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# Elevated DNA topoisomerase II activity in nitrogen mustard-resistant human cells

(drug resistance/DNA crosslinking/DNA repair/topoisomerase inhibitors/glutathione)

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**ABSTRACT** **A human Burkitt lymphoma cell line, Raji-HN2, made 10-fold more resistant to nitrogen mustard (HN2) than the parental Raji cell line, exhibited the following characteristics when compared to the parental Raji cells: (*i*) decreased HN2-induced DNA interstrand crosslinking; (*ii*) increased (3-fold) DNA topoisomerase II [DNA topoisomerase (ATP-hydrolyzing), EC 5.99.1.3] activity; (*iii*) increased (4- to 11-fold) sensitivity to topoisomerase II inhibitors; (*iv*) increased (2-fold) glutathione content; and (*v*) increased (2-fold) cell doubling time. The resistant phenotype was unstable and was maintained by weekly treatment of the cells with HN2. Growing the resistant cells in the absence of HN2 resulted in a time-dependent decrease in both resistance to HN2 and topoisomerase II activity and an increase in DNA interstrand crosslinking induced by HN2. We hypothesize that HN2 resistance is due to enhanced monoadduct repair with resultant decreased DNA crosslinking and that this process is mediated by topoisomerase II.**

Historically, HN2 was the first anticancer agent to demonstrate efficacy for human cancer (1, 2). This bifunctional alkylating agent is still in active use and, in particular, has contributed to the cure of Hodgkin disease. One of the consequences of chemotherapy is the development of drug resistance by the treated neoplastic cells. The mechanism of resistance to HN2 is poorly understood. Few human cell lines are available for study because resistance to HN2 is difficult to achieve *in vitro* (3, 4). Recently, a human Burkitt lymphoma cell line (Raji-HN2) was established with a 10- to 20-fold increase in resistance to HN2 compared to the parental Raji cell line (3). The HN2-resistant cells were not cross-resistant to other alkylating agents, demonstrating that specific mechanisms are involved in the resistance to specific alkylating agents. This type of resistance is in contrast to the phenomenon of multidrug resistance, in which cells made resistant (usually several thousand-fold) to one drug or metabolite are also resistant to a broad spectrum of drugs (5).

To further our understanding of the mechanism of resistance to HN2, we investigated the HN2-resistant Raji cells. We found that resistance was associated with increased DNA repair and topoisomerase II [DNA topoisomerase (ATP-hydrolyzing), EC 5.99.1.3] activity.

## MATERIALS AND METHODS

**Cells.** Cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum. Raji-HN2 cells were treated weekly with 10 $\mu$M HN2 to maintain the resistant phenotype. For experiments, cells were incubated with HN2 in serum-free medium for 30 min unless otherwise stated. Because HN2 has a short half-life of $\approx$10 min (2), cells were usually diluted with medium supplemented with 10% fetal bovine serum after HN2 treatment. All cells used were in the exponential growth phase at a density of $1 \times 10^6$ per ml. Cell viability was carefully monitored by trypan blue staining to ensure reproducibility between experiments.

**Clonogenic Assay.** Cell survival was determined by colony formation in soft agarose. After drug treatment, cells were diluted 1:500 into 0.15% low-melting-point agarose (Sea Plaque, FMC, Rockland, ME) prepared in RPMI medium containing 20% fetal bovine serum. Cells were stained with tetrazolium salt (6) 7 days after incubation at 37°C, and colonies were counted in a Biotran counter 2 days later. Each treatment was assayed in triplicate, and the mean value (SEM $\approx$ 10%) was calculated.

**Alkaline Elution.** Labeling of cells with $^{14}$C- or $^{3}$H-labeled thymidine and subsequent analysis of the radioactive DNA were performed as described (7). All of the cells received 300 rads (3 Gy) of $\gamma$ radiation from a Gammacel 40 cesium source (Atomic Energy of Canada, Ottawa) and were collected on membrane filters. After lysis with sodium dodecyl sulfate and treatment with proteinase K, DNA was eluted in the presence of 0.1% sodium dodecyl sulfate. Under these conditions, only DNA interstrand crosslinks were assayed.

**Glutathione Measurement.** Cells were washed and resuspended in phosphate-buffered saline at $1 \times 10^7$ per ml. An aliquot was removed for protein determination (8). Trichloroacetic acid (60%) was added to another aliquot to a final concentration of 6%. The mixture was chilled on ice for 30 min and centrifuged (2000 $\times$ *g* for 10 min). The glutathione content of a 150-$\mu$l aliquot of clarified supernatant was determined by mixing it with 600 $\mu$l of 0.3 M sodium phosphate (pH 8.4) and 150 $\mu$l of 1 mM 5′-dithiobis(2-nitrobenzoic acid). Absorbance of the reaction mixture at 412 nm was measured spectrophotometrically (9). Reduced glutathione was used as a standard.

