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DNA DAMAGE INDUCED IN HT-29 COLON CANCER CELLS BY EXPOSURE TO 1-METHYL-2-NITROSOIMIDAZOLE, A REDUCTIVE METABOLITE OF 1-METHYL-2-NITROIMIDAZOLE

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Abstract—Exposure of HT-29 colon carcinoma cells to 1-methyl-2-nitrosoimidazole (INO), a reductive metabolite of a model 2-nitroimidazole, induced concentration-dependent DNA damage detectable by conventional alkaline (single-strand breaks) and neutral (double-strand breaks) filter elution techiques. Elution of DNA from the filters under alkaline conditions was distinctly biphasic. No evidence of DNA damage was detected when cellular DNA was incubated directly with INO prior to filter elution. DNA damage was enhanced markedly in HT-29 cells incubated with buthionine sulfoximine to deplete intracellular glutathione levels prior to INO treatment. The biphasic shape of the elution profiles was not attributable to loss of labeled thymidine mononucleotides or to the formation of DNA–protein crosslinks. Rather, the data suggest the existence of two subpopulations of cells differing in sensitivity to the DNA-damaging effects of INO exposure. Based upon differential adherence, two populations of cells, differing with respect to the rate and extent of elution from the filters during alkaline elution assays, were detected, although they could not be purified sufficiently by this technique to permit biochemical characterization. The results suggest that the nitroso intermediate is either an active metabolite, or a proximate form of the ultimate DNA-reactive species, responsible for DNA damage in cells exposed to 2-nitroimidazoles under reducing conditions.

Exposure of prokaryotic and eukaryotic cells to nitroimidazoles under anaerobic conditions is associated with a diverse range of biological effects including thiol depletion, DNA-binding, DNA damage and cytotoxicity. Many of these biological effects are thought to be mediated by one or more intermediates generated by nitroreduction of the parent nitroimidazole [1, 2] according to Scheme 1.

In the presence of oxygen the nitro-radical anion (the one-electron reduction product) is back-oxidized, creating a futile cycle in which reactive reduction products are not generated. Presumably this back-oxidation accounts for the relative inactivity of these compounds under aerobic conditions. Although the data currently available do not permit unambiguous assignment of biological activity to a specific reduction product, it has been suggested that the hydroxylamino [3] or nitroso [4–9] intermediates may mediate the biological effects of reduced nitroimidazoles.

Varghese and Whitmore have provided chemical and biological evidence to suggest that the hydroxylamine derivative of misonidazole (MISO§) was the intermediate responsible for toxicity, macromolecular binding and thiol depletion in cultured cells incubated with MISO under hypoxic conditions [3, 10]. They further demonstrated that this compound interacted with glutathione (GSH) to form a stable product and formed specific covalent adducts in cellular DNA [3, 11]. Ehlhardt and co-workers have shown the nitroso derivatives of 4- and 5-nitroimidazoles to be more toxic than the parent compounds in bacterial systems and demonstrated the ability of the 5-nitroso product to bind DNA [4, 8]. Additional data supporting the role of nitroso intermediates in the action of nitroimidazoles has been provided by Noss *et al.* [5, 7] and our laboratory [6, 9]. Collectively these studies indicate that 1-methyl-2-nitrosoimidazole (INO) is extremely toxic to mammalian cells under aerobic and hypoxic conditions, is mutagenic, and produces a concentration-dependent depletion of intracellular glutathione.

In this report, we describe the results of experiments designed to quantitate DNA damage induced in HT-29 colon cancer cells following exposure to INO. The extensive DNA damage detected in these studies further supports the hypothesis that the nitroso intermediate may mediate at least some of the biological effects of nitroimidazole exposure under hypoxic conditions.

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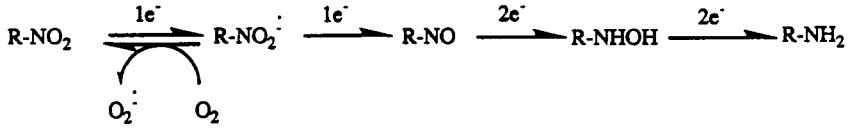
§ Abbreviations: MISO, misonidazole; INO, 1-methyl-2-nitrosoimidazole; BSO, L-buthionine sulfoximine; αMEM, α-Minimum Essential Medium; DMEM, Dulbecco's Modified Eagle's Medium; GSH, glutathione; and PBS, phosphate-buffered saline.

