Correction. In the article "In vitro assembly of intermediate filaments from baby hamster kidney (BHK-21) cells" by Robert V. Zackroff and Robert D. Goldman, which appeared in the December issue of Proc. Natl. Acad. Sci. USA (76, 6226–6230), the authors request that the following corrections in the legend to Fig. 7 be noted. In line 11 "lane d" should replace "lane e," and in line 16 "lane f" should replace "lane d".

Correction. In the article "Major intracellular cations and growth control: Correspondence among magnesium content, protein synthesis, and the onset of DNA synthesis in BALB/c3T3 cells" by A. H. Rubin, M. Terasaki, and H. Sanui, which appeared in the August 1979 issue of Proc. Natl. Acad. Sci. USA (76, 3918–3922), the right-hand vertical axis label in Fig. 5 was incorrectly placed during relettering. The correct figure is:

![Graph showing the correction for Fig. 5.](image)

FIG. 3. Survival of colony-forming ability of IMR-90 (solid symbols) and VA-13 (open symbols) cells treated with various nitrosoureas for 2 hr. Linear regression lines: ---, IMR-90; --, VA-13. The results of four independent experiments are shown. FCNU, 1-(2-fluoroethyl)-1-nitroso-3-cyclohexylurea; MNU, 1-methyl-1-nitrosourea.
DNA crosslinking and cytotoxicity in normal and transformed human cells treated with antitumor nitrosoureas

(DNA repair/alkaline elution/chloroethylnitrosoureas/chlorozotocin)

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ABSTRACT Normal (IMR-90) and simian virus 40-transformed (VA-13) human embryo cells were treated with antitumor nitrosoureas, and the effects on cell viability and cell DNA were compared. All six nitrosoureas tested were more toxic to VA-13 cells than to IMR-90 cells as measured by decrease in cell proliferation or in colony formation. The nitrosoureas capable of generating alkylisocyanates produced a smaller difference between the cell types than did derivatives lacking this capacity. DNA damage was measured by alkaline elution in cells treated with four chloroethylnitrosoureas. Whereas VA-13 cells exhibited dose-dependent interstrand crosslinking, little or none was detected in IMR-90 cells. The IMR-90 cells, however, exhibited at least as much DNA-protein crosslinking as did VA-13 cells. The results can be interpreted in terms of a possible difference in DNA repair between the cell lines.

The chloroethylnitrosoureas are reactive compounds that are highly effective against malignant neoplasms in experimental animals (1) and have significant clinical activity against human cancer (2). The compounds decompose spontaneously under physiological conditions into two types of reactive products that can alkylate or carbamoylate nucleophilic sites (3, 4). Although the alkylation function is crucial to the antitumor action, the carbamoylation function, which is a result of an isocyanate decomposition product, is not essential (5). The isocyanate products of some chloroethylnitrosourea derivatives react intramolecularly so that little or no carbamoylation takes place; yet antitumor activity is retained (6, 7). Carbamoylation may nevertheless play a significant secondary role by interference with DNA repair (8–10), DNA replication (11–13), or RNA metabolism (14, 15).

The major alkylating product, chloroethyl diazohydroxide or chloroethyl carbonium ion (16, 17), may form chloroethyl adducts with nucleic acids and proteins. The chloroethyl adducts would themselves be alkylating agents, capable of reacting with a second nucleophilic site by eliminating CF-. This mechanism may generate covalent crosslinks between complementary strands of DNA (18, 19) and between DNA and protein (20). Both types of crosslinks have been detected in chloroethylnitrosourea-treated mammalian cells by using the alkaline elution technique (20, 21).

Cell types differ in sensitivity to chloroethylnitrosoureas. In previous studies, human colon carcinoma cells having different sensitivities to 4-trans-methyl-1-(2-chloroethyl)-1-nitros-3-cyclohexylurea (22) showed corresponding differences in the formation or repair of DNA interstrand or DNA-protein crosslinks (23, 24). In the current work, a normal and a transformed line of human embryo cells are found to differ in biological sensitivities to various chloroethylnitrosoureas and to exhibit a marked difference specifically with respect to interstrand crosslinking.