**Topoisomerase II.** Enzyme activity was assayed by a modification of described procedures (10, 11). Cells ($10^7$) were swollen in 1 ml of hypotonic buffer (5 mM potassium phosphate, pH 7.0/2 mM $MgCl_2$/1 mM phenylmethylsulfonyl fluoride/1 mM 2-mercaptoethanol/0.1 mM EDTA) for 30 min on ice and then disrupted in a Dounce homogenizer (30 strokes). Nuclei were collected by centrifugation (200 $\times$ *g* for 10 min) and resuspended at $3 \times 10^7$ per ml in 5 mM potassium phosphate, pH 7.5/100 mM NaCl/10 mM 2-mercaptoethanol/0.5 mM phenylmethylsulfonyl fluoride. NaCl (5 M) was added to a final concentration of 0.35 M, and the nuclei were mixed gently in a Vortex, incubated on ice for 60 min, and sedimented (1000 $\times$ *g* for 10 min). More than 90% of topoisomerase II activity was recovered in the supernatant

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Abbreviations: HN2, nitrogen mustard [2-chloro-*N*-(2-chloroethyl)-*N*-methylethanamine]; *m*-AMSA, amsacrine [4′-(9-acridinylamino)-methanesulfon-*m*-anisidide].

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fraction. A 5-μl aliquot of the supernatant fraction was mixed with a 5-μl solution containing 100 mM Tris·HCl (pH 7.5), 200 mM KCl, 20 mM $MgCl_2$, 1 mM EDTA, 0.3 μg of bovine serum albumin, 200 ng of bacteriophage P4 DNA, and 2 mM ATP. After incubation at 37°C for 60 min, the DNA was electrophoresed in a 0.7% agarose gel and visualized by staining with ethidium bromide after electrophoresis. Specific activity is expressed as units of enzyme per μg of protein of the nuclear extract, determined by the method of Bradford (8).

## RESULTS

**Properties of Raji Cells.** The resistant cell line, Raji-HN2, had been established after 20 months of intermittent treatment of the parental Raji cells with HN2 (3). Because the resistant phenotype is unstable (3), resistance was maintained by weekly treatment of Raji-HN2 cells with 10 μM HN2. Raji-HN2 cells were ≈10-fold more resistant to HN2 than Raji cells, and their growth rate was 50% slower (Table 1). Increased glutathione levels have been detected in a number of drug-resistant cell lines and may play a role in drug resistance. Raji-HN2 cells also had elevated glutathione levels (Table 1).

**DNA Interstrand Crosslinking.** HN2, a bifunctional alkylating agent, interacts with DNA to form monoadducts which, if not removed, form lethal DNA interstrand crosslinks (2, 12). Therefore, resistance to HN2 could result from reduced crosslinking of Raji-HN2 DNA. DNA interstrand crosslinking was assayed by alkaline elution. From the DNA elution profiles, the crosslink index (a measure of the extent of DNA crosslinking) was calculated (7). Raji-HN2 DNA was crosslinked to a much lower extent than that of Raji cells (Fig. 1). HN2-induced DNA crosslinks have been shown to be repaired rapidly (13). Thus, it was of interest to determine whether resistance to HN2 was due to rapid repair of DNA crosslinks. When Raji and Raji-HN2 cells were treated with nonlethal concentrations of HN2 to produce equivalent DNA crosslinking, both cell lines repaired crosslinks at similar rates 6 hr after HN2 treatment (Table 2). Thus, reduced DNA interstrand crosslink formation and not the rate of crosslink repair correlates with HN2 resistance.

**Topoisomerase II.** Our results on DNA crosslink formation suggested that Raji-HN2 cells are more efficient than Raji cells in monoadduct repair, leading to reduced DNA crosslink formation. Because topoisomerase II is postulated to play a role in DNA repair (14), it was of interest to compare the topoisomerase II activity of Raji and Raji-HN2 cells. Topoisomerase II, extracted from nuclei with 0.35 M NaCl, was assayed by the P4 DNA unknotting assay (Fig. 2). Raji-HN2 cells contained ≈3 times as much enzyme activity as did Raji cells (Table 1).

