Sister Chromatid Exchange Induction in Patients with Anaplastic Gliomas Undergoing Treatment with Radiation plus Diaziquone or 1,3-Bis(2-chloroethyl)-1-nitrosourea

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ABSTRACT

Diaziquone (AZQ) (NSC 182986), a lipid-soluble benzoquinone derivative, is presently being tested in a Phase III clinical trial to determine its efficacy in patients with anaplastic gliomas compared to the more standard 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU) treatment following whole-brain irradiation. These patients on single-drug chemotherapy allowed us to evaluate the effects of each agent on sister chromatid exchange (SCE) induction in vivo. Eight weeks following the final radiation treatment, patients were randomly assigned to one of two groups: (a) 200 mg BCNU/m², i.v., every 8 weeks; or (b) 15 mg AZQ/m²/day, i.v., for 3 consecutive days, every 4 weeks. Blood (5–10 ml) was drawn by venipuncture before treatment, within 10 h after treatment, and for two BCNU-treated patients at various other times. Peripheral blood lymphocytes were cultured by standard techniques for analysis of SCE. Eight weeks after irradiation but before chemotherapy, the mean SCE frequency was 9.6 SCEs/metaphase. Following treatment with AZQ or BCNU, the baseline SCE frequency was increased more than 2-fold or 3-fold, respectively. Two months after BCNU treatment, there was less than a 25% reduction in SCE levels compared to samples taken and cultured within 10 h after treatment. These data show that lesions leading to SCE in human peripheral blood lymphocytes are relatively long-lived, and that on a mg/m² basis, AZQ is a more potent inducer of SCE in vivo than is BCNU.

INTRODUCTION

One of the major problems in evaluating the validity of short-term genotoxicity assays for human risk assessment is the paucity of information available from humans exposed to known genotoxicants under precisely defined conditions. One means to examine such a population is to study cancer patients on single-drug chemotherapy. As standard treatment at Duke University Medical Center, patients with anaplastic gliomas are being treated with BCNU \(^*\) 8 weeks subsequent to radiation therapy. As part of a randomized Phase III clinical trial (NCI No. T83-1073), some patients are being treated with AZQ instead of BCNU following the radiation therapy (Table 1).

AZQ is a rationally synthesized benzoquinone derivative having good lipid solubility and low ionization characteristics, which are intended to promote its efficacy against tumors of the central nervous system (1). Presently, it is being tested clinically against several central nervous system malignancies (2–4) as well as other tumor types (5–9). We selected brain tumor patients treated with either AZQ or BCNU to obtain data on the effects of in vivo exposure to genotoxicants in humans under controlled conditions.

\(^*\) The abbreviations used are BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; AZQ, diaziquone, (2,5-diaziridinyl-3,6-bis(carboethoxyamino)-1,4-benzoquinone); HFC, high frequency cell; PBL, peripheral blood lymphocyte; SCE, sister chromatid exchange.

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In this paper we report on the comparative SCE-inducing potential of AZQ and BCNU in PBLs of brain tumor patients undergoing single-drug therapy. In addition, we have followed two of the patients over several months to determine the persistence of lesions leading to SCE and to examine the frequency distribution of SCEs/cell.

MATERIALS AND METHODS

Treatment. AZQ was obtained from the Division of Cancer Treatment of the National Cancer Institute, Bethesda, MD. BCNU was purchased from Bristol-Myers (Syracuse, NY). Patients in the study were diagnosed as having either glioblastoma multiforme or anaplastic astrocytoma (10). Whole-brain and tumor-bed irradiation, consisting of approximately 6000 rads given in 30 fractions over a 35-day period, was begun within 3 weeks of diagnosis. Eight weeks after irradiation, patients were placed randomly into one of two groups: Group 1 received 15 mg/m² AZQ i.v. over a 260-min period for 3 consecutive days once every 4 weeks. Group 2 was given 200 mg/m² BCNU i.v. over a 30-min period once every 8 weeks.

