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Dissociation of cytotoxicity and DNA cleavage activity induced by topoisomerase II-reactive intercalating agents in hamster-human somatic cell hybrids

Bonnie S. Glisson[1], Ann M. Killary[3], Philip Merta[3], Warren E. Ross[4], Jeanette Siciliano[2], Michael J. Siciliano[2]

The Departments of [1] Medical Oncology, [2] Molecular Genetics, and [3] Laboratory Medicine, University of Texas M. D. Anderson Cancer Center, Houston, Texas, USA
[4] The Department of Medicine, Gainesville, FL 32610, USA

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Summary. Previous studies using the mutant Chinese hamster ovary cell line Vpm^R-5 indicate that its resistance to epipodophyllotoxins and intercalating agents is likely to be mediated through a qualitative change in type II topoisomerase that confers resistance to drug-stimulated DNA cleavage activity. In a further investigation of the genetic basis of drug resistance in Vpm^R-5 cells, we fused a hypoxanthine-guanine phosphoribosyl transferase-deficient subline of Vpm^R-5 (Vtgm-6) with normal human lymphocytes and analyzed the resultant hybrid lines (HL) for altered drug sensitivity. In all, 3 of 16 hybrid clones exhibited partial reconstitution of sensitivity to etoposide, mitoxantrone, doxorubicin, and 5-iminodaunorubicin while retaining complete resistance to *m*-AMSA. However, enhanced sensitivity to drug-induced DNA cleavage activity was observed only for etoposide. Biochemical and molecular-marker analysis of the hybrids failed to identify human chromosome 17 (the provisional location of *TOP2*) or any other human chromosome that is consistently and uniquely associated with drug sensitivity. We therefore sought to verify the chromosomal assignment of *TOP2* by Southern blot hybridization of *TOP2* cDNA on a human hybrid mapping panel and confirmed its location on chromosome 17. However, no hybridizing sequence to the *TOP2* cDNA was found in any of the 16 Vtgm-6 hybrid lines. Efforts to select more directly for human chromosome 17 Vpm^R-5 hybrids using microcell fusion of mouse A9 cells carrying human 17 linked to pSV$_2$neo were unsuccessful. None of the five hybrid clones thus obtained had 17q markers, including the gene for *TOP2*. Although the mechanism underlying partial reversion to a drug-sensitive phenotype in the original Vtgm-6 hybrid lines has yet to be defined, the data obtained in these lines indicate that anthracycline- and anthracenedione-induced cytotoxic effects can be dissociated from DNA cleavage activity. This suggests that pathways distal to cleavable-complex formation or, alternatively, independent of interactions with topoisomerase II that involve other intracellular targets are important in mediating the cytotoxicity produced by these drugs.

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Abbreviations: *m*-AMSA, 4′,9-acridinylaminomethanesulfon-*m*-anisidide; CHO, Chinese hamster ovary; HPRT, hypoxanthine-guanine phosphoribosyl transferase; WT, wild type; DMSO, dimethylsulfoxide; HAT, hypoxanthine (100 μM), aminopterin (10 μM), thymidine (10 μM); HEPES, hydroxylethyl piperazine ethanesulfonic acid; SDS, sodium dodecyl sulfate; TBS, TRIS (20 mM, pH 7.5) plus NaCl (550 mM); TTBS, TBS plus 0.05% Tween-20

Correspondence to: B. S. Glisson, Department of Medical Oncology, Box 80, U. T. M. D. Anderson Cancer Center, 1515 Holcombe Blvd., Houston, TX 77030, USA

Introduction

The nuclear enzyme DNA topoisomerase II (EC 5.99.1.3) is recognized as one of the critical intracellular targets for the antitumor activity of the epipodophyllotoxins and several diverse intercalating agents [16]. These drugs stabilize reaction intermediates normally formed by the enzyme with DNA. On protein denaturant treatment, these drug-stabilized intermediates can be detected as protein-associated breaks in DNA; they have thus been termed cleavable complexes [16]. The mutant CHO cell line Vpm^R-5 is resistant to various cytotoxic agents that interact with topoisomerase II [14, 15]. Its resistance to etoposide and several intercalating agents (i.e., *m*-AMSA, mitoxantrone, and doxorubicin) has been correlated with a loss of drug-induced cleavable-complex formation in whole cells; it is not associated with altered drug accumulation or with changes in topoisomerase II content [14, 29]. Purified enzyme from Vpm^R-5 cells, although heat-sensitive, ex-

hibits normal decatenating activity that is as sensitive to inhibition by etoposide as is WT enzyme. However, Vpm^R-5 topoisomerase II is quite resistant to the DNA-cleavage activity induced by etoposide or *m*-AMSA [29]. These data indicate that resistance in this cell line is likely mediated through a qualitative alteration in topoisomerase II that confers resistance to drug-induced DNA-cleavage activity.

This concept and the role of drug-induced DNA-cleavage activity in cytotoxic effects may be studied in somatic cell hybrids. M_1J_27 cells, the product of fusion of Vpm^R-5 cells with drug-sensitive CHO cells, exhibit nearly complete reconstitution of sensitivity to the cytotoxic effects of etoposide and, in parallel, a marked enhancement of drug-induced DNA strand-breaking activity [14]. Although these data suggest that conversion to a sensitive phenotype occurs when normal topoisomerase II becomes available for recruitment by drug to form cleavable complexes with DNA, the precise genetic basis for this conversion cannot be identified in hamster-hamster hybrids that contain a pseudotetraploid karyotype. In contrast, human-hamster hybrids rapidly segregate human chromosomes and therefore retain only a minority of the human chromosomal complement, making this system more suitable for the association of specific chromosomes with a change in phenotype.