MATERIALS AND METHODS

Drugs. Nitrosourea compounds were obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. The drugs were dissolved in ethanol immediately before use, except that chlorozotocin (CHLZ) was dissolved in 0.01 M sodium citrate (pH 4.5). The ethanol concentrations resulting from drug addition to culture media never exceeded 0.7% and did not affect the plating efficiencies of control cells.

Cells. IMR-90, a strain of normal human embryo cells (25), was obtained at passage 4, population doubling level 10, from Warren Nichols, Institute of Medical Research, Camden, NJ. The cells were grown to passage 8, population doubling level 14, and stored frozen in ampules. Cells used in colony formation experiments were at passage 10; for other experiments, cells were at passages 10–30. The VA-13 line, a simian virus 40 transformant derived from the normal human embryo cell strain, WI-38 (26), was subcultured at low density in order to increase the plating efficiency of the line (27). Cells were cultured in Eagle’s basal medium, supplemented with 10% calf serum, 1 mM l-glutamine, and 50 μg of gentamycin per ml in an atmosphere of 7.5% CO2 in air at 37°C. The cultures were tested for Mycoplasma by Flow Laboratories, McLean, VA, and were free of contamination.

Survival Studies. For colony-formation assays, both cell types were used 2 days after plating 104 cells per 25-cm2 flask, at which time the cells were in logarithmic growth phase. Single-cell suspensions were prepared by treating monolayers with 5% chicken serum/0.1% trypsin/25 units of collagenase per ml/0.02% EDTA in Hanks' balanced salts solution. This mixture produced higher plating efficiencies than 0.25% trypsin or 0.05% trypsin/0.02% EDTA. For controls, 100 cells per 100-mm-diameter plastic dish; for drug treatment, 1000–6000 cells were used. The dishes were incubated for 20 hr to allow the cells to attach. The cells were then exposed to drug for 2 hr. After 2 weeks of incubation in fresh media, the plates were fixed with methanol and stained with Giemsa, and colonies (≥32 cells) were counted. This procedure avoids replating of cells after drug treatment. Plating efficiencies were 18–45% for IMR-90 cells and 58–67% for VA-13 cells.

In the cell proliferation studies, 5 X 104 IMR-90 or VA-13 cells were seeded in 25-cm2 flasks and incubated for 24 hr. The cells were then treated with drug for 2 hr in fresh medium supplemented also with 0.02 M Heps. Cells were harvested at

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Abbreviations: BCNU, 1,3-bis(2-chloroethyl)nitrosourea; CCNU, 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea; CNU, 1-(2-chloroethyl)-1-nitrosourea; CHLZ, chlorozotocin.

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Various times by trypsinization, and cell number was determined by means of an electronic cell counter.

**Alkaline Elution Measurements of DNA Damage.** Cells (5 × 10⁵ per 25-cm² flask) were labeled with 0.02 μCi of [2-14C]thymidine (New England Nuclear) per ml (1 Ci = 3.7 × 10¹⁰ becquerels) for 24 hr and then grown for 24 hr in the absence of label. The cells were exposed to drug for 2 hr at 37°C in fresh medium buffered with 0.02 M Hepes. Cells were harvested by rinsing with ice-cold 0.02% EDTA in Hanks’ balanced salts solution and subsequent gentle scraping. This cell detachment procedure avoided cell damage indicated by increased elution rates of control cells harvested by trypsinization (28). For DNA crosslinking assays, the cells were exposed to 300 R of x-ray at 0°C (1 R = 2.58 × 10⁻⁴ C/kg). The alkaline elution assay procedures have recently been described in detail (28) and the physical basis of the method has been discussed (29). In brief, approximately 5 × 10⁵ cells were deposited on 2-μm pore-size polyvinylchloride filters (Millipore, type BSWP) and lysed with 2% sodium dodecyl sulfate (99% purity, BDH Biochemicals)/0.02 M EDTA/0.1 M glycine, pH 10. In the interstrand crosslink assays, proteinase-K at 0.5 mg/ml (EM Laboratories, Elmsford, NY) was also included. The DNA was eluted by pumping a tetrapropylammonium hydroxide/0.02 M EDTA solution, pH 12.1, through the filters at 2 ml/hr and fractions were collected at 1.5-hr intervals. In the interstrand crosslink assays, 0.1% sodium dodecyl sulfate was included in the eluting solution. Internal standard cells labeled with [3H]thymidine and irradiated with 150 R were included to monitor the elutions (28). Internal standard corrections were applied only to the data in Fig. 7; the corrections improve the internal consistency of the data but do not alter the conclusions.

