Biochemical Pharmacology, Vol. 46, No. 5, pp. 851–861, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

ALTERED TOPOISOMERASE I ACTIVITY AND RECOMBINATION ACTIVATING GENE EXPRESSION IN A HUMAN LEUKEMIA CELL LINE RESISTANT TO DOXORUBICIN

JEAN-FRANÇOIS RIOU,*† LUCILE GRONDARD,* ODILE PETITGENET,* MARC ABITBOL‡§ and FRANÇOIS LAVELLE*

*Rhône-Poulenc Rorer, Unité de Cancérologie, Département Biologie, Centre de Recherche de Vitry-Alfortville, 94403 Vitry-sur-Seine, and ‡Laboratoire de Neurobiologie Cellulaire et Moléculaire, CNRS, 91198 Gif-sur-Yvette, France

(*Received* 11 *January* 1993; *accepted* 24 *May* 1993)

Abstract—We examined the expression of the genes encoding topoisomerases I and II and those associated with V(D)J [variable(diversity)joining] recombination in two human T-cell acute lymphoblastic leukemia (T.ALL) cell lines, CEM and CEM/DOX. In CEM/DOX cells, which are resistant to doxorubicin, the topoisomerase I gene was found to be 4-fold overexpressed and nuclear topoisomerase I relaxation activity was 2-fold greater in CEM/DOX than in CEM cells. Furthermore, the cleavable complex reaction induced by camptothecin, a specific topoisomerase I inhibitor, was found to be 2.5-increased in the presence of topoisomerase I extracted from CEM/DOX, in comparison to that in CEM cells. Conversely, the topoisomerase II mRNA levels, nuclear decatenation activities and (mAMSA) 4′(9-acridinylamino)methanesulfon-*m*-anisidide-induced cleavable complex formation in CEM/DOX were similar to those of the doxorubicin-sensitive cells. The results indicate that topoisomerase I activity is elevated in CEM/DOX cells. Nevertheless, CEM/DOX cells were 11-fold more resistant to camptothecin than were CEM cells, and cross-resistance to camptothecin was not reversed by verapamil. Furthermore, using an intact cell assay for DNA–protein complexes, we found that camptothecin-stimulated cleavable complexes formed in CEM/DOX cells were increased in correlation with the elevated topoisomerase I activity. These results suggest that camptothecin resistance in CEM/DOX cells is due to different mechanism(s) than topoisomerase- or P-glycoprotein-associated multidrug resistance. The recombination activating gene, RAG1, which is one of the components of the site-specific V(D)J recombination complex, was 20-fold overexpressed in CEM/DOX cells. In contrast, RAG2 and T160 gene transcripts, other components of the V(D)J complex, were at best poorly detected in both sensitive and resistant cells. No specific V(D)J recombinase activity was found in CEM or CEM/DOX cells when the pJH201 transfection assay was used. The results indicate that CEM/DOX cells failed to generate V(D)J recombination although RAG1 gene is overexpressed. The mechanism of the RAG1 gene activation was not gene amplification, and no rearrangement was detected in the RAG1 gene locus. RAG1 presents homology with the yeast gene HPR1, itself homologous to yeast topoisomerase I and responsible for the control of recombination in somatic cells. Since DNA topoisomerases are themselves involved in the control of DNA topology, recombination and DNA repair, the possible coactivation of RAG1 and topoisomerase I genes in CEM/DOX cells is discussed.

The assembly of functional T-cell receptor and immunoglobulin genes is carried out by a series of site-specific recombination events known as V(D)J [variable-(diversity)-joining‖] recombination [1]. The recombination activity occurs at specific signal sequences that flank the possible coding regions of the immunoglobulin and T-cell receptor genes in pre-B and pre-T lymphocytes [1]. Recently, three genes (recombination activating genes) RAG1, RAG2 and T160 were identified as playing a crucial role in the recombination process [2–4]. RAG1 is homologous to the yeast gene HPR1, itself homologous to yeast topoisomerase I and responsible for the control of somatic recombinations in yeast [5, 6]. The RAG1 gene is thus thought to encode the active nicking–closing enzyme involved in V(D)J recombination, and its homology with HPR1 and topoisomerase I and the relationship between these enzymes in the control of genome integrity are currently being investigated [6–8].

DNA topoisomerases are enzymes that modulate DNA topology. Two DNA topoisomerases, type I and type II, in eukaryotes have been well characterized. They have important functions during transcription, replication, recombination and DNA repair (for a review see Ref. 9). DNA topoisomerases I and II have been identified as major targets in

† Corresponding author: Rhône-Poulenc Rorer, CRVA 14, 13 quai Jules Guesde, 94403 Vitry-sur-Seine cedex, France. Tel. (33) 1 45 73 74 70; FAX (33) 1 45 73 74 71.

§ Present address: MEEI, 243 Charles Street, Boston, MA 02114, U.S.A.

‖ Abbreviations: V(D)J, variable (diversity) joining; RAG, recombination activating gene; SDS, sodium dodecyl sulfate; mAMSA, 4′(9-acridinylamino)methane sulfon-*m*-anisidide; T.ALL, T-cell acute lymphoblastic leukemia; SSC, standard saline citrate buffer; topoisomerase I, type I DNA topoisomerase; topoisomerase II, type II DNA topoisomerase; BSA, bovine serum albumin; Amp, ampicillin; Cam, chloramphenicol; DTT, dithiothreitol.

cancer chemotherapy and their inhibitors include a variety of structurally diverse antitumor agents [8, 9]. The mechanism of action of topoisomerase inhibitors involves the transient formation of protein-associated DNA single- or double-strand breaks, which could be converted to irreversible DNA lesions during replication or into chromosome aberrations by recombination during mitosis [9, 10]. Resistance to anticancer agents is frequently observed during the clinical treatment of cancer. Many cell lines resistant to topoisomerase inhibitors have been characterized by the wide diversity of their resistance phenotypes. The most frequently observed mechanism of resistance is the elimination of the drug due to an overexpression of the P-glycoprotein ATP-dependent pump encoded by the mdr1 gene [11, 12] and/or alteration of the nuclear topoisomerase I or II activities [13–16]. Other mechanisms of resistance, such as alterations of DNA repair, have been reported for topoisomerase inhibitors and other antitumor agents that directly or indirectly damage DNA (reviewed in Ref. 17).

We examine here the modulation of the expression of topoisomerase I and II and of the genes associated with V(D)J recombination in CEM/DOX, a doxorubicin-resistant subline of the T-cell acute lymphoblastic leukemia (T.ALL) cell line CEM, in which the mdr1 gene is overexpressed and amplified.