The data reported herein represent the results of an effort to define the genetic basis of the resistance of the Vpm^R-5 line by studying somatic cell hybrid lines formed when human lymphocytes were fused with an HPRT-deficient subline of Vpm^R-5. Through analysis of these hybrid lines for reconstitution of drug sensitivity and for human chromosomal complement, we hoped to identify a specific human chromosome whose presence wa onsistently associated with drug sensitivity and whose absence was associated with stable resistance. As part of that analysis, we confirmed the location of the human gene for topoisomerase II (*TOP2*) on human chromosome 17. Although our original hypothesis was that reversion to drug sensitivity would be accompanied by expression of the normal human topoisomerase II gene in the Vpm^R-5 milieu, our data show that partial reversion occurs in the absence of normal topoisomerase II expression and, in the case of two anthracyclines and one anthracenedione, without an increase in drug-induced DNA strand breaks. This dissociation of DNA cleavage and cytotoxic activity underscores the complexity of cellular responses to these agents and suggests that pathways distal to or, alternatively, independent of cleavable-complex formation are altered in the hybrid lines.

Materials and methods

Chemicals. Cell-culture medium, fetal bovine serum, antibiotics, and trypsin were purchased from Grand Island Biological Co. (Grand Island, N. Y.). [^{14}C]-Thymidine (51 mCi/mmol), [^{3}H]-thymidine (28 Ci/mmol), and [^{3}H]-hypoxanthine (29 Ci/mmol) were obtained from ICN (Irvine, Calif.). [^{3}H]-Etoposide (900 mCi/mmol) was purchased from Moravek Biochemicals (Brea, Calif.). [^{3}H]-Daunomycin (4.9 Ci/mmol) was obtained from New England Nuclear (Boston, Mass.). Etoposide was a gift from Bristol Laboratories (Syracuse, N. Y.); mitoxantrone was a gift from Lederle Laboratories (Pearl River, N. Y.); doxorubicin was donated by Adria Laboratories (Columbus, Ohio); *m*-AMSA was obtained from the National Cancer Institute (Bethesda, Md.); and 5-iminodaunorubicin was a gift from Dr. L. A. Zwelling (Houston, Tex.). Etoposide and *m*-AMSA were dissolved in DMSO; the remaining drugs were dissolved in water. Tetrapropyl ammonium hydroxide was obtained from RSA Corp. (Ardsdale, N. Y.). All other chemicals were supplied by Sigma Chemical Co. (St. Louis, Mo.) unless otherwise specified.

Cell lines and culture techniques. CHO and CHO human hybrid lines were grown in monolayers in alpha-minimal essential medium (without ribonucleosides) supplemented with 5% fetal bovine serum, penicillin, neomycin, and streptomycin. The Vpm^R-5 cell line was formed by mutagenesis of parental WT CHO cells with ethylmethane sulfonate and was selected for teniposide resistance as described by Gupta [17]. The Vtgm-6 line, an HPRT-deficient subline, was isolated by mutagenesis of Vpm^R-5 cells with ethylmethane sulfonate (20 mM × 30 min) and subsequently selected for 6-thioguanine resistance (continuous exposure, 10 μM). HPRT deficiency was confirmed by an HPRTase assay that measures the formation of [^{3}H]-inosine monophosphate from [^{3}H]-hypoxanthine [13]. The Vtgm-5 line exhibits low reversion-frequency ($<2.5 \times 10^{-6}$) cloning in HAT-supplemented medium and has a doubling time equal to that of the parent cell line Vpm^R-5 (18 h). Cells used in colony-forming assays were trypsinized and then seeded into 100-mm plates at 18 h prior to a 1-h drug exposure at 37° C. Plates were rinsed twice with cold phosphate-buffered saline (PBS; 0.4% sodium chloride, 5 mM potassium phosphate, pH 7.0) and incubated in drug-free medium for 7 days. Colonies were stained with 2% crystal violet in methanol. Plating efficiencies varied from 50% to 90% for all cell lines.

Interspecific somatic cell hybridization. Human lymphocytes and Vtgm-6 cells were fused by a modification of the method described by Davidson et al. [6]. Human lymphocytes were obtained by culturing peripheral blood in RPMI 1640 medium containing 1% phytohemagglutinin (Burroughs Wellcome, Research Triangle Park, N. C.) and interleukin 2 (Human T-Cell Polyclone; Collaborative Research, Lexington, Mass.) at 5 half-maximal units/ml for 72 h. Lymphocytes and Vtgm-6 cells (at a ratio of 2 : 1) were incubated together on 100-mm plates for 24 h prior to fusion. Cells were harvested and rinsed twice in serum-free medium, then resuspended in 1 ml PEG 1500 (Boehringer Mannheim, Ridgefield, Conn.); this step was followed by the addition of and resuspension of cells in 9 ml serum-free medium, two rinses, and plating onto 100-mm dishes. After incubation for 24 h in nonselective medium, cells were plated in serial dilution in HAT-selective medium and grown for 2 weeks. Surviving hybrid clones were isolated with glass cloning rings.

