Inhibition of the Ligase Step of Excision Repair by 2-Chloroethyl Isocyanate, a Decomposition Product of 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

The compound 2-chloroethyl isocyanate, a decomposition product of 1,3-bis(2-chloroethyl)-1-nitrosourea, was studied for its effects on excision repair of DNA in normal human fibroblasts exposed to ultraviolet (UV) radiation. For examination of the initial step in repair (UV endonuclease), the frequency of UV-induced strand breaks was analyzed by alkaline elution. Repair polymerase activity was assessed from the rate of UV-stimulated incorporation of radioactive thymidine and from the amount of repair replication (isopycnic gradient analysis for newly synthesized patches of DNA within strands of previously synthesized parental DNA). The final step in repair (strand rejoining) was monitored by following the disappearance of strand breaks with time after UV, with the use of the alkaline elution technique. Chloroethyl isocyanate, 75 μM, did not inhibit production of strand breaks after UV; however, rejoining of breaks was inhibited. Since neither UV-stimulated incorporation of radioactive thymidine nor repair replication was affected by chloroethyl isocyanate, inhibition of strand rejoining is attributed to an effect on the ligase step. The biological significance of this effect with respect to the mechanism of action of chloroethyl nitrosoureas is uncertain; in theory this effect could amplify cytotoxicity, since an excision-type repair mechanism is involved in repair of DNA damaged by alkylation, and alkylation is considered to be fundamentally responsible for the antitumor activity of chloroethyl nitrosoureas.

INTRODUCTION

The chloroethyl nitrosoureas are compounds with clinically useful antitumor activity (for review, see Ref. 23) whose mechanism of action has been difficult to define. One feature that has complicated the study of this class of compounds is their instability: under physiological conditions they decompose quickly (22), with the portion of the molecule attached to the N-1 atom forming a chloroethyl carbonium ion capable of alkylation and the portion bound through the N-3 atom to the carbonyl group forming an isocyanate capable of carbamoylation (20, 21, 24). A simplified version of the scheme for decomposition of BCNU, the most widely studied nitrosourea, is shown in Chart 1. The available evidence now indicates that a breakdown product (rather than the parent nitrosourea compound) produces the biochemical effects responsible for antitumor activity (11).

Alkylation has for some time been regarded as the fundamental basis of nitrosourea antitumor activity (29), but until recently only monofunctional alkylation appeared to be possible, and monofunctional alkylation is usually not a source of potent antitumor activity. Findings now show, however, that nitrosoureas are capable of bifunctional alkylation, with DNA cross-linking (12, 18); the evidence suggests that it is the chloroethyl carbonium ion generated during nitrosourea decomposition that is responsible for cross-linking, and thus the role of this decomposition product in drug action appears to be reasonably well established.

The isocyanate decomposition product, on the other hand, does not have a defined role in drug action. Isocyanates carbamoylate proteins, but the degree of carbamoylating activity varies from 1 nitrosourea to the next, and some chloroethyl nitrosoureas with virtually no carbamoylating activity are potent antitumor agents (1, 26). Clearly, strong carbamoylating activity is not required for antitumor action. It has been suggested that strong carbamoylating activity might be responsible for host toxicity (29), and this view seemed to be well illustrated by the case of chlorozotocin. However, subsequent work with a chlorozotocin analog has suggested that it may not be the weak carbamoylating activity but rather the glucose carrier that is responsible for the diminished bone marrow toxicity of chlorozotocin (14).

Some consideration has been given to the possibility that strong carbamoylating activity might be a useful adjunct to the antitumor activity of chloroethyl nitrosoureas. An observation that could provide a basis for such a view is that CIC, the strongly carbamoylating decomposition product of BCNU, inhibits the repair of DNA (17). Inhibition of DNA repair might, according to a reasonable but as yet unproven line of thinking, be a way of enhancing the amount of lethality caused by a given amount of alkylation damage.

In the original studies of CIC effects on DNA repair, cells exposed to ionizing radiation were studied. In the current investigation cells exposed to UV radiation are studied. CIC effects on excision repair are examined. There are major

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4 The abbreviations used are: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; 2-chloroethyl isocyanate; dThd, thymidine; PBS, 0.15 M NaCl-0.005 M potassium phosphate, pH 7.4; BrdUrd, bromouracil deoxyriboside.
differences between excision repair following UV and the repair of damage due to ionizing radiation (10). Two differences are of particular importance to this study. First, with UV, strand breaks are not produced directly (direct breaks are produced by ionizing radiation); this means that the effect of CIC on the incision step in repair can be examined. Secondly, the length of DNA segments synthesized during repair replication is much longer after UV than it is after ionizing radiation (10); this means that, after UV, repair replication can be easily studied as a step distinct from strand rejoining.

