Topoisomerase II Levels and Drug Sensitivity in Adult Acute Myelogenous Leukemia

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The topoisomerase (topo) II-directed agents etoposide, daunorubicin (DNR), and amsacrine (m-AMSA) are widely used in the treatment of acute myelogenous leukemia (AML). In the present study, multiple aspects of topo II-mediated drug action were examined in marrows from adult AML patients. Colony-forming assays revealed that the dose of etoposide, DNR, or m-AMSA required to diminish leukemic colony formation by 90% (LD90) varied over a greater than 20-fold range between different pretreatment marrows. Measurement of nuclear DNR accumulation in the absence and presence of quinidine revealed evidence of P-glycoprotein (Pgp) function in 8 of 82 samples at diagnosis and 5 of 36 samples at first relapse, but the largest quinidine-induced increment in DNR accumulation (<2-fold) was too small to explain the variations in drug sensitivity. Restriction enzyme-based assays and sequencing of partial topo IIa and topo IIb cDNAs from the most highly resistant specimens failed to demonstrate topo II gene mutations that could account for resistance. Western blotting of marrow samples containing greater than 80% blasts revealed that the content of the two topo II isoenzymes varied over a greater than 20-fold range. However, in addition, levels of topo IIa and topo IIb in 48 of 47 clinical samples were lower than in human AML cell lines. Immunoperoxidase staining showed that these low topo II levels were accompanied by marked cell-to-cell heterogeneity, with topo IIa being abundant in some blasts and diminished or absent from others. There was a trend toward increasing percentages of topo IIa-positive cells in pretreatment marrows that contained more S-phase cells. Consistent with this observation, treatment of patients with granulocyte-macrophage colony-stimulating factor for 3 days before chemotherapy resulted in increases in topo IIa-positive cells concomitant with increases in the number of cells traversing the cell cycle. These observations have implications for the regulation of topo II in AML, for the use of topo II-directed chemotherapy, and for future attempts to relate drug sensitivity to topo II levels in clinical material.

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THE NUCLEAR ENZYME topoisomerase II (topo II) is a major target for a variety of antineoplastic agents, including epipodophyllotoxins, aminocodines, anthracyclines, and anthracenediones.5-7 These agents allow topo II to cleave DNA but slow the religation steps catalyzed by the enzyme.6 As a consequence, covalent topo II-DNA adducts accumulate. These adducts set into motion a series of events that culminate in cell death.5,8-10

Topo II-directed agents play a major role in the treatment of acute myelogenous leukemia (AML).10,15 Induction regimens commonly contain an anthracycline or anthrancenedione.10,11,13,14 Recently developed regimens also include amscarine (m-AMSA)12,16 or etoposide.14,15 These treatments induce complete responses in 50% to 70% of patients with newly diagnosed AML.10,11,13,16 The biochemical factors that determine the salutary response of some patients and not others remain largely unknown.

A number of factors that affect cytotoxicity of topo II-directed agents have been identified in model systems.6,7,9,17-32 One important factor is the cellular content of topo II.19-21,23-27,30 Cells containing less topo II form fewer topo II-mediated strand breaks and are less sensitive to topo II-directed agents; and cells containing more topo II form more enzyme-mediated strand breaks and are more sensitive.19-21,24-27,30

Initial studies indicated that topo II levels are closely linked to proliferative status.19-21,33-37 More recently, mammalian cells were shown to contain two drug-sensitive topo II isoenzymes.38 topo IIα (relative molecular weight [Mr] ~170,000) and topo IIβ (Mr ~180,000),38,39 which are products of two different genes.39,40 In certain model systems, these two isoenzymes appear to be differentially regulated, with topo IIα decreasing and topo IIβ increasing as cells become quiescent.38,41,42 However, in normal mammalian lymphohematopoietic cells, expression of both isoenzymes appears to be linked to proliferation. Topo II activity34,37 and levels of both topo II polypeptides36,37,43 increase sharply in lymphocytes after mitogen treatment. Conversely, both topo II isoenzymes decrease markedly when cells stop proliferating during the course of granulocytic maturation.43

Several studies suggest that this relationship between topo II content and cell proliferation might be altered in tumor cells. Topo II activity and topo IIα polypeptide have been detected in plateau-phase L1210 murine leukemia cells19,44 and in confuent HeLa human cervical carcinoma cells,45 for example, but not in a variety of nontransformed G0 cells.19-21,33,35,43-45

Despite these results in model systems, there have been relatively few studies of topo II levels in human malignan-

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Submitted May 10, 1993; accepted September 21, 1993.

Supported in part by National Institutes of Health Grant No. CA 50435, by the American Cancer Society (Maryland Division), and by an American Cancer Society Clinical Oncology Career Development Award to S.H.K. S.H.K. and R.J.J. are Leukemia Society America Scholars.

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0006-4971/94/8302-002253.00/0
cies.\(^ {46-50}\) Topo II polypeptide\(^ {47-50}\) and topo II activity\(^ {48}\) were readily detected in samples of aggressive lymphoma and acute lymphocytic leukemia. In clinical specimens of AML, on the other hand, etoposide-induced DNA strand breaks\(^ {46,51}\) and topo II polypeptide levels\(^ {46}\) were markedly diminished when compared with human acute leukemia cell lines. The etiology of the decreased topo II polypeptide levels in clinical AML samples was not addressed in previous studies. Likewise, the possibility that P-glycoprotein (Pgp) overexpression\(^ {52-55}\) might contribute to the decrease in etoposide-induced strand breaks was not examined.