MATERIALS AND METHODS

Materials. INO was synthesized according to the



Scheme 1.

technique published previously [5] employing 2-hydroxylamino-1-methylimidazole hydrochloride generated by electrolytic reduction of 1-methyl-2-nitroimidazole. For all experiments concentrated stock solutions of INO were prepared in ice-cold distilled water.

BSO was purchased from the Sigma Chemical Co. (St. Louis, MO).

Cell line and drug treatments. The human colon cancer cell line, HT-29, was used for these studies. Cells were maintained in exponential growth in αMEM medium supplemented with 10% fetal bovine serum and gentamycin (50 μg/mL). Cultures were incubated at 37° in an atmosphere of 97% air/3% CO_2 and transferred at weekly intervals. All cultures were determined to be *Mycoplasma* free.

The technique used to treat cells with INO has been described in detail previously [6]. Briefly, exponentially growing HT-29 cells were suspended in 10 mL of Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 1–2 × 10^5 cells/mL and transferred to glass treatment vials. To initiate INO exposure, various concentrations of the compound in distilled water were diluted 100-fold by injection into the appropriate treatment vial. Cells were incubated in the presence of INO for 1 hr at 37°. At the conclusion of the exposure interval the cell suspensions were centrifuged, washed in drug-free medium, and prepared for survival assay.

To deplete cellular levels of GSH, HT-29 cells were incubated in the presence of 1.0 mM L-buthionine sulfoximine (BSO) for 24 hr prior to INO treatment. This pretreatment reduced GSH levels to 11 ± 4% (mean ± SD of six determinations) of control HT-29 cells but did not reduce plating efficiency relative to untreated controls.

Cell survival assay. Survival was determined using a standard plating efficiency assay. Colony formation in treated and control groups was enumerated 12–14 days after plating and survival calculated as the ratio of plating efficiency of treated groups to that for controls.

Alkaline and neutral filter elution assays. Determination of DNA single-strand breakage was done by alkaline elution essentially as described by Kohn *et al.* [12]. HT-29 cells were labeled with [^{14}C]-thymidine at 0.02 μCi/mL for 48 hr and for an additional 24 hr in medium free of [^{14}C]thymidine to establish the label in high molecular weight DNA. Reference cells were similarly labeled with [^{3}H]-thymidine. Immediately after exposure 5 × 10^5 drug-treated cells were combined with an equal number of irradiated (4.0 Gy X-rays) reference cells which served as internal standards. The cells were then lysed on 0.8 polycarbonate filters with 1% sodium dodecyl sulfate, 0.020 mM EDTA (pH 10), treated for 1 hr with proteinase K (0.5 mg/mL) and eluted from the filter with 2% tetrapropylammonium hydroxide (pH 12.2) containing 0.02 M EDTA and 0.1% sodium dodecyl sulfate. DNA double-strand breaks were assayed in a similar manner using an elution buffer consisting of 2% tetrapropyl-ammonium hydroxide (pH 7.0) containing 0.02 M EDTA and 0.1% sodium dodecyl sulfate. In both cases, fractions (6 mL) were collected at hourly intervals for 6 hr. At the conclusion of the elution period, the filters were removed and heated to 60° for 1 hr in 1 N NaOH. Results were expressed as the fraction of labeled DNA retained on the filter at the conclusion of the elution period.

Alkaline sucrose gradients. DNA damage induced in INO-treated cells was also assessed by alkaline sucrose gradients essentially as described by Warters *et al.* [13]. HT-29 cells were labeled with [^{14}C]-thymidine (0.04 μCi/mL) for 48 hr and then incubated in label-free medium for an additional 16 hr. Following exposure to INO, 10^6 drug-treated cells were centrifuged and resuspended in 1.0 mL of ice-cold phosphate-buffered saline (PBS) and mixed with 0.1 mL of proteinase K (2.5 mg). Cells (4 × 10^5) were then gently layered onto 0.5 mL of lysis solution on the top of a linear 5–20% alkaline (pH = 12.2) sucrose gradient (36 mL). Cells were lysed for 3 hr in the dark at room temperature and then centrifuged at 16,000 rpm for 4 hr 45 min ($\omega^2 t = 4.9 \times 10^{10}$ rad^2/sec), 10°, using a Beckman SW28 rotor and L8-80 ultracentfifuge, slow acceleration, and no braking. Gradients were fractionated from the bottom by puncturing the tube with an 18 gauge needle. Using a peristaltic pump, ~1.4-mL fractions were collected directly into scintillation vials and radioactivity was determined by liquid scintillation counting.