MATERIALS AND METHODS

Cell Lines and Cell Labeling. Normal human fibroblasts (line CRL 1119), obtained from the American Type Culture Collection, Rockville, Md., were grown in Dulbecco's medium with 10% fetal calf serum plus penicillin and streptomycin at 37° under 5% CO2 in a humidified environment. Six to eight days prior to the experiments, 2 x 10⁶ fibroblasts were plated on 160-sq mm Petri dishes with [2-14C]dThd (0.02 µCi/ml, 57 mCi/mmol); 3 days later the medium was replaced with nonradioactive medium. On the day of the experiment, the cells were in a confluent monolayer and were no longer rapidly proliferating.

Irradiation. Confluent fibroblast monolayers were irradiated with a GE 254-nm germicidal lamp calibrated with a shortwave UV intensity meter (J-225 UV meter; Ultra-Violet Products, Inc., San Gabriel, Calif.). Prior to irradiation the cells were washed with warm PBS. The PBS was decanted, and the cells were irradiated at 37°. Low-dose UV (1.5 J/sq m) was delivered at a rate of 0.25 J/sq m/sec; high-dose UV (20 J/sq m) was delivered at a rate of 2 J/sq m/sec. Immediately after irradiation fresh, nonradioactive medium buffered with 0.01 M N' -2-hydroxyethylpiperazine-N'-ethanesulfonic acid buffer was added, and the cells were incubated at 37° for various times ("repair" time). For the achievement of single-cell suspension, the cells were washed with warm PBS (40 sec), incubated with 0.25% trypsin at 37° (60 sec), scraped free with a rubber policeman, and triturated in cold PBS.

Drug Treatment. CIC (NSC 87418), obtained from the Drug Synthesis and Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, was diluted in cold absolute ethanol immediately prior to the experiment. After irradiation of the cells, 5 ml of fresh N'-2-hydroxyethylpiperazine-N'-ethanesulfonic acid-buffered medium were added together with 0.01 ml of CIC (final concentration of CIC, 75 µM). Control samples received fresh medium plus 0.01 ml of cold absolute ethanol.

Alkaline Elution. The procedure used is a modification of that described by Kohn et al. (19). Cells were diluted in cold PBS and filtered onto a polycarbonate filter (25-mm diameter, 3-µm pore size; Millipore Corp., Bedford, Mass.) and were washed several times with cold PBS. The cells were then lysed at room temperature with 5 ml of 2 M NaCl-0.02 M sodium EDTA-0.2% Sarkosyl (pH 10.2), which was allowed to flow slowly through the filter without suction. The filters were then washed with 3 ml of 0.02 M trisodium EDTA (pH 10.3) and were eluted in the dark with 0.10 M tetrapropylammonium hydroxide-0.02 M EDTA (pH 12.2) at a pump speed of 0.04 ml/min. Fractions were collected at 90-min intervals for 15 hr. The fractions were mixed with 10 ml of Aquasol containing 0.3% acetic acid and were counted in a liquid scintillation counter. Radioactivity remaining on the membrane filters was solubilized with 0.4 M of 1 N HCl at 70° for 1 hr, followed by 2.5 ml of 0.4 N NaOH at room temperature for 30 min, and was counted after the addition of 10 ml of Aquasol. Radioactivity remaining in the filtering apparatus (ordinarily amounting to about 10% of the total radioactivity) was recovered by 5 successive washes with 0.4 N NaOH; the counts in these washes were added to the counts remaining on the filter to determine the radioactivity not eluted after 15 hr of elution.

UV-stimulated Repair Incorporation. Cells were prelabeled with [3H]dThd for 3 days and were then maintained in isotope-free medium for 1 day. Samples to be irradiated were exposed to UV at Time 0. Also at Time 0 and at 30-min intervals thereafter, the culture medium was removed and replaced with fresh, labeled medium ([3H]dThd, 4 µCi/ml, 0.5 µM dThd), with or without drug(s). Drug concentrations were 10 µM hydroxyurea and 75 µM CIC. At sequential timed intervals after UV, the amount of radioactive dThd incorporated into DNA was determined by suspension of the cells in cold PBS and precipitation with an equal volume of 10% trichloroacetic acid. The precipitate was collected on membrane filters and counted in a liquid scintillation counter.