MATERIALS AND METHODS

DNA, enzymes and chemicals. pBR322 DNA, restriction endonucleases and nick-translation kit were purchased from Boehringer Mannheim (Meylan, France). *Trypanosoma cruzi* kinetoplast DNA (kDNA) was a gift from Dr G. Riou (Institut Gustave Roussy, Villejuif, France). [α-^{32}P]dATP, dCTP and Hybond N$^+$ membranes were purchased from Amersham (U.K.). The following drugs were obtained from commercial sources: 4′-(9-acridinylamino)methane sulfon - *m* - anisidide (mAMSA) (Substancia Laboratory, France), doxorubicin (Roger Bellon Laboratory, Neuilly-sur-Seine, France) and camptothecin (Sigma Chemical Co., La Verpillière, France); mAMSA and camptothecin were dissolved in dimethylsulfoxide at 1 mM and then further diluted in water. Doxorubicin was diluted in water at 1 mM.

Cell lines and cultures. CEM (ATCC CCL 119) and CEM/DOX human T.ALL cell lines were provided by Dr M. C. Chevallier-Multon [18] and were grown in RPMI 1640 medium, supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2 mM L-glutamine, and 50 μg/mL gentamycin. Cultures were diluted twice weekly and maintained at 37° in a humidified atmosphere with 5% CO_2. The T24 human bladder carcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD, U.S.A.) and grown as monolayers in McCoy's 5a medium with 2 mM L-glutamine and 10% (v/v) fetal calf serum.

To obtain exponentially growing cell cultures, CEM and CEM/DOX cells were seeded at 2×10^5 cells/mL in complete fresh medium and collected after 48 hr of incubation. To obtain plateau-phase cell cultures, CEM and CEM/DOX cells were seeded at 5×10^5 cells/mL in complete fresh medium and collected after 6 days, when cell density had reached a plateau (~5–6 $\times 10^6$ cells/mL). Except where indicated, cells were collected in the exponential growth phase for RNA, DNA and topoisomerase preparations.

Evaluation of the antiproliferative properties. The concentration of drugs inhibiting cell growth by 50% (IC_{50}) was determined from three separate experiments performed with 96-well microculture plates. Cell lines seeded at 2×10^5 cells/mL (0.2 mL/well) were grown for 96 hr in the presence of drugs at various concentrations (each point in quadruplicate). Cells were then incubated for 16 hr with 0.02% neutral red. The cells were washed and lysed with 1% sodium dodecyl sulfate (SDS). The incorporation of the dye reflecting cellular growth and viability was evaluated by observing the optical density for each well at 540 and 346 nm, with a Titertek multiwell spectrophotometer.

RNA and DNA preparation and hybridization. Total cellular RNA from exponentially growing cells or from DBA/2 mice thymus was prepared by the guanidinium isothiocyanate/CsCl density gradient fractionation method [19]. Final RNA preparations were precipitated in ethanol and adjusted to a concentration of 1 mg/mL in DEPC-treated water, then aliquoted and stored at −80°. For northern blot analysis, 10 μg of each RNA sample were electrophoresed on 1% agarose gels containing formaldehyde and transferred to Hybond N$^+$ membranes.

DNA was extracted and resuspended in Tris–HCl pH 7.5, 10 mM, EDTA 0.5 mM at a final concentration of 1 mg/mL as described previously [19]. DNA samples (10 μg) were digested overnight by the different restriction enzymes (*Bgl* II, *Eco*R I, *Hind* III, *Pvu* II, *Pst* I, *Xba* I, and *Xho* I), electrophoresed in 1% agarose gels and transferred to Hybond N$^+$ membranes. Prehybridization and hybridization were done at 42° in 10 mL of 50% formamide, 10 × Denhardts solution, 100 μg/mL salmon sperm DNA, 1% SDS, 5 × standard saline citrate (SSC) and 25 mM $NaHPO_4$ pH 6.8. The heat denatured probe was added after 3 hr of prehybridization, and the hybridization reaction was left to run for 16 hr. Membranes were washed as follows: once at room temperature in 2 × SSC, twice at 65° in 0.5 × SSC, 0.1% SDS and twice at room temperature in 0.2 × SSC, 0.1% SDS.

Blots were autoradiographed for 1 or 2 days on Hyperfilms MP (Amersham). Autoradiograms were scanned with a Pharmacia Ultroscan® densitometer in order to quantify the transcript level.

Probes. The probes used were: the 3.4 kb *Bam*H1–*Eco*RI cDNA fragment of the human topoisomerase I gene [20], provided by Dr J. C. Wang (Harvard University, Boston, MA, U.S.A.); the 1.8 kb *Eco*RI cDNA fragment of the human topoisomerase II α gene [21], provided by Dr L. F. Liu (Johns Hopkins University, Baltimore, MD, U.S.A.), the 1.15 kb *Pst* I cDNA fragment of the mouse actin gene [22]; the 5.4 kb *Eco*RI cDNA fragment of the human RAG1 gene [2]; the 2.0 kb *Not* I cDNA fragment of the mouse RAG2 gene [3], provided by Dr D. Schatz (Whitehead Institute for Biochemical Research,

Cambridge, MA, U.S.A.); and the 0.9 kb *Hind* III–*Eco*RI cDNA fragment of the mouse T160 gene [4]. Probes were nick-translated by *E. coli* DNA polymerase with [α^{32}P]dCTP and dATP (>3000 Ci/mmol) as described [19]. The specific activities of the probes were between 5×10^8 and 10^9 dpm/μg.

Determination of topoisomerase activity. Nuclear extracts (0.35 M) were prepared in parallel from 10^8 CEM and CEM/DOX cells in the exponential phase of growth as already described [23]. Protein concentration was determined by the Biorad assay. For the determination of topoisomerase I relaxation activity, serial dilutions of nuclear extracts were incubated for 30 min at 37° with 0.1 μg of supercoiled pBR322 DNA in a final reaction volume of 20 μL containing 25 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM dithiothreitol (DTT), 30 μg/mL bovine serum albumin (BSA) and 100 mM KCl. The reaction was stopped on ice by the addition of 5 μL of stop buffer consisting of 0.1% bromophenol blue, 50 mM EDTA, 50% (v/v) glycerol. The samples were loaded onto a 1% agarose gel in 1 × TBE (Tris–borate–EDTA) buffer and electrophoresed at 40 V for 16 hr. The gel was stained with 10 μg/mL ethidium bromide for 10 min, then washed with distilled water and photographed on a UV illuminator. Negatives of the gel pictures were scanned with a Pharmacia Ultroscan® densitometer, in order to quantify the relaxation reaction. In this way, we determined the minimal amount of protein (mg) necessary to relax 50% of the supercoiled pBR322 DNA in the assay conditions, i.e. one relaxation unit.

For the topoisomerase II decatenation activity, serial dilutions of nuclear extracts were incubated for 30 min at 37° with 0.1 μg of *T. cruzi* kinetoplast DNA (kDNA) in a final reaction volume of 20 μL containing 25 mM Tris pH 7.5, 0.5 mM EDTA, 0.5 mM DTT, 30 μg/mL BSA, 150 mM KCl, 10 mM $MgCl_2$ and 1 mM ATP. The reaction was stopped and electrophoresed as described for relaxation. Decatenated minicircles were scanned on the negatives of the gel photographs in order to quantify the reaction. We determined the minimal amount of protein (mg) necessary to decatenate 50% of the kDNA network in the assay conditions, i.e. one decatenation unit.

