Differences between Melphalan and Nitrogen Mustard in the Formation and Removal of DNA Cross-Links

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ABSTRACT

The formation of DNA cross-links is thought to represent the lethal lesion following exposure of cells to bifunctional alkylating agents. Since differences in rates of formation and repair of cross-links may explain differences in activity of these agents, we have studied these events following exposure of L1210 cells to nitrogen mustard (HN2) and melphalan. With the technique of alkaline elution, it was possible to measure cross-linking at doses that result in relatively little cell kill. Following a 30-min exposure to HN2, DNA cross-links increased for 1 to 2 hr and were then removed by a process that was virtually complete in 24 hr. In contrast, following a 30-min exposure to melphalan, cross-link formation increased for 12 hr and removal was much slower than it was for HN2. Comparison of cell survival with cross-linking kinetics suggests that persistence of the cross-links with time is an important factor in determining lethality.

INTRODUCTION

Bifunctional nitrogen mustards remain an important group of agents in cancer chemotherapy. Despite the structural similarity in the alkylating moieties of the various members of this class of drugs, different substitutions on the amine can result in marked differences in chemical reactivity and clinical utility (2), as well as differences in rates and mechanisms of nucleophilic attack (17). However, the relationship of these differences to intracellular events is unclear. The lethal event following exposure of cells to these agents is thought to involve some form of DNA damage (in particular, interstrand cross-linking (12, 13, 15)). Repair of these cross-links has been described and may be important in determining resistance to the drugs (14, 15, 18). Pharmacologically meaningful comparisons between the DNA damage produced by different nitrogen mustards require quantitation of the lesions in cells treated with pharmacologically reasonable drug doses. For this purpose the new technique of DNA alkaline elution has proved to be advantageous (6-8, 11). In this study we apply this technique to a comparison between HN2 (methylmethamine, bis-2-chloroethylnitrosurea) and melphalan (L-phenylalanine mustard). These 2 agents are of interest because they provide a comparison between an aliphatic and an aromatic nitrogen mustard and because both agents are clinically useful.

A further requirement for a satisfactory molecular interpretation of pharmacological effects is characterization of the molecular lesions that are measured. This requirement, however, can only be met by degrees, since all known DNA-damaging agents produce multiple molecular lesions, and there is no general methodology to distinguish precisely the various classes of lesions in cells. The alkaline elution methodology used in this study is thought to measure a net effect of both interstrand cross-links and DNA-protein cross-links (5, 6). The question of the relative contribution of the 2 classes of DNA lesions on the effects described here will be pursued in subsequent work.

MATERIALS AND METHODS

Cell Growth and Labeling. Stock cultures of mouse leukemia L1210 cells were propagated in Roswell Park Memorial Institute Tissue Culture Medium 1630, containing 20% fetal bovine serum without antibiotics. Cultures to be used for experiments were initiated weekly from stock cultures, penicillin and streptomycin were added, and the cells grew exponentially with a doubling time of 12 hr. DNA was labeled by a 20-hr incubation with [2-14C]thymidine (43 mCi/mmol, 0.01 μCi/ml; Schwarz/Mann, Orangeburg, N. Y.) or with [methyli-3H]thymidine (20 Ci/mmol, 0.1 μCi/ml; New England Nuclear, Boston, Mass.), diluted with unlabeled thymidine to give a concentration of 10-9 M in the culture medium. By this procedure all labeled thymidine is fully incorporated into mature DNA prior to experimental manipulation.

Drug Treatments. Drugs were obtained from the Developmental Therapeutics Program of the Division of Cancer Treatment, National Cancer Institute.

One hr prior to drug treatment, log-phase cells were centrifuged at 900 rpm for 3 min at 37°, resuspended at 9 × 106 cells/ml in fresh medium, and incubated. Nitrogen mustard stock solution, 0.01 M in 0.10 N HCl, was stored frozen, and diluted 1:100 with distilled water prior to administration. Melphalan, 0.01 M in 0.10 M HCl, was similarly stored frozen, but no dilution was made at the time of administration. In neither case did the addition of the drug affect the pH of the medium. Cells were exposed to drug for 30 min and then centrifuged, washed, and resuspended in fresh medium. For experiments in which drug effect was followed with time, the cells were kept in a dark incubator at 37°, and aliquots were removed at the indicated times. Inspection of the cells by phase microscopy and by a search for free 14C counts in the medium showed that at no time during the 24 hr following treatment was there significant cell lysis.