Lymphocyte Culture. PBL cultures were established from mononuclear leukocytes isolated on a Ficol-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) density gradient as described previously (11–13). Briefly, this consists of inoculating \(10^9\) mononuclear leukocytes into 1.9 ml of complete medium composed of RPMI 1640 plus 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer, 10% heat-inactivated fetal bovine serum, 100 units of penicillin/ml, and 100 μg of streptomycin sulfate/ml, and an additional 292 μg of L-glutamine per ml. Concanavalin A (Type IV; Sigma Chemical Co., St. Louis, MO) (4 μg/ml) was added at culture initiation (0 h) to stimulate T-cell mitogenesis. The cultures were incubated at 37°C in a 5% CO₂ atmosphere for 24 h, 5 μM 5-bromo-2'-deoxyuridine (Sigma) was then added to each culture, and the cultures were returned to the incubator for an additional 44 h. The cultures were harvested after a total incubation time of 72 h, following a 4-h demecolcine (1.35 μg/ml) (GIBCO, Grand Island, NY) treatment. Between three and five cultures were established per individual at each sampling time depending upon the number of mononuclear leukocytes recovered. Culture harvest, slide preparation, and staining were as described previously (11–14).

Data Compilation and Analysis. Between 38 and 200 second-division metaphases were scored for SCE for each patient at each time point examined. Only cells with between 44 and 48 centromeres (2n ± 2) were included in the analysis.

Sampling. Because most of the patients were treated with radiation at another hospital prior to chemotherapy, in most cases preirradiation blood samples were unavailable. This has no bearing on the SCE analysis since irradiation has negligible effects on SCE frequencies of G₀ PBLs (15–18). All patients had blood drawn just prior to the start of chemotherapy. Blood samples were then drawn within 10 h after completion of the first treatment. Because AZQ was administered over a 3-day period, blood was only drawn after the first day of treatment to prevent confounding caused by toxicity and cell turnover that might occur over the 3 days. Since BCNU was administered in one 30-min period, in some cases samples were taken after several months to follow the persistence of lesions leading to SCE formation.
RESULTS

Although the sample size was quite small (N=2), the radiation treatment had no significant effect on the SCE frequency of the patients (Table 2). In addition, the SCE frequencies found in the concanavalin A-stimulated PBLs of the brain tumor patients before chemotherapy (range, 8.1-12.2 SCEs/metaphase) showed no indication of being elevated when compared to published reports of SCE frequencies found in PBLs of the population in general (19, 20) or in normal controls from our laboratory (range, 8.0-12.1 SCEs/metaphase). However, after one treatment with AZQ, the mean SCE frequency increased over 2-fold to 24.64 SCEs/metaphase. Following one treatment with BCNU, there was greater than a 3-fold increase in SCE frequency compared to pretreatment baseline levels. On a mg/m² basis, AZQ was approximately 7 times more potent an inducer of SCEs than was BCNU.

A distribution histogram showing the frequency of cells with a specified number of SCEs for the pooled data from 775 cells from 12 glioma patients prior to chemotherapy is shown in Fig. 1. The shape of the curve is similar to that found by Carrano and Moore (20) for 42 “unexposed” individuals. The curve is Poisson-shaped but has a longer than expected tail of HFCs. Using their statistical procedure (20), a cell with greater than 19 SCEs/metaphase would be termed an HFC.

A comparison of the SCE distribution histograms from pre- and first posttreatment patients shows a striking shift in the position and shape of the curves. For both BCNU (Fig. 2) and AZQ (Fig. 3) there was a large shift in the median from cells containing approximately 9-10 SCEs to those containing 25 SCEs for AZQ-treated patients and 39 SCEs for BCNU-treated patients. In addition, the curve appears much flatter with a marked skewness towards the right tail. Greater than 80% of the metaphases analyzed from patients after a single AZQ treatment and 98% of the metaphases from patients after one BCNU treatment could be categorized as HFCs. This compares to less than 5% HFCs from the patients before undergoing chemotherapy. Thus, there was little overlap between the pre- and posttreatment distributions indicating that almost all of the PBLs that were stimulated to divide in culture were affected.