The DNA-cleavage assay in the presence of camptothecin and mAMSA was performed on ^{32}P-end labeled pBR322 DNA as described previously [23].

DNA–protein formation in intact cells. Cells were incubated with radioactive leucine and thymidine as described earlier [24]. Camptothecin was added 30 min prior to the termination of the experiment, and radioactivity in washed cell pellets was determined as described [24].

Transient expression of recombinase activity. Transient expression of recombinase activity was assayed by the recombination assay described previously [25]. Approximately 10^6 cells of CEM and CEM/DOX in the exponential phase of growth were transfected by the calcium phosphate precipitation procedure with 10 μg of the recombination substrate pJH201 (a gift from Dr M. Gellert, National Institute of Health, Bethesda, U.S.A.). Plasmid DNA was recovered 48 hr later by a rapid alkaline lysis procedure. The recovered plasmid DNA was then introduced into the competent *E. coli* strain XL1 and then selected for ampicillin (Amp) and Amp-chloramphenicol (Cam) resistance. Doubly resistant colonies were picked and replated on AmpCam to control the resistance. A control plasmid, pJH201Δ, which is deleted in the 0.35 kb *Hind* III fragment containing the recombination signal sequences and the transcription terminator, was constructed and used as a positive control for Cam acetyl transferase (CAT) expression. The frequency of recombination, R, was calculated as the number of AmpCam colonies divided by the total number of Amp colonies. The number of transformants (Amp or AmpCam) was corrected for a plating dilution factor.

RESULTS

DNA topoisomerase I and II activities in nuclear extracts from CEM and CEM/DOX

Nuclear extracts from CEM and CEM/DOX cells were prepared by 0.35 M NaCl extraction of isolated nuclei [23]. Topoisomerase I ATP-independent relaxation activities and topoisomerase II ATP-dependent decatenation activities were determined with serial dilutions of the extracts (see Materials and Methods). Table 1 summarizes the specific activities of topoisomerases I and II in CEM and CEM/DOX. Results indicated that there were no variations of the topoisomerase II decatenation activity in CEM/DOX extracts compared to CEM. Topoisomerase I relaxation activity was 1.7-fold greater in nuclear extracts from CEM/DOX cells than in extracts from CEM cells. During its catalytic reaction, topoisomerase mediates a transient break in the DNA, then allows another DNA strand to pass through the break, and finally reseals the DNA [9]. At the DNA breakage step, the enzyme is covalently linked to one of the DNA termini [9]. Antitumor agents, such as camptothecin for topoisomerase I and mAMSA for topoisomerase II, are known to block the reaction by increasing the amount of enzyme covalently linked to DNA [9]. The linked enzyme can be measured in an *in vitro* reaction called "cleavable-complex reaction" in which the enzyme–DNA cross-link is transformed into a double- or single-strand break under protein denaturing conditions. The camptothecin-induced DNA cleavage activities of nuclear extracts were measured by using linear ^{32}P-end labeled pBR322 DNA as substrate [23]. The extent of DNA cleavage was quantified by densitometric scanning of the 4.3 kb pBR322 native DNA band and the results are expressed as a function of the amount of protein in the topoisomerase preparation (Fig. 1). The data show that camptothecin-induced DNA cleavage activity is 2.5-fold higher in nuclear extracts from CEM/DOX than in extracts of CEM. When the same type of experiment was performed in the presence of mAMSA, no differences could be evidenced between CEM and CEM/DOX extracts (result not shown). These data indicate that topoisomerase I activity was increased in CEM/

Table 1. Specific activities of DNA topoisomerases I and II in nuclear extracts from CEM and CEM/DOX cells

Cell line	Specific activity (U/mg) × 10⁴* Topoisomerase I† relaxation activity	Topoisomerase II‡ decatenation activity
CEM	12.5 ± 1.0	1.2 ± 0.14
CEM/DOX	21.6 ± 3.5	1.15 ± 0.23
R = CEM/DOX/CEM	1.72	0.95

* Results are the mean value (±SD) of three independent determinations.

† One topoisomerase I relaxation unit is the amount of nuclear protein able to relax 0.1 μg of supercoiled pBR322 DNA for 30 min at 37° in the conditions of the assay (see Materials and Methods).

‡ One topoisomerase II decatenation unit is the amount of nuclear protein able to decatenate 0.1 μg of *T. cruzi* kDNA for 30 min at 37° in the conditions of the assay (see Materials and Methods).

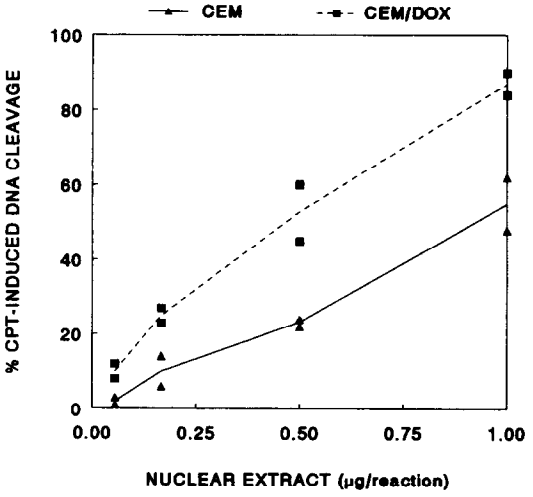

Fig. 1. Camptothecin-induced DNA cleavage in nuclear extracts from CEM (—▲—) and CEM/DOX (---■---) cells. ^{32}P-end-labeled pBR322 DNA was reacted with 10 μM camptothecin and with increasing amounts of nuclear extracts as described [23]. Camptothecin-induced DNA cleavage as a function of protein extract concentration was quantified by scanning the area of the native pBR322 DNA band (4.3 kb) on the autoradiograms. Results are expressed as the per cent of DNA cleavage compared to that in the control untreated DNA and are the mean value of two separate experiments, individual points are shown.

DOX cells, while no change was observed for topoisomerase II activity.

Formation of covalent topoisomerase I–DNA complexes in intact CEM and CEM/DOX cells

Camptothecin-mediated cleavable complex formation was also determined in intact sensitive and resistant cells by the SDS–KCl precipitation assay [24]. The ability of camptothecin to stabilize DNA–protein complexes in CEM and CEM/DOX cell lines labeled with [^{3}H]thymidine and [^{14}C]leucine is shown in Table 2. It is seen that 1 and 10 μM camptothecin caused a 1.26- and 1.93-fold stimulation, respectively, in DNA–protein complexes in CEM cells. Stabilization of complexes was found increased in CEM/DOX cells, so that 1 and 10 μM camptothecin caused a 1.69- and 3.02-fold stimulation, respectively, in DNA–protein complexes. In addition, DNA–protein complexes induced in untreated cells were found approximately double in CEM/DOX cells, compared to CEM cells. This correlated with the increased amount of topoisomerase I and camptothecin-induced cleavable complex stimulation detected in nuclear extract from resistant cells.