Microcell fusion. Vpm^R-5 cells were fused with microcells prepared from a rat hepatoma line [A9(17n)A] carrying a complete copy of human chromosome 17 marked with *neo* [21]. A9(17n)A was kindly donated by Dr. R. Leach. Microcell hybridization was performed using a modification of the procedure originally described by Fournier and Ruddle [11, 19]. Hybrids were selected in G418 (500 μg/ml) and analyzed by G-11 banding to differentiate hamster and human chromosomes [12].

Alkaline elution. Single and double DNA strand breaks were assayed by alkaline and neutral filter elution as previously described [20]. Cells were labeled with [^{3}C]-thymidine for 48 h and grown in label-free medium for 24 h prior to a 1-h drug exposure. Cells that contained [^{3}H]-thymidine-labeled DNA and that had received 150 or 1500 rads prior to elution were included on each filter as internal standards in the single-strand break assays. Low-frequency single- and double-strand breaks were measured by elution at 0.03 – 0.04 ml/min for 15 h.

Drug transport. Steady-state concentrations of [^{3}H]-etoposide and [^{3}H]-daunomycin were measured as previously described [34].

Hybrid clone mapping panels. Over the years, on the basis of studies identifying the chromosomal locations of human DNA repair genes that complement such deficiencies in CHO mutant cell lines [26, 30 – 32], we have developed somatic cell hybrid clones informative for making

human chromosomal assignments. The hybrid clone panel we have organized and characterized is useful for the assignment of genes to any human chromosome [28].

Characterization of hybrids for human chromosomal content. The presence or absence of human chromosomes in the lymphocyte-Vtgm-6 hybrids was determined using a combination of cytogenetic, isozyme, and Southern [27] blot analyses of chromosomally assigned biochemical and molecular markers as described elsewhere [25, 31]. The markers used and their chromosomal locations according to HGM10 [23] were: *ENO1*, 1pter-p36.13; *PGD*, 1p36.3-p35.13; *AK2*, 1p34; *PGM1*, 1p22.1; *PEPC*, 1q25 or 1q42; *MDH1*, 2p23; *ACP1*, 2p25; *IDH1*, 2q32-qter; *ACY1*, 3p21; *GPX1*, 3q11-q12; *PGM2*, 4p14-q12; *PEPS*, 4p11-q12; *HEXB*, 5q13; *GLO1*, 6p21.3-p21.1; *ME1*, 6q12; *PGM3*, 6q12; *SOD2*, 6q21; *EGFR*, 7p13-p12; *GUSB*, 7q21.2-q22; *GSR*, 8p21.1; *AK1*, 9q34.1-q34.2; *IL2R*, 10p15-p14; *ADK*, 10cen-q24; *CYP2C*, 10q24.1-q24.3; *LDHA*, 11p15.1-p14;, *GAPD*, 12p13; *TPI1*, 12p13; *LDHB*, 12p12.2-p12.1; *D13S1*, 13cen-q21; *D13S3*, 13q21-q34; *NP*, 14q13.1; *CKBB*, q32.3; *HEXA*, 15q23-q24; *MPI*, 15q22-qter; *PKM2*, 15q22-qter; *PGP*, 16p13; *DIA4*, 16q12-q22; *CSH*, 17q22-q24; *GALK*, 17q23-q25; *D18S1*, 18pter-qter; *PEPA*, 18q23; *PEPD*, 19qcen-q12; *MANNB*, 19q12-q13.1; *GPI*, 19q12-q13.2; *ITPA*, 20pter-cen; *ADA*, 20q13.11-qter; *SOD1*, 21q22.1; *D21S24*, 21p; *PDGFB*, 22q12.3-q13.1; *G6PD*, Xq28.

Visualization of TOP2 genomic sequences – southern hybridization. DNAs from hybrids were digested with restriction enzymes according to the manufacturer's instructions and were then analyzed (11 μg/lane) on 0.8% agarose gels (Seakem, Rockland, Me.) and transferred to Zetabind hybridization membranes (AMF Cuno, Meridian, Conn.). Approximately 25 ng of a cDNA probe for the human topoisomerase II gene (Dr. L. Liu, Baltimore, Md.) was labeled with [^{32}deoxycytidinetriphosphate] to a specific activity of approximately 10^9 cpm/μg DNA using a multiprime random priming kit (Amersham, Arlington Heights, Ill.). Hybridization of probes to filters was performed according to the method of Amasino [1] in 0.25 M $NaPO_4$ (pH 7.2), 0.25 M NaCl, 5% SDS, 10% PEG 1500, and 1 mM ethylenediaminetetraacetic acid (EDTA) after prehybridization for 15–60 min at 65°C in Seal-a-Meal bags using 1 ml hybridization buffer/10 cm^2 membrane. Filters were washed twice for 15 min at room temperature and two to four times at 65°C in a solution of 0.05 M $NaPO_4$-0.5% SDS.

Results

Preparatory to the cell-fusion experiments, human lymphocytes that were in exponential growth after undergoing mitogenesis with interleukin-2 and phytohemagglutinin were treated with etoposide and assayed for drug-induced DNA single-strand breaks. These data, which have been published elsewhere [9], document that the topoisomerase II expressed in lymphocytes after mitogenic stimulation is drug-sensitive.