Resistance of Raji-HN2 cells to HN2 is not permanent (3). When the cells were maintained in the absence of HN2, a time-dependent decrease in resistance to HN2 was observed (Fig. 3). The reduction in resistance was not complete; at 8 weeks in the absence of HN2, the cells remained about 3-fold more resistant to HN2 than Raji cells. A parallel decrease in topoisomerase II activity was observed, to a level comparable to that of Raji cells (Fig. 3). Alkaline elution analysis revealed a parallel increase in DNA crosslinking during the same time period after the placement of Raji-HN2 cells into HN2-free medium.

**Effects of Topoisomerase Inhibitors.** Raji-HN2 cells were 4- to 11-fold more sensitive than Raji cells to the cytotoxic effects of topoisomerase II inhibitors such as amsacrine, etoposide, teniposide, and novobiocin (14–16) (Table 1). The $IC_{50}$ values were only 11–28% of the comparable cytotoxic concentrations required for Raji cells. Raji-HN2 cells were also more sensitive to camptothecin (a topoisomerase I inhibitor), but the difference in sensitivity between Raji and Raji-HN2 cells was far less than that observed for topoisomerase II inhibitors (Table 1). The camptothecin result was not unexpected because we had previously demonstrated that both Raji and Raji-HN2 cells possess a similar amount of extractable topoisomerase I activity (17).

## DISCUSSION

A number of mechanisms have been proposed for the development of resistance to HN2. Studies, mostly with murine cells, have suggested that altered drug uptake, drug detoxification mediated by increased cellular glutathione levels, and increased DNA repair may be involved in HN2 resistance (18). Recent studies with Raji-HN2 cells have ruled out altered HN2 uptake as a mechanism of resistance in this cell system (3). Our results show that the rate of DNA interstrand crosslink repair was equivalent in both Raji and Raji-HN2 cells, making it an unlikely mechanism contributing to HN2 resistance.

Raji and Raji-HN2 cells, exposed to comparable concentrations of HN2, differ markedly in the degree of DNA crosslinking induced by the alkylating agent. Resistance to HN2 by Raji-HN2 cells can be correlated with reduction in DNA crosslink formation. Two mechanisms may be operative: efficient repair of monoadducts before crosslinks can form or detoxification of HN2 by the increased glutathione content of Raji-HN2 cells. Proficient monoadduct repair has

Table 1. Properties of Raji and Raji-HN2 cells

| Properties quantitated | Cells* | | Ratio Raji/ Raji-HN2 |
|---|---|---|---|
| | Raji | Raji-HN2 | |
| $IC_{50}$, μM† | | | |
| HN2 | 1.7 ± 0.2 ($n$ = 8) | 12.3 ± 1.0 ($n$ = 8) | 0.1 |
| *m*-AMSA | 8.3 (8.0–8.5) | 2.3 (2.1–2.5) | 3.6 |
| Etoposide (VP-16) | 22.0 | 3.8 | 5.8 |
| Teniposide (VM-26) | 2.2 (1.6–2.8) | 0.2 (0.2–0.2) | 11.0 |
| Novobiocin | 3400 (3320–3480) | 419 ± 53 ($n$ = 5) | 8.1 |
| Camptothecin | 1.5 | 0.6 | 2.5 |
| Topoisomerase II‡ | 18.5 ± 1.9 ($n$ = 16) | 54.1 ± 5.1 ($n$ = 12) | 0.3 |
| Glutathione§ | 52.7 ± 5.8 ($n$ = 4) | 116.6 ± 6.2 ($n$ = 4) | 0.5 |
| Cell doubling time, hr¶ | 18.0 (16.5–19.5) | 40.4 (38.5–42.3) | 0.4 |

*Mean ± SEM is cited when $n > 2$; in the case of $n = 2$, lower and upper limits are presented.
†Micromolar drug concentration required to inhibit 50% of colony formation was determined by clonogenic assays as described. Cells were treated for 30 min (HN2, novobiocin) or 1 hr (rest) at 37°C.
‡Enzyme activity in units/μg of protein was assayed by the phage P4 DNA unknotting assay.
§Quantity expressed as nmol/mg of total cellular protein.
¶Determined by counting cells in a hemocytometer at intervals over a 100-hr period.