Cytotoxic effect of mAMSA and camptothecin on CEM and CEM/DOX cells

mAMSA and camptothecin, in contrast to doxorubicin, are not recognized by the P-glycoprotein encoded by the mdr1 gene [26], and could therefore be used as biological probes to analyse variations of topoisomerase activities. We examined the cytotoxic effects of mAMSA and camptothecin on the sensitive and resistant cell lines in exponential growth after 96 hr of contact with the drugs. The results, presented in Table 3, correspond to the concentration of drugs inhibiting growth by 50% (IC_{50}). The CEM/DOX cell line was 11-fold more resistant to camptothecin than the CEM cell line, but no resistance to mAMSA was observed. The lack of cross-resistance for mAMSA is in agreement with the absence of variation in topoisomerase II activity between sensitive and resistant cells. In contrast, the cross-resistance to camptothecin is contradictory with the increased topoisomerase I activity in CEM/DOX cells. Further analysis of the cytotoxic effect of camptothecin on other cell lines bearing the P-glycoprotein and their drug-sensitive counterpart, such as KB/VLB and P388/VCR, has confirmed the lack of cross-resistance to camptothecin in cells displaying the mdr phenotype (result not shown). The cytotoxic effect of camptothecin was also examined on CEM and CEM/DOX cell lines in the presence of verapamil, a P-glycoprotein antagonist (Fig. 2). Results demonstrate that verapamil has no

Table 2. Effect of camptothecin on protein complex formation in intact cells

Cell line	DNA–protein complexes induced by camptothecin* No drug	1 μM	10 μM
CEM	0.46 ± 0.01	0.58 ± 0.02 (1.26)†	0.89 ± 0.19 (1.93)
CEM/DOX	1.12 ± 0.08	1.90 ± 0.18 (1.69)	3.39 ± 0.69 (3.02)

* Determined as described in Materials and Methods, after 30 min incubation with camptothecin. Results are ratios of [^{3}H]DNA cpm/[^{14}C]protein cpm and are the mean ± SD of three separate experiments.

† Numbers in parentheses represent fold increases in DNA–protein complexes in drug treated cells, compared to no-drug controls.

Table 3. Relative resistance to mAMSA and camptothecin of CEM and CEM/DOX cell lines

Drug	IC_{50} (nM)* CEM	CEM/DOX	Relative resistance factor†
mAMSA	4800 ± 1400	4500 ± 1100	0.93
Camptothecin	2.2 ± 0.25	25 ± 5	11.4
Doxorubicin	—	—	153‡

* IC_{50} = drug concentration, after incubation in liquid medium for 96 hr, that inhibits cell growth by 50%, determined from three separate experiments. Each concentration of drug was tested in quadruplicate. SD of the mean is indicated.

† Relative resistance factor is the CEM/DOX IC_{50} value divided by the CEM IC_{50} value.

‡ Reported from Ref. 18.

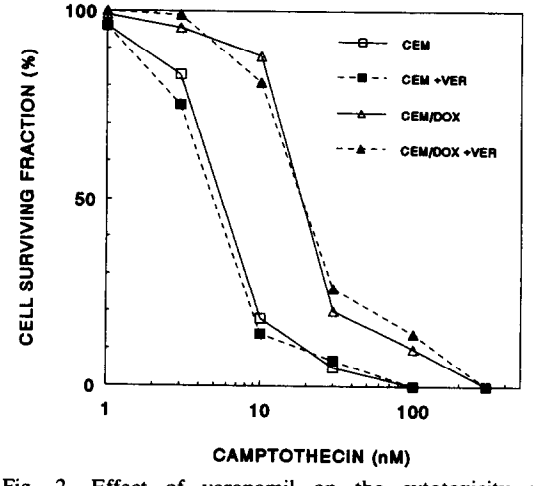

Fig. 2. Effect of verapamil on the cytotoxicity of camptothecin in CEM and CEM/DOX cell lines. Cell growth was determined after incubation in culture medium for 96 hr, as indicated in Materials and Methods, in the presence or the absence of verapamil (4 μM) for each concentration of camptothecin tested. Values are the mean of two separate experiments, each point in quadruplicate. SD are lower than 5%.

effect on the camptothecin cytotoxicity on either CEM or CEM/DOX cell lines, and suggest that P-glycoprotein is not involved in the mechanism of cross-resistance to camptothecin in CEM/DOX cells. These data indicate that CEM/DOX cells exhibit an additional mechanism of resistance towards camptothecin.

Northern blot analysis of topoisomerases I and II and RAG transcripts in CEM and CEM/DOX cells

To determine whether the expression of topoisomerases I and II and recombinase activating genes is altered in CEM/DOX cells, we used northern blots with specific probes to analyse total RNA from CEM and CEM/DOX cells (Fig. 3). The results indicated that there was no significant modulation of the topoisomerase II mRNA level while the topoisomerase I and RAG1 mRNA levels were higher in CEM/DOX than in CEM cells (Fig. 3). Hybridization with the murine β actin probe was used as an internal control for evaluating the amount of RNA loaded in each lane. Hybridization was also performed with the probes for the RAG2 and T160 genes, which correspond to other known components of the V(D)J recombination [3, 4]. No transcript could be detected for RAG2 in either CEM and CEM/DOX RNA, while the assay readily detected the RAG2 transcript in thymus RNA from mouse (Fig. 3) or from rat (data not shown). This negative result, confirmed by several independent experiments, was obtained even when the blot membrane was 10-fold overexposed to the autoradiogram. The T160 transcript was found at a low level in CEM, CEM/DOX and thymus RNA, and there was no evidence of significant variation in the transcription of this gene between sensitive and resistant cells (Fig. 3).

In order to study the influence of the cell growth variations on the RAG1 and topoisomerase I and II mRNA levels, RNA was prepared from CEM and CEM/DOX cells, collected during exponential phase and during plateau-phase (Fig. 4). The transcript levels were evaluated by densitometric scanning of the autoradiograms and values were normalized against the β actin transcript level. Results, summarized in Table 4, showed that in RNA from CEM and CEM/DOX cells, RAG1 and topoisomerase II transcripts increased by 2-fold in the exponential phase of growth compared to the