Isopycnic Gradient Analysis after BrdUrd Incorporation. These studies were performed according to the method of Cleaver (7). Cells were studied 4 days after subculturing, at a time when they were partially confluent and still proliferating. At ~2 hr the medium was removed and replaced with fresh medium containing BrdUrd, 5 µg/ml. At zero time (and again every 30 min throughout the incubation period), the medium was removed and replaced with fresh medium plus or minus CIC (75 µM); the medium also contained BrdUrd (5 µg/ml) and [3H]dThd (13 Ci/mm; 25 µCi/ml in the samples exposed to 1.5 J/sq m and 5 µCi/ml in all other samples).

At the end of the incubation period, cells were washed, resuspended, and lysed, and the density of labeled DNA was determined by CsCl centrifugation (type 65 fixed-angle
for 3 days prior to study were exposed to UV at 254 nm. 1.5 J/sq m. At timed
of normal human fibroblasts exposed to UV irradiation. Cells that had been prelabeled with [14C]dThD for 3 days and maintained in isotope-free medium
was included as a marker.

small amount of normal-density DNA labeled with [14C]dThd
determined. Odd-numbered samples were reserved (from
of "C-labeled DNA retained on the filters as a function of time of elution.

DNA. The presence of strand breaks is revealed by an increase in the rate of elution of DNA through a membrane filter. Results are conveniently expressed in terms of the fraction of the total DNA (present at the start of elution) retained on the filter as a function of the time of elution.

Elution patterns before and after UV radiation of normal human fibroblasts are shown in Chart 2. In unirradiated cells, in which there are no induced strand breaks, the rate of elution is very slow, with most of the DNA remaining on the filter after 15 hr of elution (Chart 2A). Four min after low-dose UV (1.5 J/sq m), the fraction of elution is altered (Chart 2B); the fraction of DNA retained on the filter decreases rapidly with time of elution, indicating the presence of a large number of strand breaks. This pattern of rapid elution after UV does not occur in excision-deficient cells from patients with xeroderma pigmentosum (13). With increasing time after UV, the strand breaks disappear; as seen in Chart 2C, 30 min after UV the DNA elution pattern has shifted toward a normal configuration, and after 60 min of repair (Chart 2D) the rate of elution has essentially returned to normal. The sequence of rapid elution soon after UV, followed by a return toward normal elution with increasing time after UV, is highly reproducible. It has been observed in other lines of normal human fibroblasts (13) and is in accord with the pattern that would be expected from the currently held concepts regarding mechanisms for repair of UV-damaged DNA (9).

With the normal patterns of elution after UV as a frame of reference, we now shift our attention to the effects of CIC. Four min after UV the elution of DNA from cells exposed to CIC is no different from that of the UV-irradiated controls (Chart 2B); from this it is clear that CIC does not interfere with the action of the endonuclease involved in UV repair. Thirty min after UV the pattern of elution is quite different for the CIC-treated cells compared to the control cells (Chart 2C); whereas the control cells have by 30 min begun to recover from the UV damage, rejoining some of the strand breaks that had been present earlier, no comparable recovery is apparent in the cells exposed to CIC. At 60 min this difference still holds, with DNA from the cells exposed to CIC containing as many strand breaks as were present 15 min after UV (Chart 2D). The CIC effect on the elution patterns is not a consequence of production of new strand breaks by the compound itself, since a 30-min incubation with CIC in cells not exposed to UV has no effect on the elution pattern (Chart 2A). The conclusion, therefore, is that CIC interferes with UV repair and does so at a point beyond the endonuclease step.

The observed inhibition of rejoining might be due to inhibition of repair replication or to inhibition of the ligase step. The effect of CIC on repair replication was studied by means of 2 methods. The first method involved the use of hydroxyurea, a compound that very effectively inhibits semiconservative replication of DNA but does not affect repair replication (10). The results of an experiment with hydroxyurea are shown in Chart 3. Unirradiated cells exposed to hydroxyurea are almost completely inhibited in their incorporation of radioactive dThd into DNA; however, when the hydroxyurea-treated cells are exposed to UV they are stimulated to incorporate radioactive dThd in the process of repair replication (discussed in Ref. 9). The question of interest was whether CIC would interfere with the UV-stimulated incorporation of radioactive dThd; the finding was that it did not.