Table 1 Patient diagnosis and treatment

Blood was drawn on any of six separate occasions: preradiation, prechemotherapy I, postchemotherapy I, prechemotherapy II, postchemotherapy II, and prechemotherapy III. PBLs were cultured in the presence of 5 μM bromodeoxyuridine as described in "Materials and Methods" for the analysis of SCE.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Sex</th>
<th>Age</th>
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<th>Chemotherapy</th>
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<td>82</td>
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<td>6030 BCNU</td>
<td>- - - - - -</td>
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<td>Glioblastoma</td>
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<td>4/84</td>
<td>0</td>
<td>5900 BCNU</td>
<td>- - - - - +</td>
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<tr>
<td>D. E.</td>
<td>F</td>
<td>Glioblastoma</td>
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<td>4/84</td>
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<td>Anaplastic astrocytoma</td>
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<td>- - - - - -</td>
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<tr>
<td>C. B.</td>
<td>F</td>
<td>Anaplastic astrocytoma</td>
<td>32</td>
<td>5/84</td>
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<td>5940 AZQ</td>
<td>- - - - - -</td>
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<td>Glioblastoma</td>
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<td>6000</td>
<td>+ + + + + +</td>
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<td>R. W.</td>
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<td>Gliosarcoma</td>
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<td>0</td>
<td>6020</td>
<td>+ + - - + -</td>
<td></td>
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<tr>
<td>B. R.</td>
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<td>0</td>
<td>5400 AZQ</td>
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<tr>
<td>N. M.</td>
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<td>6000 AZQ</td>
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</tbody>
</table>

Table 2 SCE frequencies (SCEs/metaphase) in PBLs of anaplastic glioma patients before and after undergoing various therapies

Blood was drawn on any of six separate occasions: preradiation, prechemotherapy I, postchemotherapy I, prechemotherapy II, postchemotherapy II, and prechemotherapy III. PBLs were cultured in the presence of 5 μM bromodeoxyuridine as described in “Materials and Methods” for the analysis of SCE.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Diagnosis</th>
<th>Sample no.</th>
</tr>
</thead>
<tbody>
<tr>
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<td>9.9</td>
<td></td>
</tr>
<tr>
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<td>9.4</td>
<td>9.9</td>
<td></td>
</tr>
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<td>D. E.</td>
<td>12.2</td>
<td>BCNU*</td>
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</tr>
<tr>
<td>P. T.</td>
<td>9.3</td>
<td>BCNU</td>
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<td>C. H.</td>
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<td>33.4</td>
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<td>BCNU</td>
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<tr>
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<td>N. M.</td>
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<tr>
<td>L. T.</td>
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<td>AZQ</td>
<td>24.9</td>
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</table>

* Chemotherapeutic agent.
SISTER CHROMATID EXCHANGE: DIAZIQUNOE

The distribution histogram is also informative when the cells from an individual patient are plotted before and after treatment or treatments with a chemotherapeutic agent. In one such patient (M. D.) treated with BCNU, there was an initial 3-fold increase in SCE frequency following BCNU administration (Table 2) compared to the pretreatment value. An examination of the SCE profiles from the pre- and posttreatment samples (Fig. 4, A and B) reveals that there were less than 5% HFCs in the pretreatment sample while greater than 95% of the metaphases from the first posttreatment sample could be classified as HFCs. Two months after treatment, the SCE profile of the patient's blood reveals a reappearance of a population of cells showing a control pattern of SCEs (Fig. 4C), but the mean SCE frequency remained substantially elevated (31 SCEs/metaphase) due to the presence of the majority of cells showing 20 or more SCEs (HFCs).

In a second BCNU-treated patient (G. T.), the differences are even sharper. The 50 metaphases from the pretreatment culture (Fig. 5A) contained no HFCs in contrast to the first posttreatment sample (Fig. 5B), in which the 100 metaphases analyzed could all be classified as HFCs. Again, 2 mo following BCNU treatment, there was a reappearance of cells displaying control SCE levels (Fig. 5C), and the mean SCE frequency declined by 25% over this time period. After the second BCNU treatment, the mean SCE frequency/metaphase was 70.5. Greater than 95% of the cells scored were HFCs, with the vast majority of the cells showing more than 65 SCEs/metaphase (Fig. 5D), indicating that the treatments are to some extent additive. Two months later the mean SCE frequency had dropped to 17.1/cell, and the SCE profile had changed dramatically (Fig. 5E). Again there was an emergence of a controllike cell population with less than 20 SCEs/metaphase. However, almost 10% of the metaphases analyzed had greater than 60 SCEs. Thus, there is apparently a rapid turnover of heavily damaged cells and a repopulation of the peripheral blood by undamaged lymphocytes; yet, many severely damaged PBLs remained in circulation.