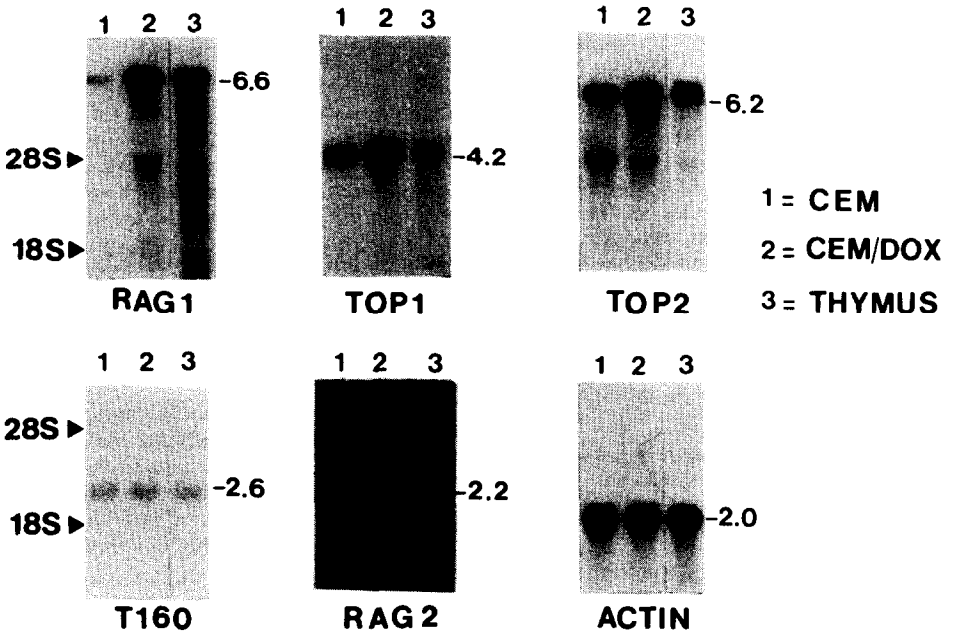

Fig. 3. Northern blot analysis of topoisomerase and recombinase gene transcripts in CEM and CEM/DOX cell lines. 10 μg of total RNA were electrophoretically resolved on 1% agarose formaldehyde gels, blotted onto Hybond N$^+$ filters and hybridized with RAG1, topoisomerase I (Top 1), topoisomerase II (Top 2), T160, RAG2 and β-actin probes (see Materials and Methods): lane 1, CEM cells; lane 2, CEM/DOX cells; lane 3, murine thymus. Numbers on the right indicate the size (kb) of the transcript detected.

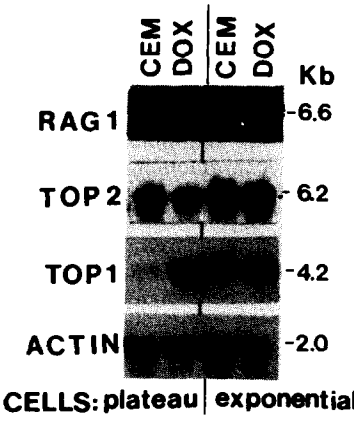

Fig. 4. Variation of transcript level in CEM sensitive and resistant cell lines in plateau or exponential phase of growth. Total RNA (10 μg) from CEM or CEM/DOX cells taken in exponential or plateau phase of growth (see Materials and Methods) was analysed by northern blot with RAG1, topoisomerase II (Top 2), topoisomerase I (top 1) and β-actin probes. Transcript size (kb) is indicated on the right of the figure. Transcript levels were measured by densitometric scanning, and the values obtained were normalized against actin values (reported in Table 4). DOX = CEM/DOX cells.

plateau phase, while no significant variation was found for topoisomerase I transcripts.

Results also indicated that RAG1 mRNA levels increased 18- and 14.7-fold in plateau and exponential CEM/DOX cells, respectively, while topoisomerase I mRNA levels increased 3.7- and 4.1-fold (Table 4). These data indicate that RAG1 and topoisomerase I mRNA levels are higher in CEM/DOX than in CEM cells, whatever the state of growth of the cells.

RAG1 gene locus

In order to search for alterations of the RAG1 gene structure which might be associated with the overexpression of RAG1, genomic DNA was digested with several restriction enzymes (*Bgl* II, *Bam*H I, *Eco*R I, *Hind* III, *Pst* I, *Pvu* I, *Xba* I and *Xho* I) and analysed by Southern blot hybridization, for which the 5.4 kb *Eco*R I fragment of the RAG1 cDNA was used as probe (see Materials and Methods). The cleavage patterns of CEM and CEM/DOX DNA produced by several restriction enzymes were compared with those obtained with the DNA from T24 human bladder carcinoma cells (Fig. 5A). No alteration of the RAG1 gene locus was detected in either sensitive CEM or resistant CEM/DOX cell lines.

In addition, no rearrangement of the topoisomerase I or topoisomerase II gene locus was detected when the same membranes were rehybridized with these probes (result not shown). As shown in Fig. 5B and confirmed by densitometric analysis, the copy number of these genes (RAG1,

Table 4. Relative levels of RAG1, topoisomerase I and topoisomerase II transcripts in exponentially growing or plateau phase CEM cells

Cell lines and culture conditions	Relative transcript level* RAG1	Topo I	Topo II
CEM plateau phase	1.0	1.0	1.0
CEM exponential	2.0 (1.0)	0.8 (1.0)	1.7 (1.0)
CEM/DOX plateau phase	18.0	3.7	1.1
CEM/DOX exponential	29.5 (14.7)	3.3 (4.1)	1.9 (1.1)

* mRNA transcript levels were determined by northern blot hybridization of total RNA. Filters were sequentially hybridized by RAG1, topoisomerase II, topoisomerase I and β-actin probes (see Materials and Methods). Autoradiograms from the experiment presented in Fig. 4 were scanned by a densitometric analyser (Ultroscan, Pharmacia) and values were normalized with the actin values.

Results are expressed relative to the mRNA level of CEM cells in plateau phase (values defined as 1), or relative to the mRNA level of CEM cells in exponential phase (number in brackets).