Expression of a stable HPRT-deficient phenotype required mutagenesis of Vpm^R-5 cells as detailed above and in Fig. 1. To exclude the possibility that mutagenesis might have altered cellular responses to topoisomerase II-targeted drugs, we carried out a direct comparison of the Vtgm-6 subline with Vpm^R-5 cells. No significant difference was found between the two lines as regards sensitivity to the cytotoxic or DNA-cleavage activities of etoposide, *m*-AMSA, mitoxantrone, doxorubicin, or 5-iminodaunorubicin (Table 1).

A total of 16 lymphocyte-Vtgm-6 hybrid lines were developed and characterized as to human chromosomal complement. Three of these lines (HL-8, HL-10, and HL-17) were chosen for detailed drug-sensitivity testing on

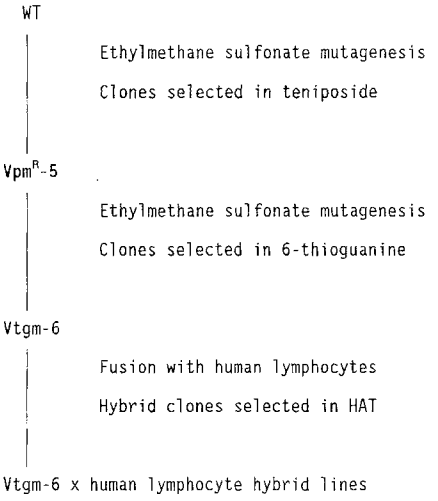

Fig. 1. Summary of cell line development

Table 1. Dose-modification factors relative to WT cells at 50% inhibition of colony formation

Drug	Cell line				
	Vpm^R-5	Vtgm-6	HL-8	HL-10	HL-17
Etoposide	16.0	15.0	7.0	6.6	7.2
Mitoxantrone	7.3	8.0	4.7	3.3	7.3
Doxorubicin	7.1	7.1	1.7	1.3	3.0
5-Imino-daunorubicin	2.1	2.1	0.9	0.6	1.3
m-AMSA	5.0	5.3	6.6	5.1	8.0

the basis of their partial loss of resistance to etoposide relative to Vtgm-6 cells. We defined the degree of resistance as a dose-modification factor, i.e., the factor by which the drug dose must be multiplied to produce equitoxic effects (50% inhibition of colony formation) in the Vtgm-6 or hybrid lines as compared with WT CHO cells, as shown in Table 1 and Fig. 2. Whereas all three hybrid lines exhibited a 2-fold increase in sensitivity to etoposide, HL-8 and HL-10 were generally more sensitive than HL-17 to the other drugs tested. Clearly, the largest change was induced by doxorubicin, for which the HL-8 and HL-10 lines possessed a 4- to 5-fold level of enhanced sensitivity (Fig. 2B). Notably, in the case of 5-iminodaunorubicin, a drug showing a markedly reduced potential for free-radical generation as compared with doxorubicin [35], these same two lines demonstrated complete reconstitution of sensitivity relative to WT cells, although this represented only a 2- to 3-fold enhancement relative to the Vtgm-6 line. Most remarkably, all three hybrid lines retained complete resistance to *m*-AMSA.

As described in Materials and methods, we used alkaline elution with SDS/proteinase K to assay for drug-induced protein-linked DNA breaks (cleavable complex) in whole cells. Figure 3A shows the increase in sensitivity to etoposide-induced DNA-cleavage activity observed in the hybrid lines (2- to 3-fold increase relative to Vtgm-6 cells). Unexpectedly, however, we found no significant differ-

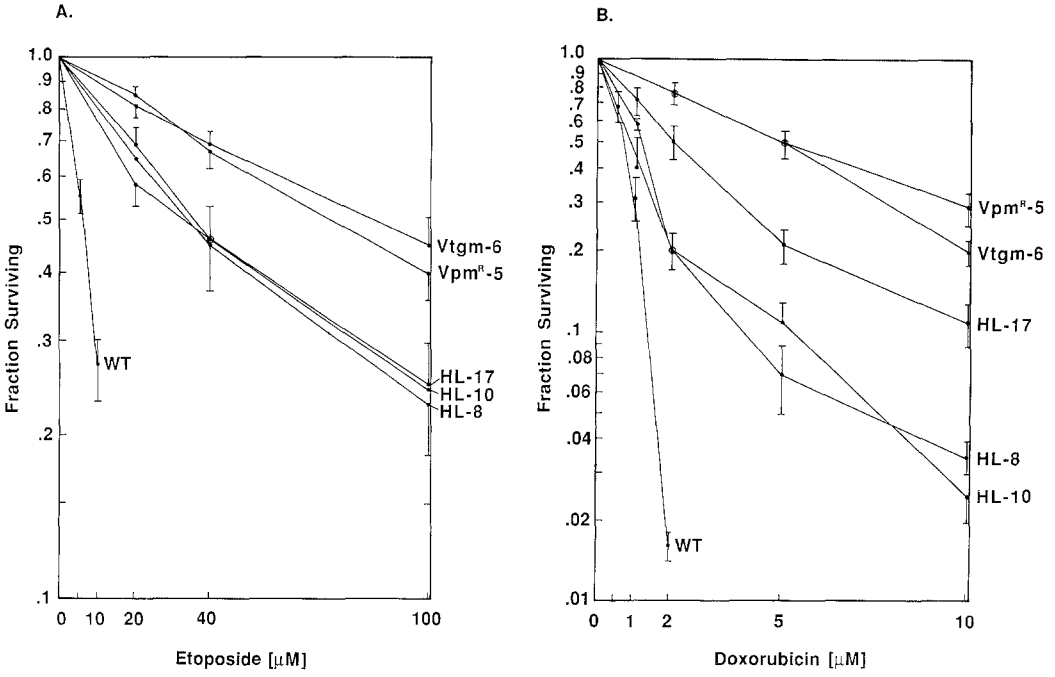

Fig. 2. A Etoposide- and **B** doxorubicin-induced cytotoxicity in a colony-forming assay