**RESULTS**

Cytotoxicity. Several chloroethylnitrosoureas inhibited the proliferation of VA-13 cells more than they inhibited that of IMR-90 cells (Fig. 1). The growth curves of the two cell types in the absence of drug were not significantly different. When examined as a function of drug concentration, the magnitude of the difference between the cell types depended on the drug; the difference was greater for 1-(2-chloroethyl)-1-nitrosourea (CNU) and CHLZ than for 1,3-bis(2-chloroethyl)nitrosourea (BCNU) or 1-(2-chloroethyl)-1-nitroso-3-cyclohexylurea (CCNU) (Fig. 2).

The difference between the two cell types was also evident in colony-formation assays (Fig. 3). Drug concentrations were chosen to show most clearly any differences between the cell types; no attempt was made to determine the exact curve shapes. The survival of IMR-90 cells was greater than that of VA-13 cells for all six nitrosoureas tested. Again, the magnitude of the difference was greater for CNU and CHLZ than for BCNU or CCNU.

**Total DNA Crosslinking.** Alkaline elution of DNA is affected both by interstrand crosslinks and by DNA-protein crosslinks (29). In the crosslink assays, cells were subjected to 300 R of x-ray at 0°C in order to introduce random single-strand breaks; the resulting single-strand population in control cells eluted with nearly first-order kinetics (Fig. 4). Crosslinks of either type decreased DNA elution in such assays. Interstrand crosslinks are presumed to do so because of the effective increase in single-strand lengths. Proteins adsorb to the filters (under the conditions of assay used in Fig. 4) and retard the elution of DNA strands crosslinked to them (30). The assays shown in Fig. 4 measure a combined effect of both types of crosslinks. In the case of chloroethylnitrosoureas, a major part of the effect is attributable to DNA-protein crosslinks (20).

![Graph 1](image1.png)

**FIG. 1.** Effect of various chloroethylnitrosoureas on the proliferation of IMR-90 (Left) and VA-13 (Right) cells. Cells were treated with 50 μM of the indicated drug for 2 hr on day 0.

![Graph 2](image2.png)

**FIG. 2.** Concentration-dependence of the effect of chloroethylnitrosoureas on cell proliferation. IMR-90 (∙) or VA-13 (△) cells were exposed to drug for 2 hr; cell number relative to control was determined on day 3 (see Fig. 1). A geometric series of drug concentrations was used, as indicated on the horizontal scale.
Chloroethylnitrosoureas produced comparable extents of crosslinking by this assay in IMR-90 and VA-13 cells (Fig. 4). [Assays done without x-ray showed only slight increases in elution rates due to drug treatment (data not shown).] Increases in elution rate, indicative of DNA strand breaks, are better exhibited in Fig. 5 in assays in which the effects of DNA-protein crosslinks have been eliminated.

DNA Interstrand Crosslinking. The effect of DNA-protein crosslinks on DNA alkaline elution can be reversed by the use of proteinase-K. The remaining decrease in DNA elution is attributed to interstrand crosslinking (20, 29). Interstrand crosslink assays disclosed a major difference between the two cell types: with all four chloroethylnitrosoureas, interstrand crosslinks were seen in VA-13 cells but not in IMR-90 cells (Fig. 5). The crosslinking in VA-13 cells was dependent on drug concentration (Fig. 6); these results were obtained 12 hr after drug exposure, at which time crosslinking was at its peak.

