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Involvement of Gene-Specific DNA Damage and Apoptosis in the Differential Toxicity of Mitomycin C Analogs towards B-Lineage Versus T-Lineage Lymphoma Cells

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ABSTRACT. Avian and mammalian B- and T-lineage lymphocytes display differential sensitivity to a variety of genotoxic agents. Specifically, T-lineage cells show a high degree of resistance to the toxic effects of exposure to chemotherapeutic drugs, whereas B-lineage cells show a high degree of sensitivity. We used a model system consisting of virally transformed B- and T-lymphoma cell lines to further define the cellular and molecular mechanisms responsible for the differential toxicity of two chemotherapeutic drugs that induce DNA-interstrand cross-links to different degrees, mitomycin C (MMC) and its aminodisulfide analog, BMY 25067. Quantification of the number of cross-links introduced in the transcriptionally active ribosomal RNA gene cluster revealed that similar levels of DNA damage were induced in B- and T-lymphoma cell lines. However, B-lymphoma cells were highly sensitive to induction of apoptosis and inhibition of growth compared with the more resistant T-lymphoma cells for both compounds. BMY 25067 induced approximately 2-fold more cross-links in rDNA than did MMC, along with a concurrent enhanced induction of apoptosis in both B- and T-lymphoma cell lines. An analysis of the persistence of DNA lesions over multiple cell cycles revealed that neither B- nor T-lymphoma cells repaired DNA cross-links to a significant extent. These data suggest that differences in the extent or persistence of DNA-interstrand cross-links are not responsible for the differential toxicity of MMC and its analog towards B- versus T-lineage cells. Rather, differential drug toxicity involves early and extensive entry into apoptosis in B-lymphoma cells contrasted to the delayed and minimal apoptotic induction in T-lymphoma cells. BIOCHEM PHARMACOL 53;6:811–822, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. mitomycin C; DNA cross-links; ribosomal DNA; lymphoma cells; apoptosis; mitomycin C resistance

Tumor cell-specific differences in resistance to killing by chemotherapeutic drugs pose a significant problem in the treatment of a variety of neoplastic diseases. Although considerable progress has been made in the treatment of various animal and human leukemias and lymphomas, resistance to chemotherapy remains a significant problem in the treatment of T-cell malignancies as well as treatment of relapsed tumors.

Differences in the sensitivity of lymphoma cells to chemotherapeutic drugs may be attributed to a variety of factors. Differential uptake, or efflux of compounds mediated by the P-glycoprotein pump, and differences in cellular metabolism, resulting in enhanced activation or detoxification of compounds, can play a significant role in determining cellular responses to chemotherapeutic drugs. In addition, differences in the levels of DNA damage and repair, as well as differences in cellular responses to the presence of DNA lesions, particularly with respect to entry into apoptosis, may also be important in determining lymphoma cell susceptibility to genotoxic drugs. Given the many mechanisms that could contribute to drug resistance, it has been difficult to determine the relative contribution of a gene-regulated apoptotic pathway in multidrug lymphoma cell resistance.

Apoptosis is a process in which, upon exposure to certain agents, cells engage in a specific metabolic pathway culminating in cell death [1]. Genotoxic chemotherapeutic drugs induce apoptosis in a variety of cell types where the induction of DNA damage is believed to be the cellular signal that initiates the apoptotic pathway. Cell death by apoptosis can be distinguished from necrosis by virtue of characteristic morphological and molecular features. The process of apoptosis involves morphological changes in the cell including cell shrinkage and blebbing, formation of segregated chromatin masses in cells, and degradation of chromatin in the internucleosomal spacer region resulting in the generation of a characteristic "nucleosomal ladder."

