DNA Damage in the Intracerebral Rat Gliosarcoma 9L Treated with 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea

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SUMMARY

Nitrosoureas are among the most effective agents for brain tumor chemotherapy, but their mode of action is uncertain. Damage to DNA in the intracerebral rat gliosarcoma 9L following in vivo exposure to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) was determined by the alkaline sucrose gradient method. Following i.p. CCNU treatment, tumors were removed and mechanically dissociated. Tumor cells were lysed upon and sedimented through an alkaline sucrose gradient. DNA content of gradient fractions was assayed fluorometrically. DNA from tumor cells exposed to therapeutic, nontoxic CCNU doses sedimented more slowly than did untreated DNA, indicating single-strand breaks or alkali-labile strand damage. At this nontoxic dose and at short lysis times, anomalous sedimentation profiles were attained with the increased damage from higher doses. Damage to tumor DNA was also investigated by measuring the fraction of DNA remaining double stranded after exposure to alkali by determining its resistance to S1 nuclease from Aspergillus oryzae. This fraction reflects its counterpart, the fraction of DNA that has completely unwound between single-strand breaks and separated irreversibly from the helix, and thus gives quantitative insight into the amount of damage present. Tumors from rats receiving a 3-hr treatment with CCNU (30 mg/kg) or methyl methanesulfonate (25 mg/kg) showed equivalent fractions of double-stranded DNA in alkali. The double-stranded DNA fraction for tumors treated with methyl methanesulfonate returned to control levels by 16 hr, demonstrating complete repair of the rat brain tumor DNA in vivo. CCNU-treated tumors showed no detectable ability to repair the damage by 16 hr.

INTRODUCTION

The nitrosourea group of antitumor agents currently offers the most active drugs for the treatment of malignant brain tumors (12). Despite their widespread use in this regard, the mode of action of these drugs has not been definitively determined. Decomposition of nitrosoureas under physiological conditions yields alkylating and carbamylation moieties (15). The alkylation of nucleic acids (and proteins to a minor degree) (4) and carbamylation of pro-tein (2, 4) have been demonstrated after in vitro and in vivo exposure to these agents. The work of Schabel et al. (19) showing that the hamster plasmacytoma is resistant to known alkylating agents and to 1,3-bis(2-chloroethyl)-1-nitrosoureas has generated speculation that the carcinostatic activity of the nitrosourea agents is principally related to their alkylating metabolites. Wheeler and Bowdon (23) offered further support for this tenet when they showed that 1,3-bis(2-chloroethyl)-2-nitrosourea has metabolic effects similar to those of alkylating agents.

Because the nitrosoureas have antitumor activity against malignant brain tumors, we chose the i.c.2 rat gliosarcoma 9L for study of the in vivo alkylating activity of CCNU. When alkylated DNA is exposed to alkali, the strands begin to separate with single-strand breaks serving as points where unwinding can begin (21). Complete unwinding of regions between breaks results in the irreversible release of single-stranded fragments from the helix, which can be detected on alkaline sucrose gradients (14) or quantitated by their susceptibility to digestion by S1 nuclease, a single-strand-specific exonuclease from Aspergillus oryzae (7, 13). Both the alkaline sucrose gradient technique and S1 nuclease determination have been used in the work presented here to demonstrate DNA damage in an experimental solid tumor treated with CCNU doses that have been shown to be nontoxic and therapeutic. Because it is difficult to effect radioactive prelabeling of the DNA of solid tissues in vivo, in both these techniques we depended upon the fluorescent assay for DNA developed by Kissane and Robbins (8). This assay has been previously applied by Wheeler and Lett (24) to a sucrose gradient analysis of radiation-induced DNA damage in a normal brain and by Zubroff and Sarma (26) to a similar analysis of carcinogen-induced DNA damage in a variety of normal tissues.

MATERIALS AND METHODS

Tumor Implantation. The gliosarcoma 9L used in this study was provided by William H. Sweet, Paul T. Kornblith, Janette R. Messer, and Beverly O. Whitman of the Massachusetts General Hospital, Boston, Mass. The tumor, originally induced in CD Fischer rats by weekly injections of N-nitrosomethylurea, has been well characterized histologically (1). It was originally described as a malignant astrocytoma; but recent reevaluation has revealed some sarcoma-

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2 The abbreviations used are: i.c., intracerebral; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MMS, methyl methanesulfonate; TCA, trichloroacetic acid.