DISCUSSION

We have shown that i.v. administration of AZQ or BCNU to humans induces substantial increases in the SCE frequency in their PBLs. With both drugs, almost all the cells that are analyzed show an increase in the number of SCEs present. After 2 mo there was a reemergence of cells showing control levels of SCEs in BCNU-treated patients.

In vivo SCE induction by AZQ is not unexpected. In vitro studies without exogenous metabolic activation have indicated that AZQ is a base substitution mutagen in UV repair deficient strains of Salmonella (21); produces 6-thioguanine resistant mutants in V-79 cells (22); and induces SCEs in Chinese hamster ovary cells (22) and in human, rat, and mouse PBLs in vitro. DNA cross-links and strand breaks in various cell lines are also produced by AZQ (23–25). Recently, AZQ was found to be a potent inducer of micronuclei in mouse PBLs following in vivo exposure.4

As can be seen from the structure of AZQ (Fig. 6), it is a symmetrical molecule having three distinct types of functional

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3 A. D. Kligerman, unpublished results.
groups; each could be able to react with DNA and cause chromosome damage. Under physiological conditions without exogenous metabolic activation, the aziridine rings open yielding aziridinium ions, which have the potential to alkylate DNA (26) and form DNA-DNA and DNA-protein crosslinks (23–25). In addition, when NADPH-cytochrome c reductase is present, AZQ semiquinone-free radicals are produced concomitant with the consumption of oxygen (27). The free radicals are capable of reacting with DNA and causing strand breakage (23, 24), which could eventually lead to chromosome aberrations. The fact that AZQ-induced DNA strand breaks are rapidly repaired while AZQ-induced DNA cross-links are longer lived (25) indicates that the SCE-inducing potential of AZQ in G₀ PBLs is due to its ability to form DNA adducts.

It is also conceivable that the two ethyl carbamate groups could play a role in the genotoxicity of AZQ. Studies of ethyl carbamate indicate that this compound is negative or at best a weak SCE-inducer in vitro even with exogenous metabolic activation (28). Thus, it is unlikely that the potent SCE-inducing response seen with AZQ in vitro is due to carbamate groups alone. However, one cannot rule out the possibility that some combination of each of the groups is responsible for SCE induction by AZQ.

Similarly, BCNU can be hydrolyzed under physiological conditions to reactive products (29) that can alkylate DNA and proteins (30) and also form DNA-DNA and DNA-protein cross-links (31). BCNU is carcinogenic in rodent assays (32), and it has been shown to be a potent SCE inducer both in vitro (33–35) and in mice in vivo (36, 37). Thus, it is not unexpected that BCNU is a potent SCE inducer in human PBLs exposed in vivo.

Previously, it has been reported that following successive cycles of chemotherapy, there are peaks in SCE frequency followed by a rapid decrease, sometimes returning to pretreatment baseline frequencies (38–40) and at other times remaining somewhat elevated (40–43). By examining the histograms from the two patients, it can be inferred that the reduction in SCE frequencies with time is due to the disappearance of most of the highly damaged cells and the appearance of cells with control-like SCE frequencies. This is similar to the findings of Lambert et al. (40), who examined the SCE frequencies in PBLs of melanoma patients treated with melphalan. Whether this change in SCE distribution is due to DNA repair or actual removal of the damaged cells and replacement by undamaged cells is unknown at present. Liquid holding experiments with human PBLs show that the efficiency of removal of lesions that lead to SCEs depends upon the type of lesions the particular chemical produces (43–45). This type of study has yet to be carried out using PBLs treated with either AZQ or BCNU. However, the most likely explanation is that precursor cells that were initially damaged by one BCNU treatment have divided several times, diluting the number of lesions present,
and giving rise to new PBLs that show near-control SCE frequencies when cultured.

With these base-line in vivo data on the SCE-inducing potential of both AZQ and BCNU in human PBLs, attempts will be made to replicate these exposures using rodents and to analyze the SCE frequency in their PBLs. If this is successful, we will be able to make direct comparisons between humans and rodents exposed to the same agent, under controlled conditions, using the same cell type for analysis. This approach should give valuable information on the use of rodents as models for human genotoxic risk assessment.

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REFERENCES


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