RESULTS

DNA damage induced by exposure of HT-29 cells to INO for 1 hr at 37° was assessed by alkaline and neutral filter elution. As shown in Fig. 1, INO exposure resulted in a concentration-dependent decrease in the amount of DNA retained on the filter following elution at pH 12.2. The elution profile was consistently characterized by a distinct biphasic shape, with a slower eluting component evident following a relatively rapid initial loss of labeled DNA from the filter. The slopes of both the initial and terminal portions of the elution curve were concentration dependent, progressively increasing as INO concentrations increased. The elution profiles were not altered when the pH of the elution solution was increased to >12.4, indicating that strand

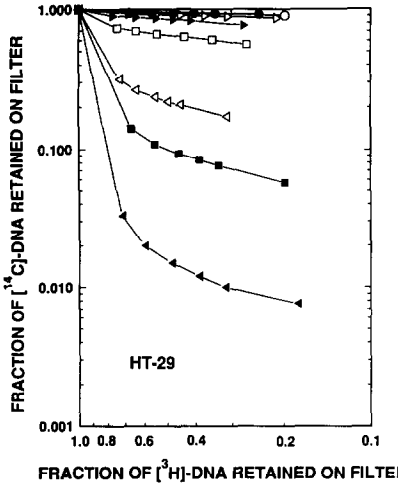


Fig. 1. Elution of DNA from filters under alkaline (pH 12.2) conditions. HT-29 cells were exposed to 0 μM (○, ●), 5 μM (▷, ▶), 10 μM (□, ■) or 15 μM (◁, ◀) INO for 1 hr at 37° at a concentration of 2×10^5 cells/mL immediately prior to alkaline elution. The elution profiles for HT-29 cells incubated with BSO prior to INO treatment (solid symbols) are also shown. BSO pretreatment reduced GSH levels to ~10% of control levels (12 fmol/cell).

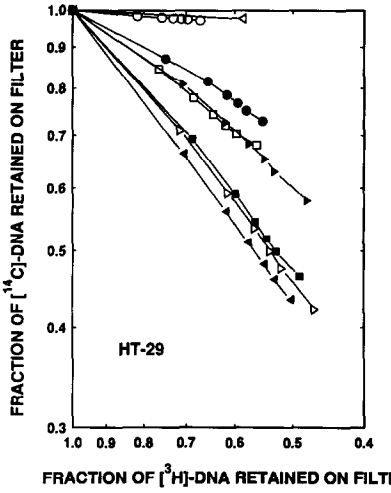


Fig. 2. Elution of DNA from filters under neutral (pH 7.0) conditions. Cells were exposed to 0 μM (◁), 10 μM (○), 20 μM (●), 50 μM (▷) and 100 μM (◀) INO as for Fig. 1. Also shown are the elution profiles for HT-29 cells exposed to 50 (□) or 100 Gy (■) of γ-irradiation. In one case, cells were exposed to 50 μM INO at a concentration of 5×10^5 cells/mL (▶).

scission was not the result of introduction of alkali-labile lesions in the DNA or INO-treated cells (data not shown).

The proportion of labeled DNA retained on the filter was greatly reduced by pretreating the HT-29 cells with BSO (Fig. 1, solid symbols), a specific inhibitor of GSH synthesis, prior to INO treatment. We have shown previously [6] that this pretreatment reduces intracellular GSH concentrations of HT-29 cells to approximately 10% of control levels (12 fmol/cell). This observation is also consistent with previous studies showing that BSO pretreatment markedly enhances the cytotoxicity of INO [6]. These earlier experiments also revealed that the cytotoxicity of INO was inversely proportional to cell density, being greatly diminished at higher cell concentrations [6]. We therefore examined the effect of cell density on INO-induced single-strand scission. As was the case for cell killing, DNA damage, as revealed by decreased rates of elution under alkaline conditions (data not shown), was likewise decreased by increasing the cell treatment density.