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tous elements, which have prompted its new classification as a gliosarcoma.

The tumor cells are grown by standard methods (25) in continuous monolayer cell cultures in Eagle's basal medium supplemented with fetal calf serum (10%), penicillin (100 units/ml), streptomycin (100 μg/ml), and L-glutamine (2 mm). Forty-eight-hr cultures in the log phase of growth were harvested by exposure to trypsin (0.25%) for 15 min. The cells were diluted to a final concentration of 5 × 10⁶ cells/ml in the supplemented Eagle's medium. Forty-two-day-old male Fischer rats (Charles River Breeding Laboratories, Wilmington, Mass.) were anesthetized with ether and affixed to a board with ear pins. The scalp was opened electrosurgically, and a hole 1 mm in diameter positioned 3 mm to the right of the midline and 3 mm posterior to the coronal suture was drilled to the extradural space. The cell suspension (10 μl) was injected into the brain through a 22-gauge needle attached to a 250-μl syringe. The needle extended 3 mm beyond the inner table of the skull. The hole was immediately filled with bone wax to prevent reflux to tumor cells, and the scalp incision was then closed with a skin clip.

**Treatment of Animals.** Approximately 3 weeks after tumor implantation, the rats showed signs of brain tumors, with increased eye secretions, weight loss, and often paralysis. Tumors at this stage of growth weigh approximately 50 mg. Symptomatic rats were chosen for drug injection. CCNU (NSC 97037) was obtained from the Drug Procurement Branch, Division of Cancer Treatment, National Cancer Institute, NIH, Bethesda, Md. The drug was diluted in ethanol at a concentration of 60 mg/ml and diluted 1:10 with fat emulsion (Intralipid; Cutter Laboratories, Berkeley, Calif.), containing, in water, soybean oil (10%, w/v), glycerol (2.25%, w/v), and egg yolk phospholipid (1.2%, w/v). MMS (Eastman Kodak Co., Rochester, N. Y.) was diluted with 0.9% NaCl to a concentration of 2 mg/ml. These agents were injected i.p. in the appropriate volume to yield doses of 30, 60, 120, 180, and 240 mg/kg for CCNU and 25 mg/kg for MMS. Untreated control animals were observed for each experiment.

**Preparation of Tumor Cells.** At intervals after treatment the rats were sacrificed by decapitation, and their brains were removed quickly. The tumor was excised from each tissue were sedimented by centrifugation for 30 sec at 2000 rpm at 4°. The supernatant solution containing the suspension of tumor cells, and the scalp incision was then closed with a skin clip.

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**Alkaline Sucrose Gradients.** A linear 30-ml 5 to 20% alkaline sucrose gradient containing 0.9 M NaCl-0.024 M EDTA to a concentration of 2 × 10⁶ cells/ml, was prepared on a Beckman density gradient former over a 2-ml cushion of 2.3 M sucrose. The tumor cell suspension, 100 μl containing 2 × 10⁶ cells, was pipetted into 0.9-ml lysis solution (0.6 M NaCl-0.1 M NaOH-0.01 M EDTA-0.2% Sarkosyl, pH 11.8) which was layered on top of the gradient. After the lysis solution was covered with 2 ml of paraffin oil, cell lysis and DNA unwinding proceeded during 45 min at room temperature (20-23°) in the dark. The gradients were then centrifuged for 90 min at 20,000 rpm at 20° in a SW 27 rotor (Beckman Instruments, Inc., Spinco Division, Palo Alto, Calif.). After centrifugation, 13 fractions, each 2.2 ml in volume, were removed from the bottom of the pierced tubes by pumping mineral oil into the top. The fractions were collected in tubes containing 100 μg bovine serum albumin, after which 0.23 ml 60% TCA was added to each. The DNA was allowed to precipitate over 15 hr at 4° before the amount in each fraction was measured. To monitor the recovery of a substantial percentage of the DNA applied to each gradient, an identical volume of cell suspension that was layered onto a gradient was precipitated separately with bovine serum albumin in 5% TCA. An assay of this sample yielded the amount of DNA that would have been ideally recovered in the sum of the 13 gradient fractions. The recoveries in the gradient studies presented here ranged from 73 to 93% of this ideal value with a mean of 83%.