Survival Studies. L1210 cells were exposed to drugs for the indicated times and then centrifuged, washed, and resuspended in fresh medium. Colony-forming ability was...
determined by the soft-agar technique described by Chu and Fischer (3). Colonies were counted 12 to 15 days after seeding.

**Alkaline Elution Assays.** The factors governing DNA alkaline elution kinetics and the procedures used in the assays have been previously described (11).

Approximately $5 \times 10^6$ 14C-labeled experimental cells plus a similar number of 3H-labeled reference cells (see below) were diluted in 20 ml of ice-cold phosphate-buffered saline (0.15 M NaCl-0.014 M KH2PO4-0.086 M K2HPO4) and collected on a polyvinyl chloride filter (pore size, 2 $\mu$m; diameter, 25 mm) (Millipore Corp., Bedford, Mass.). The cells were washed with cold phosphate-buffered saline and lysed on the filter with 5 ml of 0.2% Sarkosyl [Ciba-Geigy (U. K.) Ltd., Duxford, Cambridge, England]-2 M NaCl-0.04 M EDTA, pH 10, at room temperature (22-24$^\circ$). The lysis solution was allowed to flow through by gravity, and the filter was washed with 3 ml of 0.02 M EDTA, pH 10. No suction was applied during or after elution. Lysis was carried out in the dark at a flow rate of 0.035 to 0.045 ml/min. The eluting solution consisted of 0.02 M EDTA (acid form) plus tetrapropylammonium hydroxide (Eastman Kodak Co., Rochester, N. Y.; 10% solution in water) added in the amount required to give a pH of 12.1 to 12.2. Eluted fractions were collected at 90-min intervals and mixed with 3.3 volumes of Aquasol (New England Nuclear) containing 0.75% acetic acid for scintillation counting. Radioactivity remaining on filters was determined by treating filters with 0.4 ml of 1 N HCl at 65$^\circ$ for 1 hr followed by 2.5 ml of 0.4 N NaOH at room temperature for 1 hr and then adding 10 ml of Aquasol. Radioactivity remaining in the funnel was recovered by 5 washes with 2.5 ml of 0.4 N NaOH, and the counts were added to those remaining on the filter.

Just prior to elution the cells were placed on ice and exposed to 600 rads of X-irradiation as a means of introducing a controlled number of single-strand breaks. As a result of the cross-linking by the drugs, there is an apparent reduction in the effect of X-ray-induced breaks, thus increasing retention of labeled DNA on the filter. Since all of the 14C label has been incorporated into mature DNA prior to drug exposure, subsequent DNA synthesis does not quantitatively affect the results. The 3H-labeled reference cells (henceforth referred to as internal standard DNA) receive 150 rads prior to elution, thereby providing DNA within each experiment with consistent elution kinetics that is not influenced by the elution rate of the experimental 14C-labeled DNA. Normalization of 14C-labeled DNA to this internal standard reduces quantitative variability. Qualitatively, the shapes of the 14C-labeled DNA elution curves are the same whether plotted against time or against elution of the internal standard, because the elution of the 3H-labeled DNA is approximately first order with respect to time (7).

Since the elution curves are nonlinear, it was necessary to choose an arbitrary point at which to assess the extent of cross-linking. The point chosen for this was the fraction of 14C-labeled DNA remaining on the filter when 60% of the internal standard remained on the filter; this value is called "relative retention." The conclusions drawn do not critically depend on the choice of the point of reference used to define relative retention.

**RESULTS**

The survival of L1210 cells following treatment with various doses of HN2 or melphalan as determined by cloning in soft agar is presented in Chart 1.