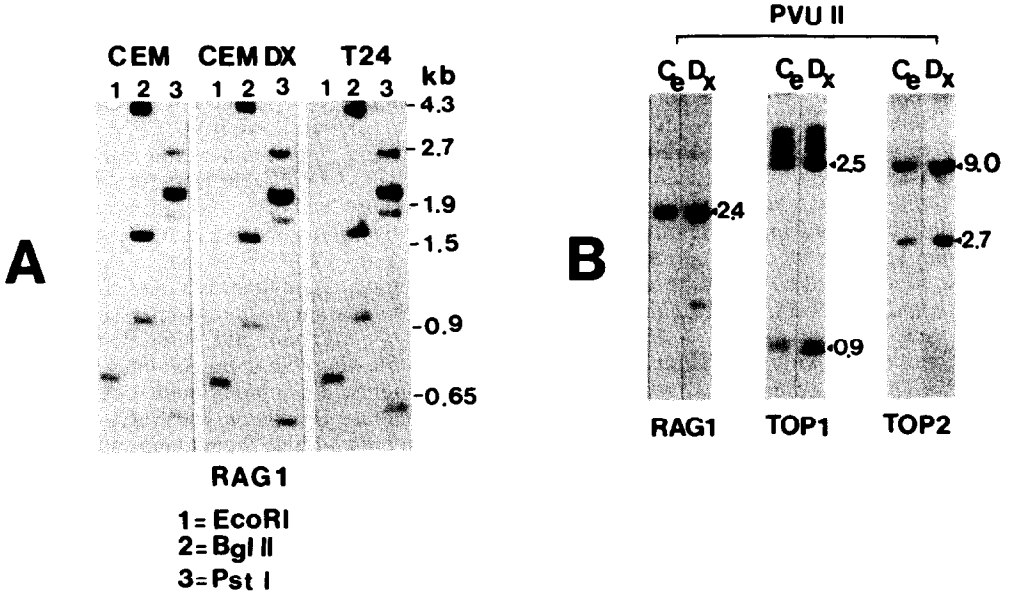

Fig. 5. Southern blot analysis of the genomic DNA from CEM, CEM/DOX and T24 cells. (Panel A) Genomic DNA (10 μg) from CEM, CEM/DOX (CEMDX) and T24 cell lines was digested with *Eco*R I (lane 1), *Bgl* II (lane 2) and *Pst* I (lane 3) and analysed by Southern hybridization with the human RAG1 probe (see Materials and Methods). No difference in the restriction cleavage pattern was observed for these three cell lines. Numbers on the right indicate the size (kb) of markers. (Panel B) DNA (10 μg) from CEM (C_e) or CEM/DOX (D_x) cell lines was digested with *Pvu* II and analysed by successive Southern hybridization with the RAG1, topoisomerase I (Top 1) and topoisomerase II (Top 2) probes. Numbers on the right indicated the size (kb) of the corresponding DNA fragment. Densitometric scanning indicates that the copy number of these three genes is identical in CEM and CEM/DOX cells.

topoisomerases I and II) is identical in CEM and CEM/DOX cells. These results indicated that the RAG1 mRNA overexpression in CEM/DOX cells is not caused by a gene amplification, nor by a readily detectable structural alteration, such as a rearrangement or deletion, of the RAG1 gene locus.

Lack of V(D)J recombination activity in CEM and CEM/DOX cells

To assay the V(D)J recombination activity in CEM and CEM/DOX cells, we used plasmid pHJ201 as the V(D)J recombination substrate. On this plasmid, which carries the Cam and Amp resistance

Table 5. V(D)J recombination activity in CEM and CEM/DOX cells

Cell line	Number of colonies Amp	CamAmp	R†
CEM*	4781	0	$<2.10^{-4}$
CEM/DOX*	2890	2‡	$<3.410^{-4}$
Control pJH201 (1 ng)	4534	0	—
Control pJH201Δ (1 ng)§	1360	920	—

* The number of transformants was corrected for a plating dilution factor and corresponds to 3% of the total DNA recovered after transfection of the pJH201 plasmid (see Materials and Methods).

† R, the frequency of recombination, corresponds to the number of CamAmp colonies divided by the number of Amp colonies.

‡ The two CamAmp colonies were false positives, since they did not grow after replating on CamAmp medium.

§ pJH201Δ, a deletion mutant of the pJH201 plasmid lacking the transcription terminator, was used as positive control for the CamAmp resistance.

genes, the V(D)J recombinational signal sequences flank a prokaryotic transcription terminator between the *E. coli* lack promoter and the CAT gene [25]. Correct V(D)J rearrangement induces the deletion of the transcription terminator and the expression of Cam resistance [25]. In order to demonstrate whether the RAG1 overexpression in CEM/DOX corresponds to an increased V(D)J recombination, pJH201 was transiently transfected into CEM and CEM/DOX cells, recovered after 48 hr, and then introduced into bacteria which were plated on Amp and AmpCam resistant media. The recombination frequency was calculated as the number of colonies resistant to both Amp and Cam divided by the total number of Amp colonies (Table 5). No AmpCam colonies were obtained with pJH201 recovered from CEM cells. This represented a recombination frequency of $< 2 \times 10^{-4}$. The recombination frequency obtained with pJH201 recovered from CEM/DOX cells was $< 3.4 \times 10^{-4}$.

These results indicate that no efficient V(D)J recombination activity occurred in either CEM or CEM/DOX cells.

DISCUSSION

CEM/DOX cells have been previously shown to overexpress and amplify the mdr1 gene, which is responsible for an active efflux of doxorubicin and other anticancer drugs [18]. Since topoisomerase II is one of the major targets of doxorubicin, we examined the mRNA transcript level and the activity of topoisomerase II in CEM/DOX cells. Our results indicate that (i) the topoisomerase II transcript level is not quantitatively modified, (ii) the topoisomerase II decatenation activity of CEM/DOX nuclei is identical to that of sensitive cells, (iii) the topoisomerase II inhibitor mAMSA is able to cleave the same amount of DNA with topoisomerase II preparations from CEM and CEM/DOX cells. These data strongly suggest that topoisomerase II is not qualitatively or quantitatively modified in CEM/DOX cells. On the other hand, we found that topoisomerase I activity is modified in CEM/DOX cells: (i) topoisomerase I transcript level is 4-fold increased, (ii) the topoisomerase I relaxation activity of CEM/DOX nuclei is 1.7-fold higher than in CEM cells, (iii) the topoisomerase I inhibitor camptothecin induces 2.5 times more DNA cleavage with topoisomerase I preparations from CEM/DOX cells than with preparations from CEM cells, (iv) the camptothecin-mediated cleavable complex formation in intact CEM/DOX cells is about 2-fold increased, compared to CEM cells. According to these data, it is likely that topoisomerase I activity is quantitatively increased in CEM/DOX cells. An increase of topoisomerase I activity was already found in several cell lines resistant to topoisomerase II inhibitors, such as Calc18/AM and P388/AMSA cell lines [27, 28]. However, in these cell lines the increase of topoisomerase I was associated with a decrease of topoisomerase II activity, and it was suggested that topoisomerases I and II could partially complement each other's biological functions. The topoisomerase I gene activation in CEM/DOX does not result from a gene amplification, nor from a rearrangement of the topoisomerase I gene locus (result not shown). However, despite the increase of topoisomerase I activity, we found CEM/DOX cells to be 11-fold more resistant than CEM cells to camptothecin. Camptothecin is not recognized by the mdr1 P-glycoprotein [26]. Accordingly, the lack of cross-resistance to camptothecin was confirmed in P388/VCR and KB/VLB resistant cell lines, which overexpress the P-glycoprotein (result not shown), and verapamil has no effect on the camptothecin cytotoxicity in CEM/DOX cells, suggesting that cross-resistance to camptothecin in CEM/DOX is not related to overexpression of the mdr1 gene. In addition, the correlation for the camptothecin-mediated cleavable complex formation between intact cells and nuclear extracts suggest that a modification of the drug penetration in resistant cells is not probable. However, other mechanisms may be involved in the observed resistance, such as cellular alterations following stabilization of the cleavable complex by camptothecin. Topoisomerase I and II inhibitors mediate their cytotoxic activity

via a common mechanism involving stabilization of the enzyme on DNA during the catalytic reaction [9]. Formation of the cleavable complex leads ultimately to cell death during replication by transforming nicks into irreversible DNA breaks, or during mitosis, by inducing aberrant chromosome recombinations [9, 10]. A potential pathway that allows resistant cells to overcome these lesions is an increased ability to repair them. Resistance mechanisms involving alterations of DNA repair have been reported for topoisomerase inhibitors and other antitumor agents which mediate direct or indirect DNA damage [11].