**S, Nuclease Reaction.** The fraction of tumor DNA left double stranded after exposure to alkali was determined by its resistance to S, nuclease. The method of Rydberg (18) was followed for cell lysis. Fifty μl of tumor cell suspension containing 10⁶ cells were injected into 1.0 ml lysis solution (0.03 N NaOH-0.9 M NaCl-0.01 M Na₂HPO₄, pH 12.0). Following a 20-min lysis period at room temperature (20-23°), the solution was rapidly neutralized by the addition of 1.0 ml 0.034 N HCl containing phenol red (2 mg/liter). Five sec after neutralization, the size of the DNA was reduced by 15 sec of ultrasonic treatment at an output control setting of 3 using a Sonifier Model W-140 cell disrupter equipped with a microtip (Heat Systems-Ultrasonics, Inc., Plainview, N. Y.). After sonic disruption, the samples were made 0.2% (w/v) with respect to sodium dodecyl sulfate.

To the neutralized cell lysis solution were added 5.0 μmoles HCl, 0.14 mmole sodium acetate buffer (pH 4.6), 2.5 μmoles ZnSO₄, and glycogen to a final concentration of 5% (v/v) in a 3-ml final volume. Duplicate samples were incubated for 5 min at 45° before one received 100 μl water and the other received an equal volume containing 1 unit nuclease S1 type III (Sigma Chemical Co., St. Louis, Mo.). One unit is defined as the amount of enzyme that solubilizes 1 μg of nucleic acid in 5 min at 45° under these reaction conditions. After 60 min, 200 μg bovine serum albumin were added, and the reaction was terminated by the addition of ice-cold 60% TCA to a final concentration of 5% (w/v). The DNA was allowed to precipitate over 15 hr at 4°, after which it was measured fluorometrically. Precipitable DNA in the tubes exposed to enzyme reflected the double-stranded DNA only, while the tubes receiving no enzyme yielded the total amount of tumor DNA injected. The fraction of DNA left double stranded after exposure to alkali was calculated by division.

**Assay for DNA.** The assay used is an adaptation of previously described techniques (8). The DNA coprecipitated with bovine serum albumin in the gradient fractions.
and S1 nuclease reaction tubes was sedimented in an HS-4 rotor for 2 hr at 6,000 rpm in a Sorvall RC-5 refrigerated centrifuge at 4°. Each precipitate was washed twice with 3 ml of 5% TCA, once with 2 ml of 0.1 M ethanolic potassium acetate, and once with 2 ml of absolute ethanol with a 30-min centrifugation after each wash. Following the last wash, the precipitates were dried in a vacuum desiccator. 3,5-Diaminobenzoic acid dihydrochloride (99%+, Gold Label; Aldrich Chemical Co., Milwaukee, Wis.) was dissolved in water (0.17 g/ml), and 100 μl were added to each precipitate. The tubes were incubated at 60° for 30 min, and then 1.4 ml 10% perchloric acid were added to each. The DNA was measured by determining its fluorescence in an Aminco-Bowman spectrofluorometer (409 nm excitation wavelength, 500 nm emission wavelength). DNA standards and blanks were assayed concurrently with each group of gradient fractions and S1 nuclease reaction tubes. These yielded reproducible fluorescence throughout the series of experiments. Recovery of calf thymus DNA from this assay is complete.

**CCNU Toxicity and Therapeutic Effect.** The i.p. CCNU dose used in the studies of DNA damage (30 mg/kg) was chosen by extrapolation from toxicity data on p.o. CCNU in rats obtained from the Laboratory of Toxicology, National Cancer Institute, Bethesda, Md. Three groups of 10 male Fischer rats (42 days old) were given this dose i.p. to establish its nontoxic nature. Control rats receiving the drug vehicle only were given injections at the same time as each experimental group. Animals were observed for 100 days after injection. Four groups of 10 tumor-bearing rats received CCNU (30 mg/kg i.p.) 11 days after tumor implantation. Accompanying each group were 10 rats that received the drug vehicle only. Survivals were studied for 100 days after tumor implantation.

**RESULTS**

**CCNU Toxicity and Therapeutic Effect.** CCNU (30 mg/kg i.p.), was not lethal to any of the 3 groups of 10 non-tumor-bearing rats given injections. All appeared healthy throughout the 100 days of observation. This 30-mg/kg dose given to the 4 groups of tumor-bearing rats on Day 11 allowed, respectively, 8 of 10, 7 of 10, 7 of 10, and 5 of 10 survivors after 100 days, while all control animals in all groups were dead by Day 60 with median survivals of 35, 43, 38, and 32 days for the 4 groups, respectively. All animals dying in this survival experiment had signs of brain tumor (increased eye secretions, weight loss, and paresis) prior to death. Autopsies were done on some animals in each group to confirm the presence of tumor. These data indicate that the CCNU dose of 30 mg/kg used in these experiments has an antitumor effect, since it significantly prolonged the survival of animals bearing the i.c. gliosarcoma 9L.