DNA cross-linking was assessed in terms of the reduction in X-ray sensitivity of intracellular DNA as measured by alkaline elution kinetics (5, 8, 11) (see "Discussion"). The DNA single-strand breaks generated by irradiation of cells would normally produce a large increase in elution rate. In cells treated with a cross-linking agent, however, the effect of this dose of X-ray is diminished. This is illustrated in Chart 2 for cells treated with various concentrations of HN2 for 30 min. The elution of DNA is seen to be reduced as a function of HN2 concentration. Elution curves obtained with melphalan were similar in shape to those obtained with HN2. The extent of DNA elution was evaluated at a selected end point, as described in "Materials and Methods" and in the chart legends.

For the measurement of DNA cross-linking with this method, it is desirable to have an experimental measure that is proportional to the frequency of drug-induced cross-links. The true quantitative relationship between DNA elution and DNA cross-link frequencies is still unknown. A reasonable conjecture is possible, however, based on the observed linearity of log r (where r is relative retention; see "Materials and Methods") as a function of single-strand break frequency (11).

Thus

$$-\log r = c_1 + c_2 P_s$$

where $r_s$ is relative retention in cells exposed to X-ray but no drug; $P_s$ is single-strand break frequency, produced by the irradiation; and $c_1$ and $c_2$ are constants. The conjecture is that relative retention depends, to a first approximation, on the ratio of break frequency to cross-link frequency.

Thus

$$-\log r = \frac{c_1 + c_2 P_s}{c_1 / P_s}$$

![Chart 1. Colony-forming ability following exposure of L1210 to HN2 and melphalan at various doses for 0.5 hr. Each point represents the mean of duplicate tubes set up from a single drug exposure. Cloning efficiency of untreated cells was 85 to 95%.](chart1.png)
where \( r \) is relative retention in cells exposed both to drug and to X-ray, \( p_x \) is cross-link frequency, and \( c_x \) is a constant. (The \( c_x \) term is included in the numerator because of the presumption that it reflects breaks arising from sources other than the X-ray exposure and that all breaks, however derived, will be affected by cross-linking in the same way.) Combining the 2 equations, we obtain

\[
\frac{\log r_a}{\log r} = \frac{p_x}{c_x}
\]

However, this reasoning assumes that drug treatment does not itself add DNA breaks. Drug-induced strand breaks would be detected in our experiments by an increase in elution rate in assays without X-ray. No such increase in elution rate was produced either by HN2 or by melphalan under the dosage conditions considered here. Drug-induced breaks, if any, are therefore negligible compared with the magnitude of cross-linking in our experiments.

For testing of Relation C, it is necessary to have a means to produce a graded frequency of cross-links in cells. It is reasonable to expect that cross-linking frequencies would be proportional to drug concentration under certain conditions. These are that drug concentration must be low enough so that membrane transport is not limiting, that the pool of chemical inactivating species is not depleted, and that no DNA damage arises from secondary effects. Furthermore, treatment time must be short enough to avoid secondary effects such as repair, yet long enough to allow cross-links to be completed (reaction at both arms of the bifunctional agent). A good possibility for meeting these requirements was judged to be treatment of L1210 cells with 0.1 to 0.4 \( \mu M \) HN2 for 0.5 hr, under which conditions survival exceeded 30% (Chart 1) and elution effects (Chart 2) were clearly measurable. This range of conditions was tested and yielded a satisfying linearity of \( \log r_a/\log r \) as a function of HN2 concentration (Chart 3). Essentially similar results were obtained with 300 R instead of 600 R as the X-ray dose and with \( ^3H \) retention intercepts of 0.75 or 0.5 instead of 0.6. Because of this linearity an experimentally derived value of \( \log r_a/\log r \) can be related to the dose of HN2 that would produce the number of cross-links necessary for such an effect. This value is referred to as “HN2-cross-linking equivalents.”

The formation and removal of DNA cross-links was then studied as a function of time following drug removal (Charts 4 and 5). Cells were treated with either HN2 (0.2 \( \mu M \)) or melphalan (15 \( \mu M \)) for 30 min and washed, and aliquots were taken at varying times of posttreatment incubation ranging from 0 to 24 hr. These doses of drug resulted in 30 and 65% cell kill, respectively. For approximately 1.0 to 1.5 hr after removal of HN2, there was a slight increase in the number of cross-links, although most had been formed by the time of drug removal. Following this period cross-links decreased exponentially with a half-time of about 7 hr. By 24 hr virtually no cross-links remained.