The dependence of interstrand crosslinking on time after drug exposure is shown in Fig. 7. The increase in crosslinking during 6–12 hr of postincubation in VA-13 cells is consistent with previous work and is attributed to the time required for the conversion of chloroethyl-DNA monoadducts to interstrand crosslinks (18–21). At 12–24 hr after drug exposure, small reductions in interstrand crosslinks were seen with BCNU, CNU,

FIG. 4. Total crosslinking (alkaline elution assays without proteinase-K) in IMR-90 (Left) and VA-13 (Right) cells treated with chloroethylnitrosoureas (50 μM) for 2 hr and then incubated in the absence of drug for 12 hr. O, Controls assayed without x-ray (in all other assays, cells were exposed to 300 R at 0°C); ●, no drug; ∆, BCNU; □, CCNU; ■, CNU; ●, CHLZ.

FIG. 5. DNA interstrand crosslinking (alkaline elution assays with proteinase-K) in IMR-90 (Left) and VA-13 (Right) cells treated with chloroethylnitrosoureas for 2 hr and then incubated in the absence of drug for 12 hr. O and ●, No drug; ∆ and △, 100 μM BCNU; □ and ■, 125 μM CCNU; ○ and ●, 125 μM CHLZ. Open symbols, no x-ray; filled symbols, 300 R at 0°C. The lines are drawn through the data for cells not treated with drug. [The slight differences in the 300-R controls (●) compared to Fig. 4 are attributable to the elimination of x-ray induced DNA-protein crosslinks by proteinase-K (20)].
and CHLZ; in the case of CCNU, the data are too scattered to permit a conclusion on this point. The IMR-90 cells in this set of experiments showed barely detectable interstrand crosslinking with BCNU, CCNU, and CHLZ.

**Single-Strand Breaks.** Alkaline elution assays performed without the use of x-ray permit an assessment of drug-induced single-strand breaks (29, 31). Single-strand breaks (or alkali-labile lesions) have been noted in cells treated with chloroethylnitrosoureas (20, 32). In the current work, we see low levels of single-strand breaks, indicated by small increases in elution rate, especially in IMR-90 cells treated with BCNU or CCNU (Figs. 5 and 6). Breaks were less evident in IMR-90 cells treated with CNU or CHLZ or in VA-13 cells treated with any of the four chloroethylnitrosoureas.

**DISCUSSION**

We have compared two human cell types and observed differences in biological sensitivity and DNA crosslinking in response to several chloroethylnitrosoureas. Both cell types are of human embryo origin. The IMR-90 strain is diploid and exhibits density-dependent growth inhibition and limited life span, and is considered to be a normal cell type. The VA-13 line was derived from another normal human embryo cell strain, WI-38, by simian virus 40-induced transformation. This line is aneuploid and has an unlimited life span in culture, but does not produce virus.

The VA-13 line was consistently more sensitive than the IMR-90 strain at various chloroethylnitrosoureas, whether measured by cell proliferation rate or colony-forming ability. The VA-13 line is also more sensitive to BCNU than is its parent strain, WI-38 (8, 32). The magnitude of the difference between VA-13 and IMR-90 cells was substantially greater for two of the compounds, CNU and CHLZ, than for BCNU and CCNU. The two pairs of compounds differ in certain biochemical effects. BCNU and CCNU have carbamoylating capabilities and inhibit the strand rejoining step of DNA repair (8-10) and the processing of RNA transcripts (14, 23), whereas CNU and CHLZ do not inhibit these steps (33). The difference appears to be related to differences in the decomposition chemistry, in that CHLZ lacks carbamoylating activity (because of intramolecular inactivation of the isocyanate), whereas CNU generates cyanate instead of alkylisocyanate. The difference between the two pairs of chloroethylnitrosoureas was evident both in the effects on cell proliferation rate and on colony formation. The colony-forming assays also showed that fluoroethylnitrosocyclohexylurea, the fluoroanalogue of CCNU, produced a relatively small difference between the cell types, similar to CNU. Methylnitrosourea, which is similar to CNU with the chloroethyl group being replaced by a methyl group, exhibited a substantial difference between the cell types. These observations are consistent with the idea that the alkylating activity of the nitrosoureas is responsible for the toxicity difference between the two cell types and that carbamoylation reduces the magnitude of the difference.