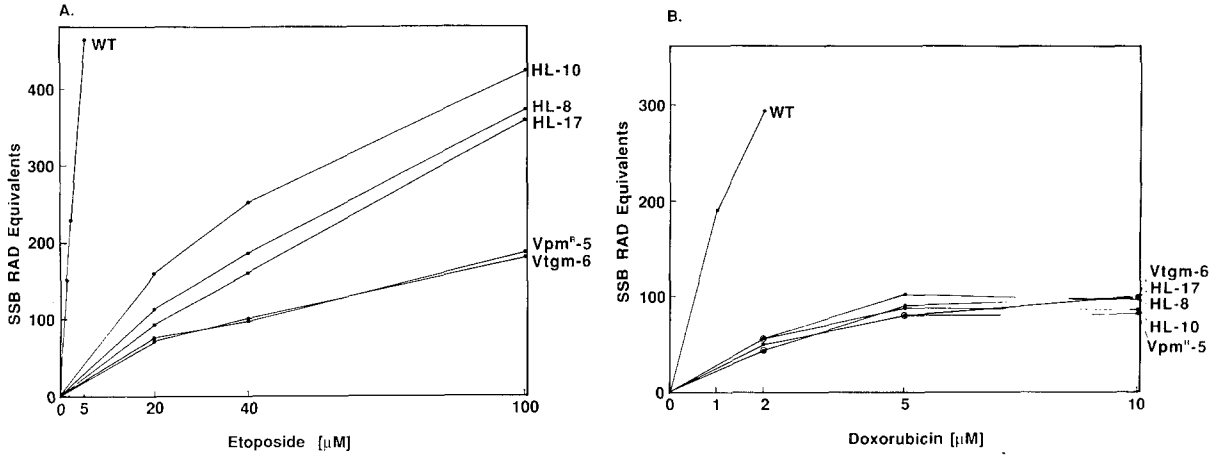

Fig. 3. A Etoposide- and **B** doxorubicin-induced DNA single-strand break frequency in whole cells as measured in rad equivalents, i.e., the amount of radiation required to produce equivalent DNA breaks. Cells growing in monolayers were treated with drug for 1 h at 37° C, rinsed with cold PBS, and harvested by scraping in 1 ml HBSS-0.02% EDTA. DNA was eluted at pH 12.1 at a rate of 0.03–0.04 ml/min for 15 h. Proteinase K was used in the lysis step. Points represent mean values for at least three separate experiments

ence between these lines as regards intercalator (doxorubicin, 5-iminodaunorubicin, and mitoxantrone)-induced single- or double-strand break frequency as compared with Vtgm-6 cells. The data for doxorubicin are presented in Fig. 3 B.

As a further demonstration of the dissociation of intercalator-induced cytotoxic and DNA-cleavage activities in the hybrid lines, the relationship between these two parameters is plotted in Fig. 4, with HL-8 as a representative hybrid line being compared with WT and Vtgm-6. The data for Vpm^R-5 are identical to those for Vtgm-6 and are not shown separately. The results obtained using etoposide (Fig. 4 A) can be compared with those obtained using doxorubicin, 5-iminodaunorubicin, and mitoxantrone (Figs. 4 B, C, and D, respectively). First, the Vtgm-6 cells exhibied a steeper slope than did the WT cells following treatment with each of the drugs, indicating a greater cell kill per cleavable complex formed. If HL-8 cells were to become more drug-sensitive through complementation of the defect responsible for resistance in Vtgm-6, one would predict at least a partial reduction in the slope and a movement toward WT. Although this was observed to a very slight degree for etoposide, the converse, an increased slope, was observed for the intercalating agents. These data suggest that HL-8 either possesses enhanced sensitivity to the cleavable complex per se or is hypersensitive to the cytotoxic effects mediated through a pathway that is independent of these intercalators' interaction with topoisomerase II. In the case of the HL-10 and HL-17 cell lines, the relationship observed between cytotoxicity and strand breaks induced by etoposide and the three intercalators was qualitatively similar that found for HL-8, with the excep-