In the present study, freshly isolated marrow samples from newly diagnosed adult AML patients were examined for their sensitivity to topo II-directed agents using colony-forming assays\(^ {56,57}\). In agreement with earlier studies,\(^ {51,58-65}\) these assays revealed that the dose required to diminish leukemia colony formation by 90% (LD\(_ {90}\)) for daunorubicin (DNR), m-AMSA, or etoposide varied over a 22,000-fold range. Assays of nuclear DNR accumulation failed to show that Pgp activity could account for these sensitivity differences. When other aspects of topo II-mediated drug action were examined, topo II levels were observed to vary widely between different leukemic marrow samples, but no correlation between topo II levels and drug sensitivity in vitro or in vivo could be shown. Instead, immunoperoxidase staining revealed marked cell-to-cell heterogeneity of topo IIa content that appears to reflect, at least in part, cell cycle variations in topo II expression. This heterogeneity must be taken into account when designing future studies to investigate the relationship between topo II levels and drug sensitivity in clinical material.

**MATERIALS AND METHODS**

**Materials**

Fluorescein-conjugated anti-BUDR, chicken erythrocytes, and bovine thymocytes were from Becton Dickinson (Mountain View, CA). Sources of all other reagents were previously identified.\(^ {44,45}\)

**Buffers**

Alkylation buffer contained 6 mol/L guanidine hydrochloride, 250 mmol/L Tris-HCl (pH 8.5 at 21°C), 10 mmol/L EDTA, 1% (vol/vol) β-mercaptoethanol, and 1 mmol/L freshly added phenylmethylsulfonyl fluoride (PMSF).\(^ {43}\) Phosphate-buffered saline (PBS) consisted of 137 mmol/L NaCl, 2.7 mmol/L KC1, 1.5 mmol/L KH\(_ 2\)PO\(_ 4\), and 8 mmol/L Na\(_ 2\)HPO\(_ 4\). Medium A contained RPMI 1640 medium, 10% (vol/vol) heat-inactivated fetal bovine serum, 100 U/mL penicillin G, 100 μg/mL streptomycin, and 2 mmol/L glutamine. Buffer B consisted of RPMI 1640 medium and 10 mmol/L HEPES (pH 7.4). Buffer C contained 145 mmol/L NaCl, 1.0 mmol/L KH\(_ 2\)PO\(_ 4\), 6.0 mmol/L Na\(_ 2\)HPO\(_ 4\), and 1% (wt/vol) bovine serum albumin. Buffer D consisted of 0.5% (wt/vol) Tween 20 in buffer C.

**Marrow Samples and Tissue Culture Cells**

Bone marrow aspirates from the posterior iliac crest were harvested before chemotherapy on protocols approved by the Joint Committee on Clinical Investigation of the Johns Hopkins Medical Institutions in accordance with policies of the US Department of Health and Human Services. Marrow mononuclear cells were isolated by ficoll-Hypaque sedimentation.\(^ {44}\) Differential counts were performed on Wright's-stained cytopsins. Marrow samples for diagnostic cytochemistry, cell surface markers, and karyotype analysis were simultaneously procured. Leukemias were classified according to the French-American-British (FAB) scheme.\(^ {66}\) Logarithmically growing HL-60 and KG1a human AML cells cultured in medium A were harvested by sedimentation on ficoll-Hypaque step gradients.\(^ {45}\)

**Colony-Forming Assays**

Aliquots containing 1 × 10\(^ 6\) T-cell-depleted marrow mononuclear cells\(^ {56,57}\) were incubated with increasing concentrations of DNR (0.02 to 2 μmol/L), m-AMSA (0.1 to 10 μmol/L), or etoposide (20 to 200 μmol/L) for 1 hour and washed. Quadruplicate aliquots containing 2 × 10\(^ 3\) cells were plated in 1 mL α-minimal essential medium (α-MEM) containing 1.32% (wt/vol) methylocellulose, 30% (vol/vol) fetal bovine serum, 1% (wt/vol) bovine serum albumin, 10% (vol/vol) conditioned medium from human mononuclear leukocytes stimulated with phytohemagglutinin for 7 days, and 100 μmol/L β-mercaptoethanol. Leukemic colony-forming units (CFU-L) were quantitated on days 3 through 6. Previous results have indicated that cells in these colonies have the genetic and morphologic features of the bulk leukemia from which they are derived.\(^ {56,57,61}\)

**Nuclear DNR Accumulation**\(^ {57,68}\)

To assess DNR accumulation in nuclei of intact cells, samples were incubated for 15 minutes at 37°C with 17.7 μmol/L DNR in medium A containing 10 mmol/L HEPES (pH 7.4) and 100 μmol/L quinidine (added from a 100 mmol/L stock in dimethylsulfoxide [DMSO]), sedimented at 3,200g for 1 minute, incubated for 20 minutes at 37°C in buffer B containing 100 mmol/L quinidine, sedimented, resuspended in PBS containing 100 μmol/L quinidine, and immediately fixed by the addition of formaldehyde to a final concentration of 3.7% (wt/vol). Duplicate aliquots were treated identically except that quinidine was replaced by 0.1% (vol/vol) DMSO. Flow cytometry was performed as previously described.\(^ {49}\) Samples displaying a ≥20% increase in nuclear DNR accumulation in the presence of quinidine were considered to have evidence of Pgp function.