Extensive DNA damage was also evident by neutral filter elution (Fig. 2), which measures the extent of DNA double-strand breakage induced by treatment. For comparison, the elution of DNA from HT-29 cells exposed to 50 and 100 Gy (open and closed squares, respectively) of ionizing radiation is also shown. Once again, the level of DNA damage associated with INO exposure was increased by BSO (data not shown) and reduced by increased cell density (compare open and closed triangles).

In a related series of experiments, cellular DNA was directly incubated with INO in PBS on elution filters for periods of up to 2 hr immediately before alkaline elution. Under these buffer conditions INO has a half-life of ~60 min [5], yet INO concentrations of up to 240 μM for 2 hr failed to induce strand breakage in excess of that detected in control DNA incubated in the absence of INO (Fig. 3).

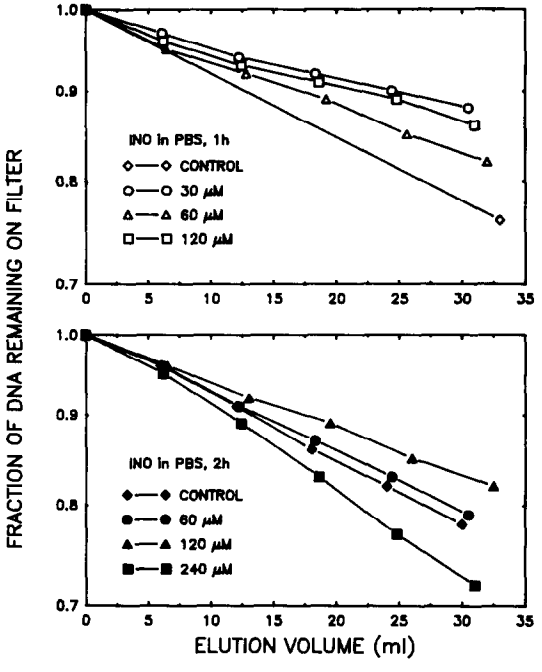


Fig. 3. Elution of DNA exposed to INO on the filter. HT-29 cells were lysed on elution filters and the cellular DNA was incubated for 1 (upper panel) or 2 (lower panel) hr with various concentrations of INO in PBS. At the conclusion of the incubation, the DNA was eluted from the filters in elution buffer at pH 12.2.

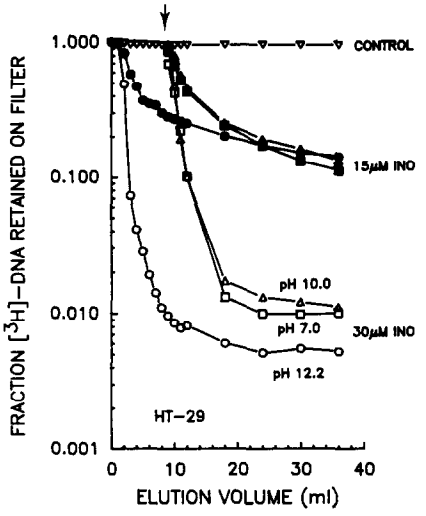


Fig. 4. Effect of pH on the elution of DNA from INO-treated cells. HT-29 cells were exposed to 15 (solid) or 30 (open) μM INO for 1 hr at 37°. After cell lysis DNA was eluted from the filters in elution solutions at pH 7.0 (□, ■), 10.0 (△, ▲) or 12.2 (○, ●). After 1 hr, elution solution at pH 12.2 was substituted for the pH 7.0 and 10.0 solutions (arrow) and elution continued. For clarity, the elution profiles for the pH 7.0 and pH 10.0 groups were not included for the first hour. The profiles were indistinguishable from that for the control group (▽).