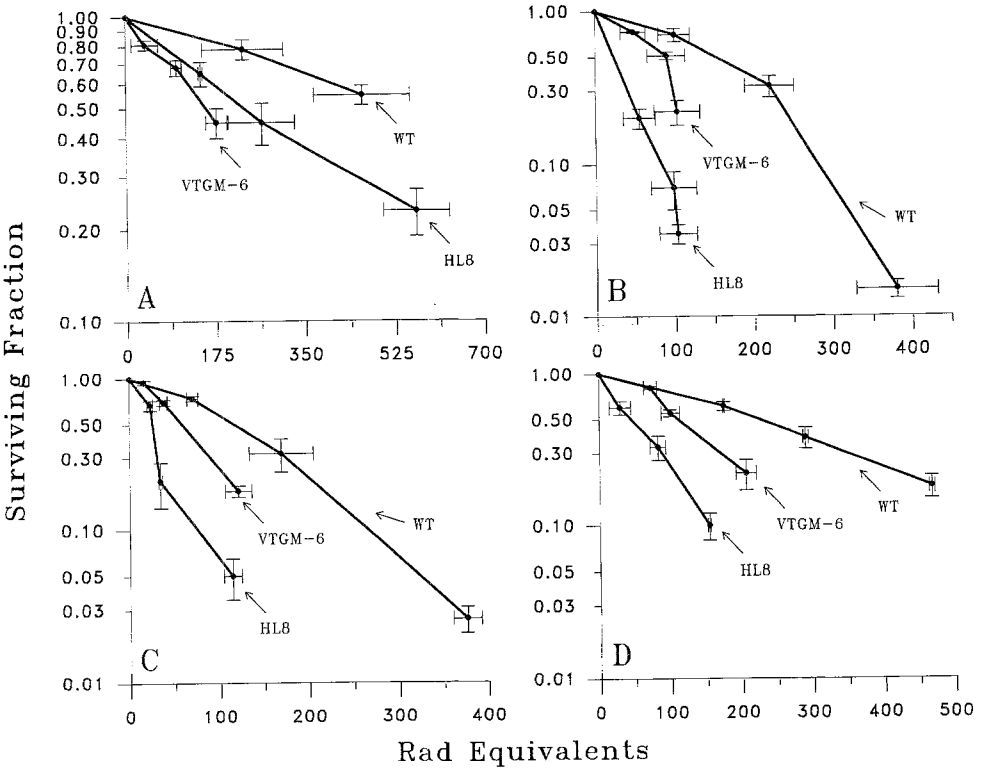

Fig. 4 A–D. Relationship between drug-induced cytotoxicity and DNA single-strand breaks. The points plotted reflect the results obtained using the following drugs and concentrations. **A** Etoposide: WT, 2 and 5 μM; Vtgm-6 and HL-8, 20, 40, and 100 μM. Slopes derived from linear regression: WT, –0.0978; Vtgm-6, –0.2970; HL-8, –0.2128. **B** Doxorubicin: WT, 0.05, 1, and 2 μM; Vtgm-6 and HL-8, 2, 5, and 10 μM. Slopes: WT, –0.2615; Vtgm-6, –0.6871; HL-8, –0.9053. **C** 5-Iminodaunorubicin: WT, 1, 2, and 5 μM; Vtgm-6 and HL-8, 1, 2, and 5 μM. Slopes: WT, –0.2554; Vtgm-6, –0.6183; HL-8, –0.7361. **D** Mitoxantrone: WT, 0.01, 0.02, and 0.05 μM; Vtgm-6 and HL-8, 0.05, 0.1, and 0.2 μM. Slopes: WT, –0.1400; Vtgm-6, –0.2674; HL-8, –0.5437

Table 2. Steady-state accumulation of [^{3}H]-etoposide and [^{3}H]-daunomycin[a]

Cell line	Extracellular [etoposide] 10 μM	Extracellular [daunomycin] 1.4 μM
WT	0.91±0.19	5.20±0.01
Vpm^R-5	1.11±0.15	6.39±0.20
Vtgm-6	1.07±0.15	6.36±0.11
HL-8	0.77±0.11	6.79±0.14
HL-10	1.04±0.33	6.92±0.62
HL-17	0.85±0.50	6.17±0.04

[a] Data are expressed in nanomoles of [^{3}H]-etoposide or [^{3}H]-daunomycin per gram (dry weight) of cells and represent mean values ± SD for at least three separate experiments

tion of HL-17 and mitoxantrone (data not shown). The latter cell line showed no reconstitution of sensitivity to mitoxantrone (Table 1) and no change in strand-break frequency; thus, the relationship between these two parameters was identical to that observed for Vtgm-6.

The data shown in Table 2 demonstrate that partial reconstitution of sensitivity in the hybrid lines was not associated with enhanced accumulation of etoposide or daunomycin at steady state.

Representatives of human chromosomes present in the lymphocyte-Vtgm-6 hybrids as determined by the 50 biochemical and molecular markers studied, as well as a concordancy analysis determined the relationship of each chromosome's presence or absence to drug resistance or sensitivity, are indicated in Table 3. As expected, because of the selection protocol for the presence of human HPRT, all hybrids containing at least a portion of the X chromosome. Markers for some chromosomes (2, 3, 4, 10, 17, and 19) were not present in any of the hybrids. The concordancy analysis (Table 3) failed to identify markers for any chromosome related to viability in drug selection; discordancies ranged from 19% to 81%, with eight chromosomes sharing the lowest level.

The apparent absence of human chromosome 17 in any of the hybrids, and notably in the three hybrids (HL-8, HL-10, and HL-17) that showed partial reconstitution of drug sensitivity, was particularly interesting in view of the recent provisional assignment of *TOP2* [33] to a region (q21-22) of the chromosome close to the marker we used for identification of that chromosome in the hybrid cells: *CSH* at 17q22-q24 [18]. Therefore, we sought to confirm the *TOP2* location on human chromosome 17 by hybridizing a cDNA for the enzyme to a separate hybrid clone (CHO-HeLa) panel developed specifically for mapping purposes. Only 2 hybrids among our 33-hybrid mapping panel retained human chromosome 17, and these were the only 2 whose DNA hybridized to the *TOP2* probe (Fig. 5) for a discordancy level of 0. Discordancies for all other human chromosomes ranged from 19% to 70%. This confirmed the presence of *TOP2* on human chromosome 17 and suggested that the human gene was not present in the lymphocyte-Vtgm-6 hybrids that showed reconstitution of drug sensitivity.