**Electrophoresis and Western Blotting**

Marrow mononuclear cells were washed in buffer B and solubilized by sonication in alkylation buffer.\(^ {44}\) Sample preparation, electrophoresis, and Western blotting were performed as described\(^ {57,64}\) using rabbit serum IIa, which recognizes both isoforms of topo II.\(^ {49}\) The resulting autoradiographs were digitized at high magnification (5 to 6 gel lanes/screen). The signal for each topo II isoform (area × intensity) was compared with a standard curve of HL-60 cells, as previously described.\(^ {43}\) The reproducibility of this method was assessed by reacting duplicate blots with anti-topo II antiseraum and digitizing the blots independently. Comparison of the signals obtained on these blots showed a correlation coefficient (R) of .99. To correct for differences in loading, blots were subsequently reacted with an IgM monoclonal antibody to histone H1, a protein that is proportional to DNA content.

**Analysis for Potential Topo II Gene Mutations**

To assay for a G-A base change at position 1493 of the topo II gene,\(^ {49}\) genomic DNA\(^ {3}\) from marrow mononuclear cells was amplified by polymerase chain reaction (PCR) using primers that encompass nucleotides (nt) 1395-1628. The product was digested with
were enrolled in a pilot study that examined recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; IV TOPOISOMERASE II IN AML 519

ture or a regular RNA/PCR kit (Perkin-Elmer, Norwalk, CT) and separate primer pairs (24mer to 27mer) that encompassed the putative ATP binding B regions (topo IIα, nt 1246-1539; topo IIβ, nt 897-1206) or active-site tyrosine regions (topo IIα, nt 2049-2648; topo IIβ, nt 1641-2264). After two isopropanol precipitations, the PCR products were sequenced using the Circumvent sequencing kit (New England Biolab, Beverly, MA) and analyzed on a denaturing polyacrylamide sequencing gel.

Immunoperoxidase Staining for Topo IIα

Affinity-purified antibodies against topo IIα were used for immunoperoxidase staining as previously described, except that incubation with the primary antibody was shortened to 12 to 16 hours at 4°C. Three hundred to 800 cells in each sample were examined. Cytospins containing a mixture of HL-60 cells and peripheral blood granulocytes were included in each experiment to provide positive and negative controls, respectively. Preliminary experiments showed that these control cytospins maintained their immunoreactivity for ≥4 weeks.

Cell Cycle Analysis

Marrow mononuclear cells were incubated with 100 μmol/L BUdR for 1 hour at 37°C in RPMI 1640 buffer containing 15% (vol/vol) autologous serum, 2 mmol/L glutamine, and 20 μg/mL gentamycin. Cells were washed twice in buffer C and fixed for 30 minutes in 70% (vol/vol) ethanol at −20°C. All further steps were performed at 20°C to 22°C. Cells were incubated with 2 N HCl containing 0.5% (wt/vol) Triton X-100 for 30 minutes, washed with 0.1 mol/L sodium tetraborate followed by buffer D, reacted for 30 minutes with fluorescein-labeled monoclonal anti-BUdR in buffer D, washed once in buffer D, and treated with 17 μg/mL propidium iodide in buffer C. Flow cytometry was performed on a Becton Dickinson FACScan flow cytometer equipped with R-Fit analysis software. Calibrations were performed using chicken erythrocyte and calf thymocyte nuclei. Logarithmically growing KG1a cells were used as a positive control for BUdR incorporation in all experiments.

Patient Treatment

Protocol 8410. Unless otherwise indicated, patients with newly diagnosed AML received cytosine arabinoside (Ara-C; 667 mg/m²/d by continuous infusion, days 1 through 3), DNR (45 mg/m²/d intravenously [IV] bolus, days 1 through 3), and m-AMSA (200 mg/m²/d IV over 4 hours, days 4 through 6), and etoposide (400 mg/m²/d over 6 hours, days 1 through 13). In conjunction with this study, topo IIα staining was examined in 14 paired marrow samples, the first harvested before initiation of GM-CSF treatment and the second harvested immediately before administration of cytotoxic chemotherapy. Each of the 14 patients studied received 5 μg/kg GM-CSF.

Data Analysis

To avoid excluding leukemic samples from patients with antece- dent myelodysplastic syndromes (a group reported to have a high frequency of Pgp expression), DNR accumulation studies were performed on all leukemic samples. Western blotting was also performed prospectively on all samples, but samples containing less than 80% blasts + progranulocytes + myelocytes were excluded from analysis. Samples used for alkaline unwinding assays contained greater than 90% blasts. Linear regressions and Student's t-tests were performed using a microcomputer equipped with an Instat2 program (GraphPad Software, San Diego, CA).