The second method used to study repair replication involved the use of BrdUrd and isopycnic gradient analysis (7). In this technique the growing end of the DNA chain is effectively marked, by an increased buoyant density, as the

![Chart 2. Effect of CIC on incision and on rejoining of strand breaks in DNA of normal human fibroblasts exposed to UV irradiation. Cells that had been prelabeled with [14C]dThD for 3 days and maintained in isotope-free medium for 3 days prior to study were exposed to UV at 254 nm, 1.5 J/sq m. At timed intervals after UV, the cells were lysed on membrane filters, and the frequency of DNA strand breaks was assessed by alkaline elution. CIC (or solvent alone) was added immediately after UV; in the case of the 60-min repair samples, medium was removed after 30 min and replaced with medium to which fresh CIC (or solvent) was added. The unirradiated samples were exposed to CIC (or solvent) for 30 min. A, no UV; B, 4-min repair after UV; C, 30-min repair after UV; D, 60-min repair after UV. • samples exposed to CIC 75 µM; O, samples exposed to solvent. Values represent the fraction of [14C]-labeled DNA retained on the filters as a function of time of elution.](chart2.png)

![Chart 3. Effect of CIC on UV-stimulated incorporation of radioactive dThd. Cells prelabeled with [14C]dThD were exposed to [3H]dThd beginning at Time 0. Relative incorporation of [3H]dThd is defined as [3H dpm/[14C] dpm. Each point represents the value for cells from an individual Petri dish (average [14C] dpm/Petri dish = 7500).](chart3.png)
result of the incorporation of BrdUrd in place of dThd during a preincubation period. Following the BrdUrd preincubation radioactive dThd is incorporated into DNA of greater-than-normal density when semiconservative replication is taking place (incorporation occurs only at the growing end of the DNA chain, which will be contiguous with the segment into which BrdUrd was just incorporated), but it is incorporated into DNA of normal density when repair replication is taking place (incorporation occurs anywhere along the DNA molecule, wherever repair is occurring).

Cells preincubated with BrdUrd and then exposed to radioactive dThd in the continuing presence of BrdUrd incorporated radioactivity into DNA of greater-than-normal density (Chart 4A). Note that the normal density for DNA is 1.700; isotope incorporation was into DNA with a density of 1.725. In Chart 4A, bottom, an alkaline reband of single-strand DNA from the same experiment shows that the radioactivity is incorporated into DNA with a density greater than that of the normal-density marker. Thus, in these cells DNA replication is predominantly semiconservative; no repair replication is evident. The effect of CIC on the DNA replication of cells not exposed to UV is shown in Chart 4B.

The amount of incorporation is reduced compared to that in the control cells (Chart 4A), but incorporation is exclusively into DNA of greater-than-normal density; in other words no detectable repair replication is occurring.

The effect of UV (20 J/sq m) on DNA replication is shown in Chart 4C. Incorporation of radioactive dThd into the high-density DNA is greatly reduced. Incorporation into DNA of normal density (1.700) is evident, representing repair replication. On the alkaline reband (Chart 4C, bottom), most of the radioactivity can be seen to be present in DNA of a density identical with that of the normal-density marker. Thus, in these cells exposed to UV much of the [H]dThd incorporation is due to repair replication. The main question was whether this repair replication would be inhibited by CIC. The experiment with UV plus CIC is shown in Chart 4D. The results are essentially the same as those without CIC; repair replication is evident and is proceeding at a normal or nearly normal rate. (On the alkaline rebands incorporation into DNA of normal density was 600 cpm in the cells not exposed to CIC after UV and 525 cpm in the cells exposed to CIC.)

Results after exposure to lower-dose UV (1.5 J/sq m) are...
shown in Chart 4, E and F. Here the UV inhibition of semiconservative replication is less extensive than it is after 20 J/sq m, and repair replication is less evident. However, on the alkaline rebands (Chart 4, E and F, bottom) the presence of labeled DNA of normal density is evident; repair replication is occurring and is not inhibited by CIC.

**DISCUSSION**

Current concepts regarding the process of excision repair have been derived primarily from studies with UV (for review see Ref. 9). The repair process may be quite similar with alkylating agents as with UV, but the effects of UV have been studied much more extensively and are better characterized. Accordingly, UV was selected as the DNA-damaging agent in this study of CIC effects on excision repair.