**Alkaline Sucrose Gradients.** The alkaline sucrose gradient technique was applied purely qualitatively. No attempt at gradient calibration or subsequent calculation of numbers of single-strand breaks was made. Brain tumor DNA from rats treated with CCNU (30 mg/kg) for 3 hr sedimented slowly compared to DNA from rats receiving the drug vehicle only (Chart 1). The treatment with CCNU, then, results in single-strand breaks or damage that progresses to breaks when exposed to the alkaline lysis conditions of the gradients. Twelve hr after treatment with the same CCNU dose, the sedimentation profile changes little (Chart 2).

The sedimentation profile at this 30-mg/kg dose is monodisperse and suggests the abnormal sedimentation behavior that is seen by others when high-molecular-weight (mammalian) DNA is centrifuged through sucrose gradients (9, 11, 16). It has been shown that DNA from mammalian cells exposed to low-dose irradiation sediments as a partially duplex DNA complex, and sharp, nonrandom sedimentation profiles can be avoided only by variations in
alkaline lysis time (11), rotor speed (9, 16), and extent of chain damage (radiation dose) (9, 16).

Holding lysis time and rotor speed constant, we investigated the ability of increasing CCNU doses to cause increasing chain damage and produce more conventional random sedimentation profiles. After a 3-hr exposure to CCNU (60 mg/kg), the DNA sedimentation profile was still largely monodisperse, but the peak was shifted to the middle of the gradient, suggesting a lighter complex (Chart 3). At the 120-mg/kg dose level, DNA fragments were distributed in a random fashion through fractions near the top of the gradient (Chart 3). Further studies with CCNU doses of 180 and 240 mg/kg showed no increase in this damage.

S1 Nuclease. Another measure of in vivo DNA damage is based upon the specificity of S1 nuclease from A. oryzae for digestion of the single-stranded portions of DNA helices (7, 13). The single-stranded fragments that are released in alkali by unwinding between single-strand breaks are digested, while the DNA remaining double stranded is resistant to digestion and remains acid-precipitable for fluorescent assay. Since the rate of strand separation is proportional to the number of single-strand breaks (18), the fraction of DNA remaining double stranded after a timed exposure to alkali reflects the number of single-strand breaks induced by therapy. DNA damage in the rat brain tumor caused by CCNU treatment was compared to that induced by treatment with MMS, an agent with accepted monofunctional alkylating activity (3). The fraction of CCNU-treated (30 mg/kg for 3 hr) tumor DNA remaining double-stranded in alkali was equivalent to that after MMS treatment (25 mg/kg for 3 hr), implying equivalent chain damage at these respective doses (Table 1).

The ability of the rat brain tumor to repair the single-strand breaks induced by MMS (25 mg/kg) and CCNU (30 mg/kg) was also investigated. Tumors were removed 16 hr after the rats were treated with either of these agents. After

![Chart 3. Alkaline sucrose sedimentation of rat gliosarcoma DNA exposed in vivo for 3 hr to CCNU.](image)

**DISCUSSION**

DNA strand damage is presumed when cells are exposed to alkaline lysis conditions and the released DNA sediments slowly through an alkaline sucrose gradient (14). Methods that demand exposure to alkali, however, cannot differentiate between a preexisting strand break and a region of strand damage that becomes a break only after alkaline lysis. The DNA of the rat gliosarcoma 9L exposed in vivo to CCNU doses shown here to be nonlethal to normal animals and to be therapeutic to tumor-bearing animals sediments more slowly than DNA from untreated tumors. In view of the existing evidence for the alkylating activity of CCNU (2, 4, 15, 19, 23), this slowly sedimenting DNA species may be the result of single-strand breaks in alkylated DNA helices. In any case, these data demonstrate the ability of CCNU in nontoxic, therapeutic doses to damage the DNA of a solid brain tumor in vivo.