Treatment of mammalian cells with chloroethylnitrosoureas produces DNA interstrand and DNA-protein crosslinks, both of which affect DNA alkaline elution (20, 21). In the direct elution assay, which measures the combined effect of the two classes of crosslinks, the major part of the crosslinking effect is due to DNA-protein crosslinks. Differences in total crosslinking, assayed in this way, accompanied differences in cytotoxic sensitivity of human colon carcinoma lines to 4-trans-methyl-CCNU (23, 24).

Interstrand crosslinking was measured by assays in which proteinase-K was used to remove DNA-protein crosslinks. The argument that the proteinase-resistant component of the DNA elution assay measures interstrand crosslinking has been re-
viewed elsewhere (29). Although this inference has not been unequivocally proven, it is clear that the proteinase-sensitive and resistant components of the elution assay measure distinct classes of crosslinks. Alternatives to interstrand crosslinking would have to invoke other classes of links between DNA and non-DNA constituents. The formation of interstrand crosslinks in purified DNA treated with chloroethylnitrosourea has been demonstrated and has been shown to occur with two-step kinetics, as is inferred by the proteinase-resistant elution assay in cells (19–21, 29).

In the current work, four different chloroethylnitrosoureas were tested by the total crosslink assay and produced no significant difference between IMR-90 and VA-13 cells. However, assays for interstrand crosslinking, performed under conditions that eliminate the effect of DNA-protein crosslinks, showed interstrand crosslink formation in VA-13 cells whereas there was little or none in IMR-90 cells. DNA-protein crosslinks, therefore, must have been formed to at least as great an extent in IMR-90 cells as in VA-13 cells. The lack of interstrand crosslinking in IMR-90 cells, therefore, cannot be due to lack of drug uptake or to increased chemical inactivation of intracellular drug.

Interstrand crosslinking did not, however, correlate quantitatively with cytotoxicity. IMR-90 cells show less crosslinking than VA-13 cells even when the two cell types are compared at equitoxic doses. Also, the cytotoxicity differences among the different chloroethylnitrosoureas was not reflected by differences in interstrand crosslinking. Interstrand crosslinking is therefore not the only factor determining cytotoxicity.

Either chloroethylnitrosourea treatment in IMR-90 cells fails to form interstrand crosslinks or these crosslinks are repaired very rapidly. Because little or no interstrand crosslinking was detected at any time after treatment, even with high drug doses, we lean towards the idea that the crosslinks are not formed. The absence of crosslink formation may be due to (i) inaccessibility of certain DNA sites to chloroethylation, (ii) interference with the reaction that converts DNA-chloroethyl monooadducts to interstrand crosslinks, or (iii) removal of DNA-chloroethyl monoadducts prior to their conversion to interstrand crosslinks. Of these three possibilities, the last is easiest to reconcile with current information. According to the current picture of crosslink formation, the conversion of chloroethylated DNA to interstrand crosslinks occurs slowly over a period of several hours (18–21). This would provide time for chloroethyl monooadduct removal prior to crosslinking. DNA-protein crosslinking, on the other hand, may form by initial chloroethylation of protein sites followed by reaction with DNA sites (20), so that these lesions may not be affected by repair mechanisms that act on the DNA monooadducts. Methylnitrosourea is much less cytotoxic than the haloethylnitrosoureas and does not crosslink DNA (21, 34). The cytotoxicity may nevertheless be affected by repair of certain methylated DNA sites, so that the difference in sensitivity of the two cell types to this agent could be based on the same mechanism as in the case of the chloroethyl compounds. Chloroethylnitrosourea-treated WI-38 cells were noted to have a higher frequency of single-strand breaks than VA-13 cells, and these single-strand breaks are repaired (32). The breaks could be a consequence of excision repair of chloroethyl monooadducts, occurring more rapidly in WI-38 than in VA-13 cells.

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