RESULTS

Patient Population

Factors that might affect response to topo II-directed agents were analyzed prospectively in bone marrow aspirates from adult patients with newly diagnosed, relapsed, or

Table 1. AML Patient Population Analyzed

<table>
<thead>
<tr>
<th>Samples from patients with newly diagnosed AML</th>
<th>No. of Samples</th>
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<tbody>
<tr>
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</tr>
<tr>
<td>M0</td>
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</tr>
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<td>Refractory</td>
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</tr>
<tr>
<td>Paired samples available†</td>
<td>18</td>
</tr>
</tbody>
</table>

*Classification of AML according to the FAB system in newly diagnosed AML samples analyzed.
† Treated with Ara-C, DNR, and m-AMSA on protocol 8410.16
‡ Treated with GM-CSF, Ara-C, DNR, and etoposide on protocol 8914. Responses of these patients were not considered in Fig 7.
§Treatment with Ara-C, DNR, and etoposide using the same schedule as protocol 8914, but without GM-CSF. Responses of these patients were not considered in Fig 7.
¶† For the indicated number of patients, samples were analyzed before antileukemic therapy and again at the time of recurrence.

by continuous infusion, days 1 through 13) in conjunction with Ara-C (667 mg/m²/d continuous infusion, days 4 through 6), DNR (45 mg/m²/d IV bolus, days 4 through 6), and etoposide (400 mg/m²/d over 6 hours, days 1 through 13). In conjunction with this study, topo IIα staining was examined in 14 paired marrow samples, the first harvested before initiation of GM-CSF treatment and the second harvested immediately before administration of cytotoxic chemotherapy. Each of the 14 patients studied received 5 μg/kg GM-CSF.
refractory AML treated at a single institution between September 1, 1987 and December 31, 1992 (Table 1). Specimens adequate for one or more assays were obtained from 140 of 182 patients with newly diagnosed AML admitted during this period. Marrow aspirates were also obtained at the time of leukemic regrowth in patients with refractory (17 patients) or relapsed (40 patients) disease, 36 of whom were studied at the time of diagnosis. These samples provided an opportunity to compare pretreatment and clinically resistant blast populations.

Patients with newly diagnosed AML received therapy that contained the antimetabolite Ara-C and two topo II-directed agents, m-AMSA and DNR (107 patients on protocol 8410) or etoposide and DNR (8 patients on protocol 8914, 8 patients on Ara-C/DNR/etoposide individual therapy without GM-CSF).

Of 94 evaluable patients treated on protocol 8410, 57 (61%) achieved a CR, including 43 of 68 patients with de novo leukemia (CR = 63%), 8 of 14 patients whose leukemia evolved after an antecedent hematologic disorder (AHD; CR = 57%), and 6 of 12 patients with chemotherapy-related leukemia (CR = 50%). The present studies were undertaken to search for biochemical factors that might distinguish patients who achieve a CR with this topo II-weighted therapy from those who do not.

Sensitivity to DNR, m-AMSA, or Etoposide

Clonogenic assays (Fig 1) revealed that blasts from different individuals with newly diagnosed AML had inherent differences in sensitivity to topo II-directed agents. Figure 1A shows the results obtained after exposure of two leukemic marrows to DNR for 1 hour. The LD₉₀ was 0.07 µmol/L in one marrow and greater than 1 µmol/L in the other. Data from 22 additional samples showed that the LD₉₀ for DNR varied from 0.04 µmol/L to greater than 1 µmol/L (Fig 1D). Likewise, the LD₉₀ for m-AMSA (Fig 1B and E) and etoposide (Fig 1C and F) varied over a greater than 20-fold range. Similar variation in sensitivity to DNR, m-AMSA, and etoposide has been previously reported.

Formation of Etoposide-Stabilized DNA Strand Breaks

To begin to assess potential explanations for these differences in drug sensitivity, the formation of topo II-mediated DNA strand breaks was examined in 10 samples of newly diagnosed AML using an alkaline unwinding assay (Fig 2).

Assessment of Pgp Phenotype

This paucity of topo II-mediated strand breaks might reflect either diminished drug accumulation (eg, caused by the action of Pgp) or low levels of topo II in the clinical specimens. To evaluate the former possibility, accumulation of DNR in nuclei of marrow mononuclear cells was examined in the absence and presence of the Pgp modulator quini-

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A technique that measures drug accumulation in nuclei rather than whole cells was used in these studies to eliminate the potential confounding effect of increased cytosolic drug sequestration that can occur in Pgp-expressing cells. Controls demonstrated that Pgp-expressing CH1C5 cells, which show a 40-fold decrease in DNR LD₁₀₀ in the presence of quinidine, display a 20-fold increase in nuclear DNR accumulation (Fig 3A). Likewise, Pgp-expressing KG1a human AML cells, which show a threefold decrease in DNR LD₁₀₀ in the presence of quinidine, display a twofold increase in nuclear DNR accumulation (Fig 3B). In contrast, HL-60 cells, which do not express Pgp, show no change in DNR accumulation in the presence of quinidine (Fig 3C).