The alkaline sucrose gradient analysis of damage to tumor DNA by therapeutic, nontoxic doses of CCNU resulted in anomalous sedimentation of what probably are partially duplex DNA complexes (20). Lett (10) and his co-workers have demonstrated that prolonged lysis times are required to reduce these mammalian DNA complexes to their purely single-stranded form (120 to 165 S) but that low doses of irradiation (200 to 1000 rads) eliminate the need for this lytic storage. This suggests that a therapeutic dose of CCNU (30 mg/kg) produces less damage to the rat brain tumor DNA than that produced by these low doses of irradiation.

The assay for the fraction of DNA remaining double-stranded by therapeutic doses of alkylating agents is given in Table 1. The DNA from tumors treated with MMS demonstrated a fraction of double-stranded DNA in alkali which was significantly greater than that from tumors similarly treated but removed earlier (Table 1). This reflects less unwinding and strand separation, the product of repaired single-strand breaks. After 16 hr, the MMS-treated tumor DNA showed a double-stranded fraction in alkali that was not different from that of DNA from untreated tumors, implying that repair was complete (Table 1). In contrast, CCNU damage after 16 hr was unchanged from that of 3 hr, suggesting that the gliosarcoma 9L was unable to repair the DNA damage caused by this agent.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of animals</th>
<th>Repair time (hr)</th>
<th>Fraction of double-stranded DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>No drug (control)</td>
<td>12</td>
<td>0.78 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>CCNU</td>
<td>10</td>
<td>0.59 ± 0.04</td>
<td></td>
</tr>
<tr>
<td>CCNU</td>
<td>4</td>
<td>0.58 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>MMS</td>
<td>6</td>
<td>0.58 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>MMS</td>
<td>3</td>
<td>0.72 ± 0.05</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± S.D.

a Significantly different from control (p < 0.001).

b Not significantly different from control.
stroanded after exposure to alkali by determining its resistance to digestion by S1 nuclease has provided convenient access to quantitation of damage to mammalian (tumor) DNA in vivo at the levels of damage caused by single therapeuthic doses of antitumor agents. Parodi et al. (17) defined alkaline lysis conditions that yielded a complete transition of rat liver DNA from the double-stranded to the single-stranded state, and they warned that S1 nuclease hydrolyzed only 10 to 15% of this DNA. As they pointed out, however, neutralization of the DNA lysate to the pH range of the enzyme will prompt renaturation of partially unwound DNA, and only single-stranded fragments that have irreversibly separated from a helix will be digested in the subsequent exposure to S1 nuclease. The 10 to 15% digestion seen by these investigators would correspond to the approximately 20% digestion of the DNA from our untreated (control) tumor cells, which have been exposed to similar lysis conditions.

The capability of the rat gliosarcoma 9L for efficient DNA repair has been demonstrated by the MMS experiment and by current work with γ-irradiation (unpublished results). The inability of the brain tumor DNA to repair comparable damage caused by CCNU treatment is without apparent explanation. A possibility that cannot be tested is that following a single CCNU dose where there is ongoing damage and repair, and at 16-hr post-treatment our method detects only the balance of these processes. However, since the plasma half-life of CCNU in male Fischer rats is approximately 50 min (6), it seems unlikely that the drug would be actively damaging tumor DNA as late as 16 hr after therapy. Another explanation would be that CCNU caused tumor cell death with an accompanying loss of repair activity. Tobey and Crissman (22) have shown, however, that after lethal doses of CCNU Chinese hamster ovary cells in vitro remained capable of cycling through S phase until they accumulated at G2 approximately 30 hr later. Other work from our laboratory has shown that pretreatment of L1210 or HeLa cells with cytotoxic levels of CCNU has no effect on the subsequent repair of radiation damage (unpublished results). Clearly, then, tumor cells maintain their synthetic potency for a period of time following a cytotoxic insult from CCNU.

A final explanation for the inability of the rat gliosarcoma 9L to repair CCNU-induced DNA damage would be that the nature of the damage is different from that caused by alkyla tion (MMS) or irradiation. It must be reemphasized that methods demanding exposure to alkali cannot differentiate preexisting strand breaks from alkali-labile strand damage and that CCNU could act by inducing alkali lability rather than by classical alkylation. Alternative mechanisms for CCNU-induced DNA damage are under investigation.

REFERENCES

DNA Damage in the Intracerebral Rat Gliosarcoma 9L Treated with 1-(2-Chloroethyl)-3-cyclohexyl-1-nitrosourea