We have also examined the expression of the genes involved in V(D)J recombination. We found, by northern blot analysis, a 15–20-fold increase of the RAG1 gene transcript in CEM/DOX cells. The overexpression of RAG1 is not caused by a gene amplification, nor by an obvious alteration of the RAG1 gene locus, since no differences in the restriction enzyme pattern or the mRNA transcript size were found between CEM/DOX, CEM and T24 cells. This result does not exclude the possibility of sequence modifications or other alterations, such as a rearrangement in the regulatory regions of the gene, which have not yet been studied.

Interestingly, the expression of RAG1 and topoisomerase II genes in both CEM and CEM/DOX cells was 2-fold higher in exponentially growing cells than in plateau phase cells. The transcript level and the enzymatic activity of topoisomerase II were found to be directly related to the proliferative state of the cells [29, 30]. RAG1, RAG2 transcription and V(D)J recombination are transiently increased in lymphoid cells treated with agents that elevate cAMP [31]. These data indicate that cell growth may generate intra- or extra-cellular stimuli that can modulate the expression of RAG1.

Other genes, such as RAG2 and T160, participate in the formation of the V(D)J recombinase complex [3, 4]. Our results indicate that RAG2 gene expression is undetectable in both CEM and CEM/DOX cells and that T160 is expressed at a resting level which is not modified in CEM or CEM/DOX cells. Previous studies have shown that the concordant expression of RAG1 and RAG2 genes is necessary for the activation of V(D)J recombination and the resulting T cell receptor rearrangement in pre-B or pre-T cells [3]. In contrast, the T160 gene product, which probably plays a role in the sequence-recognition of the recombinase complex formation, was found to be ubiquitously transcribed in adult tissues, that do not exhibit V(D)J recombination [4]. The lack of RAG2 gene expression in our experiments suggests that V(D)J recombination is not efficient in CEM or CEM/DOX cells. Transient transfection of the recombination substrate indicated that V(D)J recombination occurs at a frequency lower than 10^{-4} in CEM or CEM/DOX cells. This figure corresponds to the frequency of recombination observed in non-lymphoid cells, such as fibroblasts, and represents a resting level of unspecific recombination [3]. According to a previous report [3], these results indicate that RAG1 gene expression is not sufficient by itself to generate efficient V(D)J recombination and suggest that in CEM/DOX cells, the RAG1 gene is diverted from its normal biological function.

Other studies have indicated that in CEM cells, an abnormal recombinase activity is responsible for the disruption of the SCL locus (also called TCL5 and TAL1) which is implicated in 25% of T.ALL neoplasms [32–34]. It is, however, unclear whether this translocation is mediated by an abnormal RAG1 function or by another factor associated with the recombinase complex [35]. As shown by our results, a discordant expression of RAG1 and RAG2 genes, which is originally observed in CEM sensitive cells, may explain by itself a recombination error at the SCL locus which is not a normal V(D)J site.

In order to determine the putative function of RAG1 in the resistance to topoisomerase inhibitors in CEM/DOX cells, Cos-7 fibroblasts were transiently transfected with eukaryotic RAG1 expression vectors. These experiments indicated that transfected cells had an increased topoisomerase I activity and displayed a resistance phenotype relative to the cytotoxic effect of doxorubicin and camptothecin (J. F. Riou, manuscript in preparation). Therefore, increased topoisomerase I activity in CEM/DOX cells might be the direct or indirect consequence of increased RAG1 gene expression. Topoisomerases and recombinases have a common function in controlling genome stability [6, 7], and the modulation of DNA topology is probably an important factor which controls the complex processes involved during DNA recombination [36, 37]. Thus, the concerted co-activation of RAG1 and topoisomerase I genes may ensue from their functional regulation. Topoisomerase inhibitors are known to promote illegitimate recombination *in vitro* [38, 39] and chromosome aberrations by recombination during mitosis [9, 10]. The unusual resistance to camptothecin in CEM/DOX cells and to topoisomerase I and II inhibitors in Cos-7 cells transfected with RAG1 might be related to increased recombination, driven by RAG1, which remove the drug-induced DNA lesions before their transformation in lethal events. On the other hand, V(D)J recombination was found impaired in several double-strand break repair mutants and Taccioli *et al.* [40] recently provided evidence that several factors encoded by independent genes are each involved in both DNA repair and V(D)J recombination. Thus, it is also possible that one of these factors is activated by RAG1 overexpression and participates to remove the DNA lesions.

In conclusion, our result is the first evidence that both topoisomerase I and recombinase gene transcription may be altered in a T.ALL cell line resistant to a topoisomerase II inhibitor. Since 25% of human T.ALL neoplasms have aberrant recombinase functions [33–35], it would be interesting to determine whether other T.ALL cell lines resistant to anticancer agents similarly overexpress the RAG1 gene.

Acknowledgements—We are very grateful to Dr M. C. Chevallier-Multon for the CEM/DOX cell line. We thank Dr M. Gellert for the gift of the pJH201 vector and Dr D. Schatz for the RAG probes. Corrections of the manuscript by Dr K. Pepper are greatly appreciated. We also thank

Drs C. J. Larsen and O. Bernard for helpful discussion and L. Ramdani for typing this manuscript.