Results obtained in 131 AML marrow samples are summarized in Fig 3D through F. Quinidine did not detectably increase DNR accumulation in the majority of clinical samples (Fig 3D and E). Only 8 of 82 specimens from newly diagnosed patients showed a detectable quinidine-induced increase in DNR accumulation, the largest increment being the 60% increase shown in Fig 3F. Consistent with previous reports, 4,5,7,11 of the 8 positive samples were from the 26 patients with an AHD or secondary leukemia. Interestingly, detectable Pgp function did not appear to affect clinical outcome in evaluable patients (CR rate 57% [4 of 7] if assay positive; CR rate 58% [34 of 59] if assay negative).

Serial studies revealed evidence of Pgp function at the time of disease recurrence in samples from 2 of 18 patients (11%) whose cells were initially negative by this assay. Likewise, 3 of 18 samples (17%) from other patients in first relapse and 1 of 14 samples (7%) from patients in subsequent relapses had detectable quinidine-induced increments in DNR accumulation. Once again, all changes in nuclear DNR accumulation were less than twofold.

In summary, the small size of the quinidine-induced changes in DNR accumulation (Fig 3F) and the lack of these changes (Fig 3D and E) in highly resistant clinical specimens (upper curves, Fig 1A and B) suggest that Pgp-mediated differences in drug accumulation do not explain the sensitivity differences depicted in Fig 1.

Topo II Polypeptide Content in Newly Diagnosed AML

To assess the possibility that quantitative changes in topo II contribute to the paucity of etoposide-induced DNA strand breaks observed in Fig 2, topo II content was examined by Western blotting (Fig 4). Qualitative examination of the resulting blots leads to several observations. First, both topo II isoforms were present in most samples in similar amounts. Only rare samples (eg, Fig 4A, lanes 10 and 20) had a predominance of one isoform. Second, topo II content varied widely between samples (Fig 4A, lanes 11 and 12), spanning a greater than 10-fold concentration range when samples were compared with a serial dilution of HL-60 cells (Fig 4A, lanes 3 through 7). Third, topo II levels were substantially lower in most leukemic marrows (Fig 4A, lanes 8 through 27) than in an equal number of HL-60 cells (Fig 4A, lanes 7 and 28).

To provide a more quantitative assessment, the autora-diographic signals for topo IIα and topo IIβ in each sample were quantitated by high resolution densitometry (see the Materials and Methods). To correct for loading, blots were probed with an antibody to histone H1 (Fig 4B). From this point on, samples containing less than 80% blasts plus promyelocytes + myelocytes were excluded from analysis.

Correlation Between Levels of Topo IIα and Topo IIβ

In the clinical specimens, the relative levels of topo IIα and topo IIβ ranged from less than 5% (undetectable) to approximately 140% of the amounts found in an equal number of HL-60 cells (Fig 5A). Moreover, the levels of topo IIα and topo IIβ appeared to be strongly correlated (R = .87, P < .0001). Because this correlation seemed to conflict with earlier reports suggesting that the two topo II isoenzymes are differentially regulated in tissue culture cells, 38,41,42 control experiments were performed to confirm the identity of the M₁ ~170,000 and M₂ ~180,000 bands. Duplicate blots containing three samples with a predominance of the M₁ ~170,000 polypeptide (Fig 5B, lanes 1 through 3) and three samples with approximately equal amounts of the M₁ ~170,000 and M₂ ~180,000 polypeptides (Fig 5B, lanes 4 through 6) were probed with isoform-specific antisera. Topo IIα (Fig 5D) was detected in all of the samples, whereas topo IIβ (Fig 5C) was detected only in those samples that contained the M₂ ~80,000 polypeptide (Fig 5B, lanes 4 through 6). These results are consistent with the identifica-
Fig 3. Assessment of nuclear DNR accumulation by flow cytometry. Cells were treated with DNR, washed, fixed, and examined by flow cytometry as described in Materials and Methods. Control experiments (not shown) confirmed that the fluorochrome was localized exclusively in nuclei.27 Duplicate samples were prepared in the absence and presence of 100 μmol/L quinidine. (A) Hamster CH5C5 cells that contain an amplified mdr1 gene. Control experiments with this cell line demonstrated that the effect of quinidine was half-maximal at 3 to 5 μmol/L (data not shown).27 (B) Human KG1a cells that express low but detectable amounts of mdr1 message.66 (C) HL-60 cells that do not express detectable mdr1 message.66 (D through F) Samples of newly diagnosed AML. Cytotoxicity curves for samples in (D) and (E) are presented in Fig 1 A and B, respectively.

Topo II Polypeptide Content at Diagnosis and Relapse

To assess the possibility that topo II polypeptide content might be an intrinsic feature of each leukemic cell population, samples obtained at diagnosis and at relapse were analyzed on adjacent wells of sodium dodecyl sulfate (SDS)-polyacrylamide gels. The results (Fig 6) indicate a strong correlation between topo II levels at diagnosis and at relapse (R = .77, P = .02 for topo IIα; R = .81, P = .008 for topo IIβ), suggesting that topo II content might be a feature of the blast population that is relatively constant over time.

Relationship of Topo II Polypeptide Content and Drug Sensitivity

In view of the correlation between topo II levels and drug sensitivity in model systems (see Introduction), the relationship between topo II polypeptide content and response of newly diagnosed patients to therapy on protocol 8410 was examined. Despite the fact that two of the three agents used in this treatment were topo II-directed, there was no correlation between topo II content and clinical response (Fig 7). Additional analysis (not shown) also failed to show any relationship between topo II content and remission duration.