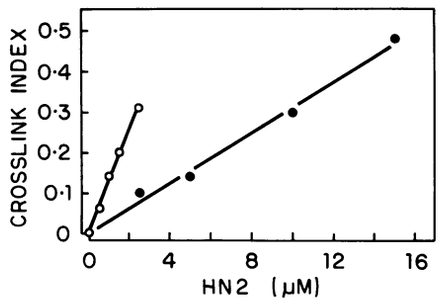


FIG. 1. DNA interstrand crosslinks induced by HN2. Cells treated for 1 hr with HN2 were analyzed by alkaline elution. The crosslink index was calculated from the formula: crosslink index = $\sqrt{(1 - R_0)/(1 - R)} - 1$, where $R_0$ and $R$ are the fractions of DNA retained on the filters of control and HN2-treated cells, respectively (7). ○, Raji; ●, Raji-HN2 cells. The mean values of two independent experiments are presented. Less than 10% variation was obtained.

been shown to be the principal mechanism of resistance to the nitrosourea class of alkylating agents. Human $Mer^+$ cells treated with chloroethylnitrosourea are able to repair $O^6$-alkylguanine monoadducts, whereas $Mer^-$ cells lack this capacity, and their DNA becomes crosslinked in a time-dependent manner (19, 20). Repair of $O^6$-alkylguanine is mediated by DNA-$O^6$-methylguanine methyltransferase (EC 2.1.1.63). HN2 forms predominantly $N^7$-alkylguanine monoadducts (21). An analogous mechanism involving efficient repair of $N^7$-alkylguanine monoadducts would reduce crosslink formation of Raji-HN2 DNA. Human cells have been shown to contain DNA glycosylase activities that excise $N^3$- and $N^7$-alkylguanines but not $O^6$-alkylguanines (22). The existence of specific repair mechanisms for different alkylating agents would explain why Raji-HN2 cells are not cross-resistant to other alkylating agents (3, 4).

Raji-HN2 cells have twice the amount of glutathione present in Raji cells. Elevated glutathione levels have been detected in several drug-resistant and tumor cell lines (23–25). Drug detoxification mediated by glutathione has been proposed as a mechanism of resistance to nitrosourea, *m*-AMSA, and melphalan (25). Resistance to melphalan (a bifunctional amino acid nitrogen mustard) was attributed to a 2-fold increase in glutathione in a melphalan-resistant human ovarian cancer cell line over that in the parental cell line (23). Raji-HN2 cells are not cross-resistant to melphalan or nitrosourea (4) and are hypersensitive to *m*-AMSA (Table 1). Because of this apparent disparity between glutathione content, resistance to HN2, and sensitivity to melphalan, nitrosourea, and *m*-AMSA, it is difficult to associate increased glutathione with HN2 resistance via HN2 detoxification in Raji-HN2 cells. Furthermore, treatment with a noncytotoxic concentration of buthionine sulfoximine (50 μM) to reduce the glutathione content of Raji-HN2 cells to that present in Raji cells did not have any effect on the HN2 killing of these cells (unpublished data).

Table 2. DNA interstrand crosslink repair

| Cells | Crosslink index after HN2 treatment* | |
|---|---|---|
| | 1 hr | 6 hr |
| Raji | 0.27 (0.25–0.28) | 0.12 (0.10–0.14) |
| Raji-HN2 | 0.27 (0.26–0.27) | 0.11 (0.09–0.12) |

*Raji cells were treated with 1 μM HN2 and Raji-HN2 cells were treated with 5 μM HN2 to produce equivalent crosslinking at 1 hr after treatment. Repair of interstrand crosslinks was determined by alkaline elution 6 hr after treatment with HN2. The crosslink index was calculated as described in the legend of Fig. 1. The mean values of two independent experiments are presented together with the lower and upper limits.

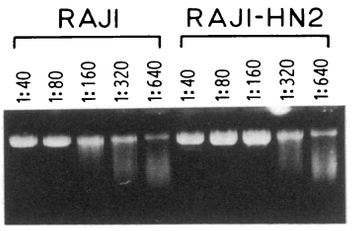


FIG. 2. Phage P4 DNA unknotting assay for topoisomerase II. The 0.35 M NaCl nuclear extract prepared as described was serially diluted (from 1:40 to 1:640) for assay of ATP-dependent unknotting of bacteriophage P4 DNA. One unit of enzyme activity is required to convert 200 ng of DNA running as a smear (knotted DNA) to DNA that runs as a single band (unknotted DNA).