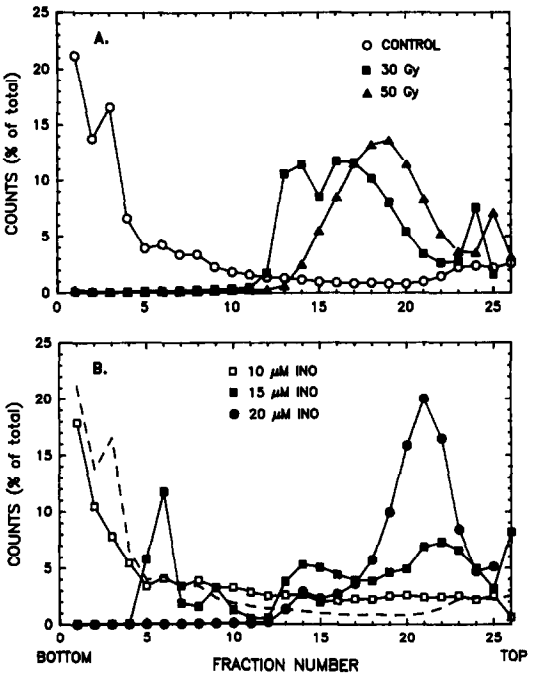


Fig. 5. Alkaline sucrose gradients for HT-29 exposed to ionizing radiation (A) or various concentrations of INO for 1 hr at 37° (B). The dotted line in panel B is the control profile redrawn from panel A. Cells were exposed to INO at a density of 2×10^5 cells/mL.

Knox *et al.* [14] demonstrated that electrolytic reduction of the 2-nitroimidazole, misonidazole, results in cleavage of phosphodiester bonds around thymine residues in DNA resulting in release of thymidine mononucleotides. If labeled thymidine mononucleotides are lost from DNA following incubation with INO in an analogous way, it could account for the rapid initial loss of label from the filter detected in our experiments. To address this possibility, the elution experiments were repeated as before with the exception that elution solutions buffered to pH 7.0 or 10.0 were used in addition to the standard pH 12.2 elution solution. As shown in Fig. 4, after cells exposed to either 15 or 30 μM INO were lysed on the filter, the DNA was eluted with one of the three elution solutions until ten 1-mL fractions were collected. At that time the elution solution was changed to pH 12.2 for the remainder of the elution period. No radiolabel was detected in the eluates of the groups eluted at pH 7.0 or 10.0 until the pH of the eluting solution was changed to pH 12.2, at which point typical elution profiles were detected. These results suggest that preferential loss of labeled-thymidine was not accountable for the initial rapid elution. This conclusion was also supported by subsequent alkaline elution experiments employing [^{3}H]cytidine (data not shown).

DNA–protein crosslinks were also ruled out as possible contributors to the reduced elution rate of the terminal portion of the elution profiles. Treatment with proteinase K failed to change the shape of the elution curves or alter the individual elution rates of the initial or final portion of the profile (data not shown).

The magnitude of DNA damage was also measured by alkaline sucrose gradients. As shown in Fig. 5B, the sucrose gradient profile for cells treated with 10 μM INO was similar to that for untreated controls, while at 20 μM the gradient profile revealed extensive DNA fragmentation. The median DNA fragment size in this latter case was smaller than that produced by exposing the cells to 50 Gy X-rays (compare with panel A). At this dose the DNA size distribution was unimodal and, consequently, no evidence for two levels of DNA damage was detected. However, in contrast to the unimodal distribution expected for DNA-damaging agents, such as ionizing radiation, which induce random strand breaks, the gradient for DNA from cells exposed to 15 μM INO was characteristically bimodal.

Both the alkaline elution and alkaline sucrose gradient data were consistent with the co-existence of two populations of cells expressing differing amounts of DNA damage following INO treatment. This possibility was further supported by the observation that only a proportion of the cells was capable of adhering to plastic culture dishes when replated following INO exposure. The percent of adherent cells was inversely proportional to INO concentration, and, typically, could be approximated by extrapolating the terminal linear portion of the elution profile back to the Y-axis. From these observations, we hypothesized that the terminal slope corresponded primarily to DNA damage in cells capable of adhering to the plastic substratum and that the initial slope corresponded to more