Inclusion of the antimetabolite Ara-C in protocol 8410 might have obscured an underlying relationship between topo II level and sensitivity to topo II-directed agents in vivo. To eliminate this confounding variable, the relationship between topo II content and sensitivity to DNR (Fig 8A and B) or m-AMSA (Fig 8C and D) in vitro was examined. Once again, no correlation was evident.

Lack of Evidence for Mutated Topo II in Resistant Patient Samples

One possible factor that could obscure the anticipated correlation between topo II level and drug sensitivity would be the presence of mutations in the genes encoding topo II. Tissue culture cell lines that contain abundant levels of topo II but are insensitive to one or more topo II-directed agents have been described.69,74,78 These cell lines contain mutations in the ATP-binding B or gyrase (active site tyrosine) domains of topo IIα.69,74,78 Because patients in this study received m-AMSA and DNR, we initially searched for a G-A mutation at position 1493 of the topo IIα gene that arose independently in two intercalator-resistant HL-60 cell lines.69,74,78 Genomic DNA from marrows of 23 AML patients (26 samples) was amplified by PCR using primers that encompass the nucleotide of interest and then digested with Mse I, a restriction enzyme that cleaves the amplified DNA if the mutation is present.69 This analysis was performed on 7 samples obtained at the time of diagnosis in patients who subsequently failed to achieve a CR on protocol 8410; 13 samples obtained at the time of disease recurrence in refrac-
Fig 4. Detection of topo II by Western blotting. Samples containing $5 \times 10^5$ marrow mononuclear cells (lanes 8 through 27) were separated by SDS-PAGE and stained with Coomassie blue (upper panel) or transferred to nitrocellulose and reacted with antiserum that recognizes both topo II isoforms (A). To provide a standard curve, dilutions of HL-60 cells (0.25 $\times$ $10^6$, 0.5 $\times$ $10^6$, 1.25 $\times$ $10^6$, 2.5 $\times$ $10^6$ and 5 $\times$ $10^6$ in lanes 3 through 7, respectively) were included on each blot. After autoradiography, the blot was reacted with antihistone H1 (B).

Fig 4. Detection of topo II by Western blotting. Samples containing $5 \times 10^5$ marrow mononuclear cells (lanes 8 through 27) were separated by SDS-PAGE and stained with Coomassie blue (upper panel) or transferred to nitrocellulose and reacted with antiserum that recognizes both topo II isoforms (A). To provide a standard curve, dilutions of HL-60 cells (0.25 $\times$ $10^6$, 0.5 $\times$ $10^6$, 1.25 $\times$ $10^6$, 2.5 $\times$ $10^6$ and 5 $\times$ $10^6$ in lanes 3 through 7, respectively) were included on each blot. After autoradiography, the blot was reacted with antihistone H1 (B).

To search for additional topo II mutations that have been observed in drug-resistant cell lines, RNA from six clinical AML samples with the appropriate phenotype (m-AMSA LD$_{50}$ $\geq$ 10 $\mu$mol/L in vitro) was subjected to reverse transcription PCR using primers that span the ATP B and gyrase domains of topo IIa and topo IIb. These domains encompass all currently identified mutations resulting in this phenotype. Sequencing of the cDNA obtained from these clinical samples showed only wild-type topo IIa and topo IIb sequences. There was no evidence for the mutations observed in resistant cell lines.

*Heterogeneity of Topo II Expression in AML Cells*

Another factor that could obscure any underlying correlation between topo II content and drug sensitivity is cell-to-cell heterogeneity of topo II expression. The analysis presented in Figs 7 and 8 was based on the assumption that a low signal on a Western blot reflects relatively low topo II content in each cell in the population. To evaluate this assumption, marrow mononuclear cells from 40 newly diagnosed AML patients were stained with affinity-purified anti-topo IIa antibodies. In proliferating human AML cell lines, every cell stained (Fig 9B). In contrast, expression of topo IIa in the clinical samples was heterogeneous, with a strong signal in some blasts and no signal in others (Fig 9D, F, and H). Among the 20 stained marrows that contained greater than 80% blasts, the percentage of topo IIa-positive cells varied from less than 1% to 40%.

To determine whether this heterogeneity reflects differences in cell cycle distribution, the relationship between the percentage of topo IIa-positive cells and the percentage of S cells was examined. These two pieces of data were available for 11 samples that contained greater than 80% blasts. The frequency of topo IIa-positive cells tended to increase as the number of cycling cells increased (Fig 10), but this trend did
not reach statistical significance in this small sample ($R = .46, P = .20$). In addition, it was noted that the number of topo IIα-positive cells was often larger than the number of S phase cells (Fig 10), as one would expect for an antigen that is present through other phases of the cell cycle in cycling cells.$^{53,79}$

**Increased Topo IIα Expression After GM-CSF Treatment**

To further evaluate the relationship between topo IIα and cell proliferation, we examined marrow samples from newly diagnosed and relapsed AML patients who were treated with GM-CSF for 3 days before chemotherapy on protocol 8914. Paired samples, the first harvested before GM-CSF treatment (day 1) and the second harvested before cytotoxic chemotherapy (day 4), were stained for topo IIα. The number of positive cells on day 4 increased to greater than 150% of day 1 values in 6 of 14 (43%) patients. If analysis is restricted to samples containing greater than 80% blasts, the number of topo IIα-positive cells on day 4 increased to greater than 150% of day 1 values in 3 of 4 cases (eg, Fig 9J and L). BUDR incorporation studies (available in 3 of these last 4 cases) revealed that the increase in topo IIα occurred concomitant with an increase in the percentage of S phase cells to greater than 150% of control values in 2 of 2 cases. In contrast, topo IIα expression failed to increase in the patient in whom the percentage of S-phase cells did not change. These results are again consistent with the view that heterogeneity of topo IIα expression reflects, at least in part, differences in cell cycle traverse.