In the evaluation of CIC effects on the endonuclease and ligase steps in repair, a technique capable of detecting small numbers of strand breaks was needed. The number of strand breaks present at any 1 time after UV is comparatively small (in relation to the number present after ionizing radiation) so that the usual technique of sedimentation analysis with alkaline sucrose gradients is difficult to use. Before this study began extensive preliminary work was done that showed that the technique of alkaline elution could readily demonstrate the strand breaks that are transiently present after low-dose UV (13). Confidence in the validity of those measurements came with the finding that alkaline elution analysis disclosed no strand breaks in xeroderma pigmentosum cells after UV. Alkaline elution, therefore, was the technique used for assessment of the effects of CIC on the incision and the ligase steps in repair.

We conclude that CIC inhibits only the ligase step in excision repair. The first experiment, designed to assess the effect of CIC on the incision step, showed that in the absence of CIC UV-induced strand breaks appeared with the usual frequency (Chart 2B); thus, the endonuclease step was unaffected. The subsequent rejoining of strand breaks, however, was inhibited (Chart 2, C and D), indicating an effect at some later step in the repair process. From the finding that UV-stimulated incorporation of radioactive dThd was not diminished in the presence of CIC (Chart 3), it was evident that the synthesis steps were not inhibited. A second line of evidence bearing on the DNA synthesis steps was available from the BrdUrd experiments, which clearly showed that repair replication continued in the presence of CIC, with insertion of patches of newly synthesized DNA within old segments of DNA (Chart 4). The conclusion that CIC inhibits the ligase step in DNA repair thus is based on the observations that rejoining of strand breaks is inhibited, while there is no inhibition of repair synthesis.

CIC is the only inhibitor of DNA repair currently known to act specifically at the ligase step; presumably, however, some other organic isocyanates will be found to have a similar effect. [There is 1 report in which hydroxyurea was said to interfere with strand rejoining in repair (2); others (8, 17) have been unable to confirm the finding.] It is reasonable to expect that other classes of ligase inhibitors will be found now that a reliable method is available for detecting inhibition of rejoining of UV-induced strand breaks. In the past inhibitors of DNA repair have been detected primarily on the basis of their ability to inhibit repair replication (7).

A reasonable but untested assumption is that the isocyanate effect is the result of protein carbamoylation. Previous work with another nitrosourea, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, indicated that its isocyanate decomposition product bound extensively to protein but not to nucleic acids (6). Presumably the same is true for CIC. One of the principal features of organic isocyanates is their high reactivity; these compounds are capable of reacting with many cellular constituents and are not expected to bind selectively. However, certain isocyanates have been shown to inhibit specific enzymes with a remarkable degree of selectivity (3-5, 15, 27). Inhibition was a function of the ability of the isocyanate to bind at or near the active site of the enzyme, and binding capability was determined strictly by stereochemical factors. From this, thoughts may arise regarding the possibility of identifying an organic isocyanate that would have some desirable selectivity in its reactions and then of designing a nitrosourea that would release this compound on decomposition. The degree of interest in such a possibility would depend on the results of studies of the biological significance of repair inhibition.

If the action of CIC were due to a direct inhibition of the enzyme polynucleotide ligase, the full consequences of that inhibition would not be clear because of uncertainty regarding the exact role of that enzyme. Besides its involvement in DNA repair, polynucleotide ligase may also be involved in normal replicative synthesis of DNA, joining Okazaki fragments (16), in which case inhibition of the enzyme would interfere with normal, semiconservative DNA replication as well as with repair.

The question of the biological significance of repair inhibition by CIC remains open. Already mentioned was the idea that inhibition of repair of DNA could logically be expected to amplify the amount of cytotoxicity produced by a given level of DNA damage. Two examples of this effect may have already been observed, indirectly, in the case of the parent compound of CIC, BCNU. One example is the reported therapeutic synergism between BCNU and conventional alkylating agents (25), and the other is the reported synergistic cytotoxicity from the combination of BCNU plus ionizing irradiation (28, 30). In opposition to the idea that repair inhibition by CIC is biologically important for the antitumor activity of BCNU is the fact, already mentioned, that nitrosoureas that do not form isocyanates capable of inhibiting repair [chlorozotocin and 1-(2-chloroethyl)-1-nitrosourea] have potent antitumor activity of a degree that may be quite comparable with that of BCNU.

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**REFERENCES**


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