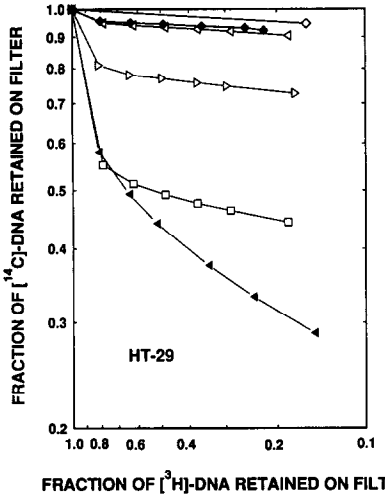


Fig. 6. Alkaline elution profiles for HT-29 cells exposed to 15 μM INO for 1 hr at 37° and separated on the basis of differential adherence to the culture dish 6 hr after drug treatment. Refer to text for details. Elution profiles for untreated cells (◇) and cells collected for elution immediately following exposure to INO (▲) are shown, as are the profiles for control cells (◆) and cells attached (◁) or unattached (□) to the dishes 6 hr after drug treatment. The elution profile for the entire population 6 hr after treatment (▷) is also included.

extensively damaged DNA, arising predominately from the non-adherent population.

Using a crude separation procedure based upon differential adherence of INO-treated (15 μM; 1 hr) cells 6 hr after treatment, the type and magnitude of DNA damage in these two populations of cells were assessed by alkaline elution. After the 6-hr attachment interval, approximately 50% of the cells were isolated by attachment to the plates. The elution rate for DNA from these cells was indistinguishable from controls, indicative of no DNA strand scission damage (Fig. 6). No evidence of rapidly eluting DNA was detected. In contrast, the elution profile for DNA from the floating population was still biphasic, indicating that the floating population did not consist exclusively of cells with severely damaged DNA. The slope of the terminal portion of the elution profile for the floating cells was not as steep as that for the entire population, however, suggesting that some repair of this damage had taken place during the 6 hr allowed for cell attachment.

The relative plating efficiencies for these populations were also determined in parallel with the alkaline elution experiments described above. The plating efficiency of control HT-29 cells was approximately 0.54 (±0.06). The mean plating efficiencies for the attached and floating populations were 0.3 (±0.03) and 0.1 (±0.04), respectively.

DISCUSSION

Exposure of cells to nitroimidazoles under hypoxic conditions induces, among other biological consequences, DNA strand breaks [15–18]. Several of these biological effects have been attributed to the activity of one or more reactive intermediate generated upon the nitroreduction of the parent compound. While the nitroso and hydroxylamino intermediates have been implicated directly in the expression of several biological effects commonly observed following exposure of mammalian cells to 2-nitroimidazoles under hypoxic conditions [3, 5–7, 9–11], the involvement of either intermediate in the induction of DNA strand breakage has not been addressed previously. It is obvious from the results presented here that cellular exposure to 2-nitrosoimidazole, INO, is indeed capable of inducing extensive DNA damage. The ability to form lesions in DNA is consistent with previous studies which demonstrated that INO is mutagenic [5].

In contrast to parental 2-nitroimidazoles, INO-induced damage is expressed in cells exposed under aerobic conditions. According to the nitroreduction pathway, the activity of INO would not be expected to be hypoxia dependent since it is located downstream of the oxygen-sensitive step in the reduction series [19]. These data therefore support the hypothesis that the nitroso intermediate is an active intermediate in 2-nitroimidazole-induced DNA strand breakage, or is at least a more proximal form of the ultimate active species. Considering experimental evidence implicating the nitroso intermediate in the expression of other biological effects commonly observed following exposure to 2-nitroimidazoles under hypoxic conditions, a similar hypothesis can be formulated for the role of the nitroso intermediate in most, if not all, of the biological effects of 2-nitroimidazoles.