**DISCUSSION**

The present study indicates that blasts from different AML patients vary in sensitivity to topo II-directed agents (Fig 1), that “average” topo II content varies between different AML marrow (Figs 4 and 5), that part of this variation arises from cell-to-cell heterogeneity of topo II expression (Fig 9), and that the percentage of blasts expressing topo II increases after GM-CSF treatment in some patients (Fig 9). All of these observations represent positive findings. Nonetheless, the correlation between topo II content and sensitivity to topo II-directed agents previously observed in model systems (see above) was not detectable in the present study (Figs 7 and 8). Our results suggest that cell-to-cell heterogeneity in the clinical samples might have obscured any underlying relationship between topo II content and drug sensitivity. This cell-to-cell heterogeneity has several important implications.

**Differences in Sensitivity to Topo II-Directed Agents In Vitro**

Before addressing the relationship between topo II expression and drug sensitivity, it is important to consider whether the clonogenic assays are measuring true differences in sensitivity to topo II-directed agents. Previous studies from our laboratories using the same colony-forming assay have indicated a strong correlation between sensitivity to 4-hydroperoxycyclophosphamide in vitro and clinical response to cyclophosphamide-containing therapy.$^{57}$ A strong correlation between sensitivity to Ara-C in vitro and response to Ara-C-containing therapy has also been observed using the same or similar assays (our unpublished results).$^{69,74,80}$ These observations suggest that the clonogenic assay is measuring intrinsic sensitivity differences$^{81}$ that have potential therapeutic importance.

The effect of some of these sensitivity differences might be difficult to discern in the context of multiagent chemotherapy. Each patient in the present study received Ara-C, an agent that is active and potentially curative in AML.$^{10,11}$ The fact that Ara-C sensitivity in vitro correlates with response to protocol 8410 (our unpublished results), but sensitivity to topo II-directed agents does not (Fig 1; for similar results, see McCulloch et al$^{60}$ and Marie et al$^{81}$) suggests that Ara-C sensitivity plays a dominant role in determining response to the regimen in question. However, these results do not
preclude the possibility that differences in sensitivity to topo II-directed agents might have a greater impact when patients are treated with other regimens. Consistent with this possibility, a correlation between anthracycline sensitivity in vitro and clinical response has been observed by other investigators.39-64

**Pgp Function in Newly Diagnosed AML**

A dominant role for Ara-C in determining clinical outcome to protocol 8410 might explain the lack of correlation between topo II levels and response in vivo (Fig 7) but not the lack of correlation between topo II levels and drug sensitivity in vitro (Fig 8). One potential explanation for our failure to observe the anticipated correlation in vitro would be the overexpression of Pgp, a transporter of DNR, m-AMSA, and etoposide,67,82,85 in some of the clinical samples. Consistent with this possibility, Pgp expression has been detected in some samples of newly diagnosed or relapsed AML using sensitive flow microfluorimetry or molecular biology techniques.52,55,71,84-86

In the present study, Pgp function was assessed by measuring nuclear DNR accumulation in the absence and presence of quinidine. Control experiments using Pgp-expressing CHF5C5 and KG1a cell lines68 confirmed a correlation between enhanced DNR accumulation and enhanced cytotoxicity in the presence of quinidine. When this assay was applied to clinical specimens, quinidine-induced increases in DNR accumulation were observed in 8 of 82 samples harvested before chemotherapy and 5 of 36 samples harvested at first relapse. However, it does not appear that Pgp expression is obscuring an underlying relationship between topo II levels and drug sensitivity. The largest quinidine-induced change observed in the AML samples, a 60% increase in nuclear DNR accumulation (Fig 3F), is similar to that observed by other investigators,66,89 but much too small to account for the 50-fold differences in DNR sensitivity observed in vitro (Fig 1A).58,62,63 These considerations suggest that one or more additional factors contribute to variations in drug sensitivity.

**Lack of Evidence for Mutated Topo II in Resistant Clinical Specimens**

The presence of mutations that alter the ability of topo II to form drug-stabilized cleavage complexes69,74-78 could also obscure any relationship between topo II levels and drug sensitivity. To assess this possibility, a restriction digest-based assay was used to examine samples of refractory/relapsed AML for a topo IIα mutation that arose in two m-AMSA-resistant HL-60 cell lines.69,76 In addition, cDNAs for topo IIα and topo IIβ regions encompassing all previously reported drug-resistant topo II mutations were isolated from six highly resistant clinical AML specimens and sequenced. None of these experiments provided any evidence for topo II gene mutations. Taken together with the results of Danks et al.,78 who recently described the lack of mutations in the ATP B and gyrase domains of topo IIα in specimens from relapsed ALL patients, these observations suggest that topo II mutations probably did not obscure an underlying relationship between topo II levels and drug sensitivity.