Since breakage and rearrangement of human genetic material has often been observed in human-CHO somatic cell hybrids [26], *TOP2* may have become separated from the marker used to identify chromosome 17. Therefore, *TOP2* cDNA was directly hybridized to lymphocyte-Vtgm-6 hybrids on Southern blots, but it could not be detected in any of them (data not shown). We conclude that the return to drug sensitivity of HL-8, HL-10, and HL-17 was not due to complementation of the Vtgm-6 phenotype by human *TOP2*.

Table 3. Presence or absence of markers representing human chromosomes and the drug-resistant or drug-sensitive phenotype in human lymphocyte-Vtgm-6 hybrid clones and the percentage of discordancy between each human chromosome and the sensitive phenotype

Clone	Human chromosome																						Phenotype
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	
1	–	–	–	–	–	+	+	–	–	–	–	+	–	+	–	–	–	–	–	–	+	+	+	R
2	+	–	–	–	+	–	+	+	–	–	–	+	–	+	–	–	–	+	–	+	+	+	+	R
3	+	–	–	–	+	–	+	+	–	–	–	+	–	+	+	–	–	+	–	+	+	+	+	R
4	–	–	–	–	–	+	–	–	–	–	–	+	–	+	+	–	–	+	–	–	+	+	+	R
6	–	–	–	–	+	–	–	–	–	–	+	+	–	+	+	–	–	–	–	–	+	+	+	R
8	–	–	–	–	–	–	–	+	–	–	+	–	–	–	–	–	–	–	–	+	–	–	+	S
9	+	–	–	–	–	+	+	+	+	–	+	–	–	+	+	–	–	–	–	+	+	+	+	R
10	–	–	–	–	–	–	–	–	+	–	+	–	–	–	+	+	–	–	–	–	–	–	+	S
11	–	–	–	–	+	–	–	+	–	–	+	–	–	–	–	+	–	–	–	+	+	–	+	R
12	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–	+	R
13	–	–	–	–	–	+	–	+	–	–	+	–	–	–	–	–	–	+	–	–	+	+	+	R
14	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+	R
15	–	–	–	–	+	–	–	+	–	–	–	–	+	+	–	–	–	+	–	+	+	+	+	R
16	–	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	R
17	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	+	–	–	+	S
19	–	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	R
Discordancy[a]	38%	19%	19%	19%	50%	44%	50%	50%	19%	19%	31%	50%	31%	56%	38%	19%	19%	50%	19%	38%	81%	75%	81%	

[a] The number of sensitive hybrids without the chromosome plus the number of resistant hybrids with the chromosome, divided by the total number of hybrids (16), × 100

+, Presence of markers; –, absence of markers; R, resistant phenotype; S, sensitive phenotype

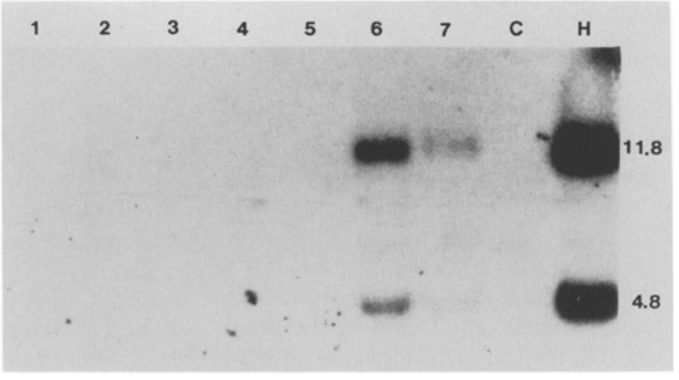

Fig. 5. Autoradiogram of a Southern blot containing *Hind*III-digested DNA from seven clones in hybrid mapping panel CHO × HeLa, a CHO cell control (*C*), and a human HeLa cell control (*H*). Note that the 11.8- and 4.8-kb bands diagnostic of human *TOP2* *Hind*III fragments are present only in the human control and in hybrids 6 and 7. The weaker human bands in hybrid 7 reflect the lower percentage of cells retaining human chromosome 17 in that cell line

The location of the human *TOP2* gene on chromosome 17 is unfortunate in that there appears to be a gene within that syntenic group that is detrimental to the survival of hybrids made with CHO cells [21]. Therefore, to study further the interaction of the *TOP2* gene in the Vpm^R-5 milieu, we next used a direct method to select for chromosome 17. Cells of the rat hepatoma line A9(17n)A, which carries a complete copy of human chromosome 17 with an integrated dominant selectable marker *neo*, were used as donors for microcell fusion into Vpm^R-5 cells [11, 19]. Selection in G418 yielded five hybrid clones (VM-13, -16, -18, -22, and -40). G-11 analysis, however, indicated deletions or rearrangements of chromosome 17 in four of these five clones. In addition, Southern analysis in all five hybrid clones was negative for both *TOP2* (17q21-22) and *CSH* (17q22-224). Not surprisingly, these hybrids retained full resistance, relative to Vpm^R-5 cells, to the cytotoxic effects of etoposide, *m*-AMSA, doxorubicin, and mitoxantrone.

