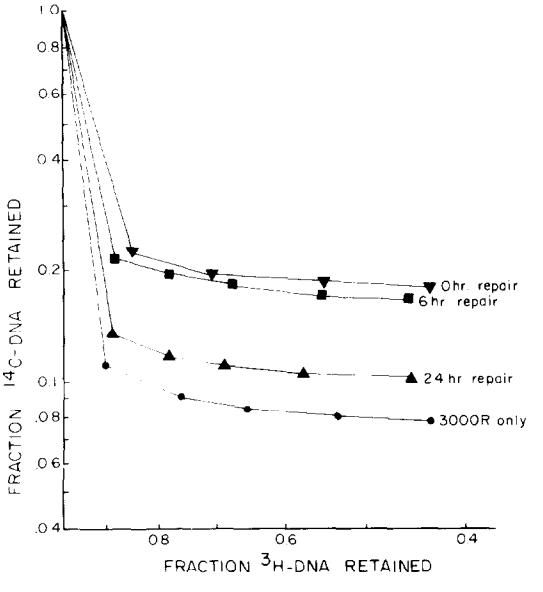

Fig. 5. Repair of DNA–protein crosslinks following a 1-hr treatment with adriamycin, 1.0 μg/ml. Experimental procedure was identical to that in Fig. 4.

Although our data, as well as those of Zwelling *et al.* [11], indicate a strong association between repair of DNA damage and maintenance of intracellular drug concentration, the relationship between the DNA effects and the mechanism of cell death remains unclear. On the basis of evidence accrued to date, the DNA breaks and crosslinks could represent a form of damage which contributes to cell death or a means of relieving the topological distortions imposed by intercalation of drug into DNA, perhaps via a topoisomerase as previously suggested [13]. With regard to the latter, it is of interest that we have shown recently that berenil, an inhibitor of the L1210 nuclear Type I topoisomerase, also partially inhibits the formation of DNA strand breaks in cells simultaneously exposed to either ellipticine or adriamycin.

Paoletti *et al.* [14] have reported that DNA single strand breaks caused by elipticine and several congeners were rapidly repaired. The time course of the repair process was not well defined, however, and the method of drug removal was not detailed. We found that repair of ellipticine-induced DNA damage was not evident unless the cells were adequately diluted following drug removal. This most likely relates to the solubility characteristics of ellipticine. In aqueous solution at physiological pH, ellipticine exists in a water-soluble protonated form and a lipophilic uncharged form [15]. The octanol:water partition ratio has been estimated to be 10^2 [16] and 10^4 [17]. The protonated species of ellipticine is responsible for DNA binding [18] while the lipophilic form binds avidly to membranes [19].

Thus, we believe that, without cell dilution following drug removal, a significant amount of ellipticine remains bound to plasma membrane in equi-

librium with the intra- and extracellular aqueous milieu. That the drug is in equilibrium with the intracellular and extracellular compartments is supported by the fact that, when untreated cells are added to a suspension of previously treated cells, DNA strand breaks appear in the cells not directly treated. In addition, because of the lower intracellular pH, some drug will be maintained in the ionized form. Dilution of the cells in drug-free medium results in movement of the drug out of the membrane (and this out of the intracellular water) by mass action. This effect is visually observable, since ellipticine-treated cells resuspended without dilution retain the bright yellow color of the drug, but upon dilution this color is lost. Since efflux of adriamycin is generally a slow process dependent on active transport (*vide supra*), cell dilution would be expected to have little effect on drug movement. In fact, dilution of adriamycin-treated cells did not influence repair of DNA breaks and crosslinks.

The repair of ellipticine-induced DNA damage is temperature dependent (Fig. 3). While our data do not allow us to unequivocally conclude whether the temperature effect is on drug efflux or on the repair process itself, we favor the latter for two reasons. First, even at 0° the cells immediately lost the yellow color of ellipticine, indicating that most drug was, in fact, removed. Second, Zwelling *et al.* [11] have found previously that, while efflux of *m*-AMSA was not affected significantly by low temperature, the repair of *m*-AMSA-induced DNA strand breaks did not occur at 4°.

The similarity in rates of repair of intercalator-induced DNA strand breaks and DNA–protein crosslinks is worthy of special note. We have shown previously that these two lesions are formed in association and that they are spatially related [3, 4]. Further, by quantitating the frequency of both lesions over broad concentration ranges of either adriamycin or ellipticine, we found that the single strand breaks and crosslinks occur with equal frequency. This has been confirmed by others using the intercalating agents *m*-AMSA [11] and anthracenedione [20]. Based on these data, we have hypothesized that the protein may represent an enzyme which nicks the DNA in response to drug binding and remains bound at the nick site. This hypothesis leads to the prediction that removal of the DNA–protein crosslinks and strand breaks would occur simultaneously. Such was our observation. This is in contrast to effects of ionizing radiation. In this case, the DNA strand breaks are repaired much more rapidly than the DNA–protein crosslinks [21].

In summary, the effects of ellipticine on the DNA of L1210 cells were repaired much more rapidly than those of adriamycin. This is consistent with the suggestion of others that cytotoxicity relates, in part, to intracellular drug retention. The precise role intercalator-induced DNA effects play in determining cytotoxicity remains an enigma.

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