**Heterogeneity of Topo II Polypeptide Content**

A correlation between topo II levels and drug sensitivity has been anticipated in clinical material because of studies demonstrating this relationship in yeast and tissue culture cells (see Introduction). In studies of these model systems, topo II mRNA or topo II polypeptide content was measured in large, actively cycling populations and assumed to accurately reflect the content of individual cells. This assumption is probably valid in genetically and phenotypically homogeneous tissue culture cell lines, in which topo II content varies within fairly narrow confines in actively cycling cells,43,79 but our results suggest that the same approaches might not be valid in clinical AML samples. Staining of topo IIα in these clinical specimens (Fig 9) revealed marked cell-to-cell heterogeneity that has several potential implications.

This heterogeneity has implications for models of topo II regulation in neoplastic cells. Previous reports suggested that topo II levels diminished when nontransformed tissue culture cells entered G0,30,31 but remained high when L1210 mouse leukemia cells46 or HeLa human cervical carcinoma cells46 reached plateau phase. These observations led to the suggestion that pathways linking topo II levels and cell cycle traverse might be deranged in neoplastic cells. Our observations do not support this model. In human AML specimens, topo II is diminished or absent from the majority of cells.
The blasts that contain detectable topo II appear to be a subset of the cells that are traversing the cell cycle (Fig 10). In short, there is no evidence that the linkage between cell cycle traverse and topo II expression is abnormal in AML.

The heterogeneous expression of topo II also has implications for current understanding of the therapeutic efficacy of topo II-directed agents. In the past it had been unclear why etoposide and m-AMSA were curative as single agents in murine leukemia models but not in human AML. Recent studies suggest that the cytotoxicity of topo II-directed agents results from the conversion of reversible drug-stabilized topo II-DNA adducts into cytotoxic lesions through an interaction with advancing replication or transcription complexes. Murine leukemia cell lines contain abundant levels of topo II. In addition, ~35% of these cells are in S phase and virtually all are traversing the cell cycle in vivo. It is not surprising that extensive cytoreduction can be achieved with topo II-directed agents under these conditions. In human AML, the percentage of cells containing topo II (Figs 9 and 10) and the percentage of cells traversing the cell cycle (Fig 10) are smaller. These factors probably contribute to the diminished efficacy of topo II-directed agents in human AML.

Finally, the heterogeneous expression of topo II has important implications for attempts to correlate topo II content and drug sensitivity in human tumors. Western blotting (Fig 4) or alkaline elution provides a measure of "average" topo II content in a large tumor cell population. Because topo II is heterogeneously expressed, this "average" topo II content does not necessarily reflect topo II levels in the subset of cells that are clonogenic in vitro and in vivo. Attempts to define a relationship between topo II content and drug sensitivity in clinical material will be fraught with difficulty until better methods are devised for identifying these clonogenic tumor cells and measuring their topo II levels on a cell-by-cell basis.

Because of these concerns, the significance of the increased topo II expression in marrows from GM-CSF-treated AML patients is unclear. Towatari et al reported that administration of G-CSF to G-CSF-dependent human leukemia cell lines in vitro resulted in increased topo II levels and increased etoposide sensitivity. Consistent with these results, we observed that treatment of two AML patients with GM-CSF resulted in concomitant increases in the percentage of blasts traversing the cell cycle and the percentage...
of cells containing detectable topo IIα. This GM-CSF-induced increase in topo II expression was observed in samples containing greater than 90% blasts (Fig 9J and L) and therefore appears to reflect changes in topo IIα expression in the leukemic cells. These findings strengthen the relationship between cell cycle traverse and topo II expression in human AML. On the other hand, the cell-to-cell heterogeneity makes it difficult to determine whether topo II expression is changing in the clonogenic cells. Consequently, it is not clear whether the administration of GM-CSF before topo II-directed therapy will provide any therapeutic benefit. Further studies of this issue are required.

In summary, we found that estimates of topo II content in human AML samples do not correlate with sensitivity to topo II-directed agents in vitro. This lack of correlation appears to result from marked cell-to-cell heterogeneity of topo II expression within any leukemic marrow, a heterogeneity that obscures differences in topo II levels in clonogenic leukemia cells. Whether similar considerations apply to other malignancies remains to be determined.

ACKNOWLEDGMENT

The encouragement of Joel Shaper, O. Michael Colvin, and Wm. T. Beck; the kind gifts of antibodies by Fred Drake, Leroy Liu, and James Sorace; the technical assistance of Sharon McLaughlin, Sandra Kiesewetter, Tim Soos, Lisa Prichard, Milada Vala, Jamie Barber, and Janice Mayes; the data retrieval by John Stryzak; and the secretarial assistance by Ann Larocca are gratefully acknowledged. This study was also made possible by the skillful care provided by Ken Hall, Louann Morrell, and the attendings, fellows, housestaff, and nurses of the Adult Leukemia Service.

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