REFERENCES

1. Blackwell TK and Alt FW, Molecular characterization of the lymphoid V(D)J recombination activity. *J Biol Chem* **264**: 10327–10330, 1989.
2. Schatz DG, Oettinger MA and Baltimore D, The V(D)J recombination activating gene, RAG-1. *Cell* **59**: 1035–1048, 1989.
3. Oettinger MA, Schatz DG, Gorka C and Baltimore D, RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science* **248**: 1517–1523, 1990.
4. Shirakata M, Hüppi K, Usuda S, Okazaki K, Yoshida K and Sakano H, HMG1-related DNA-binding protein isolated with V-(D)-J recombination signal probes. *Mol Cell Biol* **11**: 4528–4536, 1991.
5. Aguilera A and Klein HL, *HPR1*, a novel yeast gene that prevents intrachromosomal excision recombination, shows carboxy-terminal homology to the *Saccharomyces cerevisiae TOP1* gene. *Mol Cell Biol* **10**: 1439–1451, 1990.
6. Wang JC, Caron PR and Kim RA, The role of DNA topoisomerases in recombination and genome stability: a double-edged sword? *Cell* **62**: 403–406, 1990.
7. Wang JC, DNA topoisomerases: why so many? *J Biol Chem* **266**: 6659–6662, 1991.
8. Caron PR and Wang JC, DNA topoisomerases as targets of therapeutics: a structural overview. In *Molecular Biology of DNA Topoisomerases and its Application to Chemotherapy* (Ed. Andoh T), pp. 1–18. CRC Press Inc., Boca Raton, Florida, 1993.
9. Liu LF, DNA topoisomerase poisons as antitumor drugs. *Annu Rev Biochem* **58**: 351–375, 1989.
10. Charron M and Hancock R, Chromosome recombination and defective genome segregation induced in Chinese hamster cells by the topoisomerase II inhibitor VM-26. *Chromosoma* **100**: 97–102, 1991.
11. Hayes JD and Wolf R, Molecular mechanisms of drug resistance. *Biochem J* **272**: 281–295, 1990.
12. Beck WT, Multidrug resistance and its circumvention. *Eur J Cancer* **26**: 513–515, 1990.
13. Zijstra JG, de Vries EGE and Mulder NH, Multifactorial drug resistance in an adriamycin-resistant human small cell lung carcinoma cell line. *Cancer Res* **47**: 1780–1784, 1987.
14. Ganapathi R, Grabowski D, Ford J, Heiss C, Kerrigan D and Pommier Y, Progressive resistance to doxorubicin in mouse leukemia L1210 cells with multidrug resistance phenotype: reductions in drug-induced topoisomerase II-mediated DNA cleavage. *Cancer Commun* **1**: 217–224, 1989.
15. Deffie AM, Bosman DJ and Goldenberg GJ, Evidence for a mutant allele of the gene for DNA topoisomerase II in adriamycin-resistant P388 murine leukemia cells. *Cancer Res* **49**: 6879–6882, 1989.
16. Beck WT, Unknotting the complexities of multidrug resistance: the involvement of DNA topoisomerases in drug action and resistance. *J Natl Cancer Inst* **81**: 1683–1685, 1989.
17. Epstein RJ, Drug induced DNA damage and tumor chemosensitivity. *J Clin Oncol* **8**: 2062–2084, 1990.
18. Chevallier-Multon M-C, Jacquemin-Sablon A, Besselièvre R, Husson H-P and Le Pecq J-B, Comparative cytotoxicities of a series of ellipticine and olivacine derivatives on multidrug resistant cells of human and murine origins. *Anticancer Drug Design* **5**: 319–335, 1990.
19. Sambrook J, Fritsch EF and Maniatis T, In: *Molecular Cloning, a Laboratory Manual*. Second Edition. Cold Spring Harbor Laboratory Press, New York, 1989.
20. Bjornsti MA, Benedetti P, Viglianti GA and Wang JC, Expression of human DNA topoisomerase I in yeast cells lacking yeast DNA topoisomerase I: restoration of sensitivity of the cells to the antitumor drug camptothecin. *Cancer Res* **49**: 6318–6323, 1989.
21. Tsai-Pflugfelder M, Liu LF, Liu AA, Tewey KM, Whang-Peng J, Knutsen T, Huebner K, Croce CM and Wang JC, Cloning and sequencing of cDNA encoding human DNA topoisomerase II and localization of the gene to chromosome region 17q21-22. *Proc Natl Acad Sci USA* **85**: 7177–7181, 1988.
22. Alonso S, Minty A, Bourlet Y and Buckingham M, Comparison of three actin-coding sequences in the mouse; evolutionary relationships between the actin genes of warm-blooded vertebrates. *J Mol Evol* **23**: 11–12, 1986.
23. Riou JF, Helissey P, Grondard L and Giorgi-Renault S, Inhibition of eukaryotic DNA topoisomerase I and II activities by indoloquinolinedione derivatives. *Mol Pharmacol* **40**: 699–706, 1991.
24. Wolverton JS, Danks MK, Granzen B and Beck WT, DNA topoisomerase II immunostaining in human leukemia and rhabdomyosarcoma cell lines and their responses to topoisomerase II inhibitors. *Cancer Res* **52**: 4248–4253, 1992.
25. Hesse JE, Lieber MR, Gellert M and Mizuuchi K, Extrachromosomal DNA substrates in pre-B cells undergo inversion or deletion at immunoglobulin V-(D)-J joining signals. *Cell* **49**: 775–783, 1987.
26. Chen AY, Yu C, Potmesil M, Wall ME, Wani MC and Liu LF, Camptothecin overcomes MDR1-mediated resistance in human KB carcinoma cells. *Cancer Res* **51**: 6039–6044, 1991.
27. Lefevre D, Riou JF, Ahomadegbe JC, Zhou D, Bernard J and Riou G, Study of molecular markers of resistance to *m*-AMSA in a human breast cancer cell line. Decrease of topoisomerase II and increase of both topoisomerase I and acidic glutathione *S*-transferase. *Biochem Pharmacol* **41**: 1967–1979, 1991.
28. Tan KB, Mattern MR, Eng W-K, McCabe FL and Johnson RK, Nonproductive rearrangement of DNA topoisomerase I and II genes: correlation with resistance to topoisomerase inhibitors. *J Natl Cancer Inst* **81**: 1732–1735, 1989.
29. Hsiang YH, Wu HY and Liu LF, Proliferation-dependent regulation of DNA topoisomerase II in cultured human cells. *Cancer Res* **48**: 3230–3235, 1988.
30. Riou JF, Lefevre D and Riou G, Stimulation of the topoisomerase II induced DNA cleavage sites in the c-myc protooncogene by antitumor drugs is associated with gene expression. *Biochemistry* **28**: 9104–9110, 1989.
31. Menetski JP and Gellert M, V(D)J recombination activity in lymphoid cell lines is increased by agents that elevate cAMP. *Proc Natl Acad Sci USA* **87**: 9324–9328, 1990.
32. Aplan PD, Mombardi DP, Ginsberg AM, Cossman J, Bertness VL and Kirsch IR, Disruption of the human SCL locus by "illegitimate" V-(D)-J recombinase activity. *Science* **250**: 1426–1429, 1990.
33. Brown L, Cheng J-T, Chen Q, Siciliano MJ, Crist W, Buchanan G and Baer R, Site-specific recombination of the *tal*-1 gene is a common occurrence in human T cell leukemia. *EMBO J* **9**: 3343–3351, 1990.
34. Bernard O, Lecointe N, Jonveaux P, Souyri M, Mauchauffé M, Berger R, Larsen CJ and Mathieu-Mahul D, Two site-specific deletions, and t(1;14) translocation restricted to human T cell acute leukemias disrupt the 4′ part of the tal-1 gene. *Oncogene* **6**: 1477–1488, 1991.
35. Boehm T and Rabbitts TH, The human T cell receptor genes are targets for chromosomal abnormalities in T cell tumors. *FASEB J* **3**: 2344–2359, 1989.

36. Kanaar R and Cozzarelli NR, Role of supercoiled DNA structure in DNA transactions. *Curr Opinion Structural Biol* **2**: 369–379, 1992.
37. Wasserman SA and Cozzarelli NR, Biochemical topology: application to DNA recombination and replication. *Science* **232**: 951–960, 1986.
38. Bae YS, Kawasaki I, Ikeda H and Liu LF, Illegitimate recombination mediated by calf thymus DNA topoisomerase II *in vitro*. *Proc Natl Acad Sci USA* **85**: 2076–2080, 1988.
39. Shuman S, Vaccinia DNA topoisomerase I promotes illegitimate recombination in *Escherichia coli*. *Proc Natl Acad Sci USA* **86**: 3489–3493, 1989.
40. Taccioli GE, Rathbun G, Oltz E, Stamato T, Jeggo PA and Alt FW, Impairment of V(D)J recombination in double-strand break repair mutants. *Science* **260**: 207–210, 1993.