As is evident from Figs 1 and 4, the alkaline elution profiles for DNA from cells treated with INO were characterized by an unusual biphasic shape. The biphasic nature of the profiles is not attributable to preferential loss of labeled thymidine mononucleotides or DNA–protein crosslink formation and presumably reflects different levels of DNA damage in two subpopulations of cells. Based upon differential adherence, two populations of cells, differing with respect to the rate and extent of DNA elution from the filters during alkaline elution assays, could indeed be identified, although they could not be separated on this basis. In an attempt to develop an improved identification and isolation technique which does not depend upon prolonged incubation of cells following drug exposure, we adapted a flow cytometric technique based upon the alteration of the emission spectrum of the DNA-binding agent Hoechst 33342 in damaged DNA [20] While preliminary studies utilizing this technique confirmed the existence of two distinct cell populations expressing different levels of DNA damage following exposure to INO, it has still not been possible to obtain sufficienty pure populations of either to allow reliable biochemical characterization (Mulcahy, unpublished observations). Consequently, the nature of the cellular heterogeneity which accounts for the differential sensitivity to INO has not been identified. Analysis of the flow cytometric data does suggest, however, that the

difference in sensitivity is not cell cycle phase-specific.

Judging from the change in slope, the DNA strand scission responsible for the terminal portion of the biphasic alkaline elution profiles appears to be completely repairable within the 6-hr posttreatment interval used for the differential adherence separation technique. The slope of this portion of the curve for both attached and floating populations was reduced relative to corresponding portions of profiles obtained immediately after drug exposure, approaching that for untreated controls (Fig. 6). The repairability of the damage responsible for the initial slope of the elution profile, on the other hand, could not be readily established from the current experiments.

While these studies clearly document strand breakage, the mechanism of strand break induction by INO has not been identified. INO does not appear to interact directly with DNA to induce strand breakage, failing to induce any evidence of strand scission when cellular DNA was incubated with INO on filters immediately prior to elution under alkaline conditions. Direct strand break damage was likewise not observed following incubation of pBR322 plasmid DNA with INO in solution* (Mulcahy, unpublished observations). A similar lack of reactivity was also reported for a 5-nitrosoimidazole [8]. Presumably, further metabolism or interactions with other cellular substituents are required to produce DNA damage. Ehlhardt and Goldman [8] have proposed a bioactivation scheme for another model nitroimidazole, 4-phenyl-5-nitrosoimidazole, involving the generation of an electrophilic intermediate via a thiol catalyzed reaction with the 5-nitroso reduction intermediate. In support of this mechanism, they were able to demonstrate binding of the radiolabeled 5-nitroso compound to DNA in the presence of the thiols, glutathione and cysteine. The extent of binding under these conditions was sufficient to account for the bactericidal effect of therapeutic doses of 5-nitroimidazole.

Whether an analogous mechanism is operative in the case of INO remains to be determined, but in this regard it is interesting to note the modulatory effect of GSH on INO-induced cytotoxicity and DNA damage and the ability of INO to dramatically reduce intracellular GSH levels in treated cells. Based upon the evidence presented, however, the role of GSH in the biological activity of INO is likely to be complicated. For example, both the GSH depletion experiments and the studies in which the concentration of treated cells was varied suggest that GSH plays a protective, as opposed to an activating, role. It seems highly likely that the net effect of GSH on INO activity will depend upon its concentration, cellular location and drug dose. Being a good nucleophile itself, GSH might effectively compete for reactive electrophiles with critical cellular targets, such as DNA, when present at high concentrations. Once GSH is sufficiently depleted, the equilibrium would favor greater drug-induced damage. In fact, if large enough INO concentrations are utilized, this GSH depletion could result from reaction with INO itself. This concept is supported by cytotoxicity studies which have shown that GSH depletion is well correlated with cell lethality. Heterogeneity of intracellular GSH levels detected in exponentially growing HT-29 cells [21] may actually contribute to the differential cell sensitivity observed in our alkaline elution studies. While the thiol-mediated activation mechanism proposed by Ehlhardt and Goldman is very attractive, definition of the role of GSH in the activity of INO and related nitrosoimidazoles must await further evaluation of the stoichiometry of the reaction between the two compounds and its correlation with activity.

In conclusion, INO is clearly capable of inducing significant damage to cellular DNA in addition to mediating other biological effects associated with exposure to 2-nitroimidazoles under reducing conditions. The mechanism responsible for this activity and the nature of the differential cellular sensitivity observed in the current studies have not been clearly identified. Nevertheless, in conjunction with previous studies of the effects of INO, these results support the hypothesis that INO is at least a proximal form of the active species responsible for the expression of the principal biological effects of 2-nitroimidazoles.

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