Discussion

The three human lymphocyte-Vtgm-6 hybrid lines described in this report exhibited a variable degree of heightened sensitivity to the epipodophyllotoxin etoposide and to agents representing two different classes of intercalating agents (anthracyclines and anthracenediones). This change in sensitivity was accompanied by correlative increases in etoposide-induced DNA-cleavage activity, but, surprisingly, DNA cleavage induced by doxorubicin, 5-iminodaunorubicin, and mitoxantrone remained unchanged relative to the parent line Vtgm-6. Plots of DNA cleavage and drug-induced cytotoxicity demonstrate that Vtgm-6 (and Vpm^R-5) cells are killed with many fewer strand breaks than are WT cells, i.e., the line described by these two parameters in Vtgm-6 cells possesses a steeper slope (etoposide and intercalators). When these plots are constructed in the hybrid lines, one obtains a further increase in slope (with etoposide being the exception) rather than the decrease that would be predicted if partial reversion to sensitivity were to occur via alteration of the mechanism that mediates resistance in Vtgm-6. These data further support our conclusion, based on the results of the Southern blot with the *TOP2* cDNA, that expression of the human topoisomerase II gene is not responsible for the partial sensitization to etoposide and the three intercalators. We also excluded the possibility that enhanced intracellular drug accumulation might be contributory to sensitization in the hybrid lines. This mechanism has been identified in another CHO cell line selected for hypersensitivity and isolated by the replica plating technique [8].

Although our data demonstrate a dissociation of intercalator-induced cleavable-complex formation from cytotoxic effects in somatic cell hybrid lines showing partial reversion to drug sensitivity, previous investigations of cell lines made resistant to topoisomerase II-targeted drugs have generally shown quite good correlation between these two activities for any given agent [2, 7, 24]. Parallel changes in the two effects have also been documented in studies using

drug analogues [22]. However, there are clearly situations in which the drug effect on the enzyme and DNA can be dissociated from cytotoxicity. The malignant cell line HeLa and mouse fibroblasts BALB/c3T3 are most sensitive to the cytotoxic effects of *m*-AMSA and etoposide, respectively, during DNA synthesis despite maximal drug-induced cleavage in mitosis [4, 10]. Furthermore, murine leukemia L1210 cells exhibit relative resistance to etoposide when they are in quiescence because of nutrient deprivation or when they are pretreated with the protein-synthesis inhibitor cycloheximide [5]. The drug resistance observed under these conditions is not associated with inhibition of etoposide-induced DNA-cleavage activity or with decreased topoisomerase II content.

These data indicate that post-cleavable-complex processing, i.e., events occurring distal to cleavable-complex formation and involving gene products other than topoisomerase II, can influence the ultimate cellular response to drug exposure. This concept is also supported by the nature of the cleavable complex, i.e., its rapid reversibility after drug removal and the failure of the cell to recognize it as typical DNA damage [16]. These findings suggest that drug-induced stabilization of the enzyme-DNA complex is the initiator or trigger in a poorly understood process that may or may not result in cell death. Although the data obtained in the human lymphocyte-Vtgm-6 hybrid lines are consistent with altered cellular processing of intercalator-induced cleavable complexes, this represents a partial explanation at best. The stable resistance of all three lines to *m*-AMSA must be borne in mind, as must the good correlation between changes in DNA cleavage and cytotoxic effects in the case of etoposide.

Alternatively, it is conceivable that enhanced sensitivity in these hybrid lines is related to a topoisomerase II-independent mechanism that is also available to all of these drugs except *m*-AMSA. It is tempting to speculate that *m*-AMSA's quite narrow clinical spectrum represents a singular interaction with topoisomerase II and an inability to utilize other intracellular targets and pathways. Doxorubicin, for example, which enjoys broad clinical application, is clearly capable of exerting cytotoxic effects through free-radical-mediated damage, although this mechanism, at least as regards DNA effects, is observed at intracellular drug concentrations much higher than those required for its interaction with topoisomerase II [3]. The concept of an alternative pathway in the Vtgm-6 hybrid lines is also supported by the observation that the HL-10 line was in fact more sensitive than were WT cells to 5-iminodaunorubicin. This suggests that a mechanism that is operative in both WT and Vtgm-6 (and Vpm^R-5) cells, perhaps to an equal extent and not contributory to the resistance in Vtgm-6, may be altered in the hybrid lines. However, given the qualitatively similar results obtained using doxorubicin and 5-iminodaunorubicin in the hybrid lines, it is unlikely that this "second" mechanism is related to enhanced sensitivity to free-radical-mediated effects.

The question remains as to which human component(s) might be responsible for the changes in phenotypes seen in the three hybrid lines that showed partial reversion to sensitivity. This may be determined by making biotinylated probes from the human DNA retained in those hybrids and conducting in situ hybridization to human metaphase cells using fluorescence to identify a human chromosomal region that is consistently and uniquely associated with sensitivity. Such experiments are in progress.

The mechanism underlying the enhancement of drug sensitivity in the human lymphocyte-Vtgm-6 hybrid lines is not immediately apparent. However, the altered relationship observed between cytotoxic effects and DNA-cleavage activity for two anthracyclines and an anthracenedione in the hybrid lines suggests that the anticancer activity of these compounds is not likely to be based on an interaction with topoisomerase II alone. Rather, cellular responses to these agents are complex and likely reflect interaction with multiple targets.

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