In contrast, immediately following removal of melphalan very few cross-links were seen. Over the next 90 min cross-linking increased dramatically and was then followed by a more gradual rise, which appeared to reach a plateau approximately 12 hr after drug removal. Thereafter, there was a gradual decline in cross-links, but at 24 hr a significant amount of cross-linked DNA remained.

Log \( r_a/\log r \) values from the above experiments were then transformed into HN2 cross-link equivalents by the use of the linear regression line shown in Chart 3, and the cross-link equivalents were plotted against the duration of post-
Formation and Removal of Cross-links

Chart 4. Changes in DNA alkaline elution patterns with time following treatment of L1210 cells with HN2 or melphalan. Cells were treated with HN2 (0.2 µM) or melphalan (15 µM) for 30 min, and aliquots then were assayed for cross-linking at varying times following drug removal. Cells received 600 R prior to elution. The elution of experimental [14C]DNA is plotted against the simultaneous elution of [3H]DNA internal standard.

Chart 5. Changes in cross-linking following cell exposure to either HN2 (0.20 µM) or melphalan (15 µM) for 0.5 hr. Cross-linking equivalents are derived from the calibration line in Chart 3. O, HN2-treated cells; Δ, cells treated with melphalan. Cells on ice received 600 R just prior to elution.

treatment incubation (Chart 5). The differences in DNA cross-linking patterns following removal of HN2 or melphalan are again apparent.

By elution of drug-treated cellular DNA without prior irradiation, cells were examined for the appearance of single-strand breaks. At no time (0 to 24 hr) after removal of either drug were breaks seen (data not shown).

DISCUSSION

The alkaline elution procedure used in this work assesses DNA cross-linking on the basis of a reduction in the effect of X-ray on a size-dependent property of DNA single strands. The procedure is in this sense analogous to the assay for interstrand cross-links described by Jolley and Ormerod (9) in which sedimentation in alkali is the strand-size-dependent property measured. The strand-size dependence of alkaline elution has been previously characterized (11). It is reasonable to assume that interstrand cross-links would reduce the alkaline elution rate of DNA bearing a given frequency of X-ray-induced single-strand breaks (6-8, 11). However, the converse assumption, that reduced elution results solely from interstrand cross-links, is not valid. In particular, there is evidence that DNA-protein cross-links also retard DNA elution (5, 6). The assumption that reduced alkaline elution signifies cross-links of some type is supported by the observation that monofunctional alkylating agents fail to produce this effect (5, 8). The data presented by the current work are most reasonably interpreted in terms of the combined effect of these 2 classes of cross-links. Subsequent work will investigate the behavior of each of these cross-link classes individually. The focus of the current work is the demonstration of differences between different nitrogen mustards in the kinetics of formation and removal of DNA lesions in cells treated at pharmacologically relevant dosage.

As illustrated in Chart 4, HN2 and melphalan define very different time courses with respect to both formation and removal of DNA cross-links. With regard to the former, very little additional cross-linking is seen following removal of HN2. It is probable that the peak between 0 and 1.5 hr represents both continued formation and simultaneous removal of the cross-links. Melphalan, on the other hand, exhibits initial rapid cross-link formation for 0 to 1.5 hr with gradual tapering over the next 10 hr. This long period of slow cross-link formation is not easily explained for a drug with a half-life of hydrolysis of about 70 min when examined in a purely aqueous milieu (17). Recently, Alberts et al. (1) have shown that the chemical reactivity of melphalan is markedly reduced in the presence of bovine serum albumin. A similar phenomenon may be occurring in the complex intracellular milieu. Additionally, some form of intracellular metabolism of the drug could alter its reactivity. Alternatively, the delayed cross-linking by melphalan may stem from a slow second step in a 2-step reaction sequence leading to a cross-link. Delayed DNA cross-linking due to a slow second step has recently been reported for chloroethylnitrosoureas (6, 10).