The unique finding of this study is the demonstration of an increase in extractable topoisomerase II activity in HN2-resistant human cells. Preliminary results indicate that the increased enzyme activity was likely due to an increase in enzyme content (unpublished data). Topoisomerase II activity has been reported to be regulated by the proliferative state of mammalian cells in culture. Logarithmic-phase Chinese hamster ovary cells were shown to contain 4 times as much topoisomerase II activity as quiescent cells in the plateau phase (16). Similarly, when guinea pig lymphocytes were stimulated with concanavalin A, a 20-fold increase in topoisomerase II activity was observed over untreated cells (27). These findings are consistent with a major role for topoisomerase II in DNA replication. We find that topoisomerase II activity was significantly greater in the slower growing Raji-HN2 cells than in Raji cells. Since both cells were

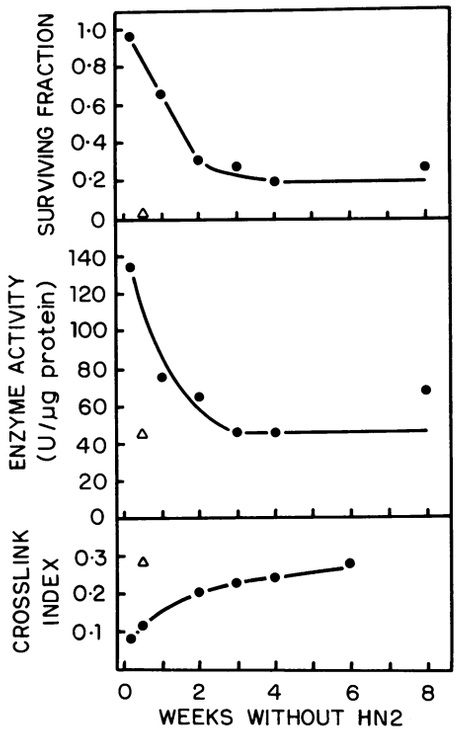


FIG. 3. Effects of growing Raji-HN2 cells in HN2-free medium. Raji-HN2 cells maintained in medium lacking HN2 for the time periods indicated were assayed for colony-forming ability after treatment with 10 μM HN2 (*Top*), and duplicate aliquots were assayed for topoisomerase II activity (*Middle*). The DNA interstrand crosslink index, calculated as described in the legend of Fig. 1, was determined from a separate experiment (*Bottom*). The data presented are representative of several experiments showing similar differences between Raji (△) and Raji-HN2 (●) cells.

studied in the logarithmic phase of growth, our results indicate that topoisomerase II in Raji-HN2 cells may be performing functions other than those related to DNA replication.

To retain their resistant phenotype, Raji-HN2 cells must be treated weekly with HN2. Cells that were initially 10-fold resistant became approximately 3-fold resistant after several weeks in culture in the absence of HN2. This reversion of phenotype was accompanied by a decrease in topoisomerase II activity and an increase in susceptibility to HN2-induced DNA interstrand crosslinking. All three characteristics of the phenotypically reverting cells changed with the same kinetics (Fig. 3). Based on this congruence, we propose that topoisomerase II participates in HN2 resistance by modulating HN2-induced DNA interstrand crosslinking. Although this hypothesis is not the only possible interpretation of our data, it is consistent with what is known about topoisomerase II. In addition to catalyzing changes in DNA linking number, this enzyme has been shown to be a major structural component of the nuclear matrix and chromosome scaffold and, thus, helps to maintain chromosomal DNA in an organized looped conformation (28–30). Evidence for its actual biological function comes from studies with yeast cells, which indicate that topoisomerase II participates in the segregation of newly replicated DNA daughter strands via decatenation-like activity (31, 32). Other roles for eukaryotic topoisomerase II have been suggested; these are based upon indirect evidence and inferences from bacterial genetic studies and include chromatin assembly, gene replication and transcription, and DNA repair (14, 15). The latter role would be consistent with, although not proved by, the data presented here. The increased topoisomerase II activity of Raji-HN2 cells may alter the structure of chromatin so as to increase the accessibility of HN2-induced monoadducts to repair enzymes and, thus, reduce the formation of interstrand crosslinks. In support of this hypothesis, we have demonstrated that the chromatin of Raji-HN2 cells is digested more efficiently by DNase I than that of Raji cells, indicating that the chromatin of these two cell lines is organized differently (17). The involvement of topoisomerase II in DNA repair has been suggested by studies that demonstrate lower topoisomerase II activity in cells from patients suffering from genetic disorders characterized by reduced DNA repair (14, 26, 33).

Two types of topoisomerases have been reported for eukaryotic cells, topoisomerase I and topoisomerase II. Our findings suggest a new role for topoisomerase II in the resistance of human Burkitt lymphoma cells to nitrogen mustard. Topoisomerase I, in contrast, is not associated with HN2 resistance because its activity is the same in both Raji and Raji-HN2 cells (17). The mechanism of resistance to HN2 by Raji-HN2 is obviously complex and may involve interactions between DNA repair enzyme(s), topoisomerase II, chromatin structure, and glutathione. The findings of this study may have important clinical implications and suggest that tumor cells that have developed resistance to HN2 therapy may be susceptible to inhibitors of topoisomerase II.

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