In addition to the differences in the formation of cross-links, it is apparent that major differences in rate of repair exist. HN2-induced cross-links appear to be repaired with approximately first-order kinetics with a half-life of about 7 hr. This is in good agreement with the data of Yin et al. (18), who studied cross-link repair in Ehrlich-Letré cells by alkaline denaturation and renaturation following treatment with HN2.

That our data represent actual removal of the cross-links and not simple fragmentation of the drug-treated DNA is supported by the failure to see strand breaks in the DNA at any time after drug removal. While a small number of breaks may have been concealed by cross-links, these would not be sufficient to account for the reduction in cross-link equivalents seen following drug removal. In contrast, Yin et al. (18) reported DNA strand scission following
treatment of sensitive Ehrlich-Lettre cells with HN2 and attributed it to repair endonuclease activity. Other mechanisms that might account for DNA strand breaks following alkylation include depurination at sites of monofunctional alkylating, thereby creating alkali-labile sites that would appear as strand breaks at the proper pH (19) and non-specific degradation of DNA during the lysis of acutely dying cells. In comparing our data with those of Yin et al. (18), several important differences are apparent. First, Yin et al. measured strand breaks by sedimentation in alkaline sucrose gradients. Recent data suggest that this technique reveals more breaks following treatment of cells with 1,3-bis(2-chloroethyl)-1-nitrosourea than are seen by alkaline elution (4). This was thought to be due to the greater efficiency of strand scission at alkali-labile sites in the higher pH used in sedimentation analysis. Second, while DNA strand breaks are obscured equally by the presence of interstrand cross-links when examined by either alkaline sucrose gradients or alkaline elution, in the latter technique breaks are also concealed by DNA-protein cross-links (5, 6). This is not true for the sedimentation technique. Interestingly, in preliminary experiments in which DNA-protein cross-links were digested with proteinase K prior to elution, strand breaks were still not seen following treatment of cells with HN2 or melphalan. Third, we used doses of alkylating agents that resulted in less than 1 log cell kill, while in the study of Yin et al. (18) there was greater than 99% cell kill. The difficulties of interpreting data obtained 6 and 24 hr following an injury lethal to such a large fraction of the cell population are obvious. Finally, it is conceivable that the differences in cell lines (L1210 versus Ehrlich-Lettre tumor cells) could account for the discrepancies in break appearance. In this regard it is worth noting that strand breaks were not seen in a resistant subline of Ehrlich-Lettre cells following HN2 treatment (18).

In contrast to HN2, melphalan-induced cross-links are repaired very slowly. Whether this is related to differences in the chemical structures of the drugs or the type of cross-link formed is unclear. Recent work in this laboratory (5, 6) has suggested that bifunctional alkylating agents produce 2 kinds of cross-links; proteinase sensitive, interpreted as DNA-protein links, and proteinase resistant, presumably DNA interstrand links. These may not be removed at the same rate. We are currently investigating the relative role that each of these 2 types of cross-links plays in the results described in this paper.

Roberts et al. (15) have suggested that the lethal event following treatment of cells with bifunctional alkylating agents involves inactivation of the DNA template as a result of cross-linking. This results in inhibition of DNA synthesis. Further, they propose that repair of the cross-links and attempted DNA synthesis are in effect 2 competing processes, such that, if the former occurs before the latter, the cells may replicate normally. If this is correct, then the persistence of the cross-links following treatment with melphalan should result in greater toxicity than the HN2 produces despite the fact that no difference in the maximal cross-linking is seen. While the data suggest that the melphalan dose used in the experiments represented in Charts 4 and 5 produced more cell kill than did the HN2 dose (Chart 1), more data will be required to confirm this point. In addition, it will be necessary to distinguish quantitatively between DNA-protein and interstrand cross-links when correlating drug-induced DNA damage and repair with cytotoxicity.

In summary, we have described differences between nitrogen mustard and melphalan with respect to both formation and removal of DNA cross-links. Further investigations into the chemical nature of these cross-links (e.g., DNA-protein versus DNA-DNA) and their relationship to attempted DNA synthesis may clarify differences in their cytotoxic potential.

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