Resistance of cells to the induction of apoptosis may be

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acquired during the process of tumorigenesis due to the accumulation of mutations in specific genes involved in the apoptotic pathway, such as the tumor suppresser gene *p53*. Conversely, cellular resistance to apoptosis may be an intrinsic property of specific cell types. For example, in a number of experimental systems, B-lineage lymphocytes show enhanced sensitivity to the cytotoxic and genotoxic effects of chemotherapeutic drugs compared with T-lineage lymphocytes. Selective toxicity of drugs towards B-lineage lymphocytes was first demonstrated in the chicken embryo [2] and subsequently in mammalian systems [3]. Differential toxicity of genotoxic drugs, including cyclophosphamide, aflatoxin B_1 and MMC,† to B-lineage lymphocytes and the concurrent resistance of T-lineage lymphocytes in the chick embryo are evidenced by an increased rate of SCE and by the specific depletion of bursal resident B-lymphocytes [4, 5]. Subsequent studies showed that the observed B-cell depletion was due to preferential induction of apoptosis in this cell population [6, 7]. Such differences in susceptibility to the induction of apoptosis are also observed in virally transformed chicken cell lines of B- and T-cell origin. Several chemotherapeutic agents preferentially induce apoptosis in B-lymphoma cells, whereas T-lymphoma cells show extreme resistance to induction of apoptosis‡ [6]. Sensitivity of human B-lymphoma cell lines over that of T-lymphoma cell lines to MMC-induced cytotoxicity also has been demonstrated [8]. The refractory nature of T-cell leukemias and lymphomas to standard chemotherapy has been shown [9, 10], but it is not known if this drug resistance involves failure to induce an apoptotic response as a primary mechanism.

The induction of DNA damage by certain chemotherapeutic drugs can be an early step in the cellular signaling pathway for induction of apoptosis. Recently, techniques that allow for the detection of DNA damage in specific genes have been developed [11]. These studies show that the induction and repair of DNA damage in transcriptionally active genes differs from that of bulk DNA. Specifically, the DNA of actively transcribed genes is repaired preferentially over that of non-transcribed genes [12, 13]. Deficiencies in the ability to repair actively transcribed genes have been associated with increased sensitivity to genotoxic drugs in cells from individuals with DNA-repair deficiency syndromes [14]. It is therefore of interest to determine the relationship between the levels of gene-specific damage and repair in tumor cells, and the propensity of such cells to undergo apoptosis.

In the present investigation, we examined several candidate mechanisms underlying B-lymphoma cell sensitivity and T-lymphoma cell drug resistance that may be part of a cascade leading to cell demise. We used established lymphoma cell lines derived from virus-induced cancers including avian leukosis virus-induced B-cell lymphoma and Marek's disease virus-induced T-cell lymphoma. The latter is a particularly aggressive lymphoma. The mechanisms we studied included differential induction of gene-specific interstrand cross-links and their persistence, differential downstream triggering of apoptosis (as distinguished from necrosis), and the influence of a distal detoxification system, mediated by GST. We used the RAGE assay [11] to determine the extent of MMC-induced DNA-ICL formation in the ribosomal RNA gene cluster (rDNA) of B- and T-lymphoma cell lines, which were used previously to demonstrate differential MMC resistance [6]. Additionally, we studied an aminodisulfide MMC analog, BMY 25067, which has been reported to be more toxic than MMC [15, 16] and, unlike MMC, BMY 25067 is insensitive to detoxification by GST [16]. The GST/GSH enzyme system is known to modulate drug resistance in some cell types, but its role in differential B- versus T-lymphoma cell toxicity is unknown. These studies with MMC and its analog also permitted a test for an association between the extent of gene-specific DNA-ICL formation (modulating the DNA damage signal) and potency for engaging the apoptotic process in the respective lymphoma cell types.

MATERIALS AND METHODS

Chemicals

MMC was obtained from the Sigma Chemical Co. (St. Louis, MO). The aminodisulfide MMC analog BMY 25067, *N*-7-[2-(4-nitrophenyldithio) ethyl]-MMC, was provided by the Bristol–Myers Squibb Co. (Princeton, NJ). BMY 25067 was dissolved in DMSO. MMC was dissolved in PBS (GIBCO, Grand Island, NY) or, alternatively, in DMSO for the experiments involving direct comparisons of the two compounds. Fresh solutions of drugs were prepared for each experiment.

Cell Lines and Growth Curves

Six virally transformed B- and T-lymphoma chicken cell lines were selected based on the similarities of their phenotypes and functions with differentiating B- and T-lymphocytes. The LSCC-DT40 cell line is derived from an early B-cell, expressing IgM and Ia surface markers [17]. This cell line is particularly valuable since it retains the B-cell function of diversification of rearranged immunoglobulin light-chain genes. The LSCC-DT95 [17] and LSCC-RP9 [18] cell lines were both derived from late B-cells. The DT95 cell line expresses IgM, and additionally has a plasma cell phenotype, including secretion of IgM. In contrast to the above B-cell lines, RP9 does not express IgM and is considered to have de-differentiated. The *myc* oncogene is activated in all three B-cell lines as a result of insertion of the LTR region of the avian leukosis virus.

The T-lymphoma cell line MDCC-CU159 is derived from a partially differentiated T-cell, expressing TCR2 re-

† *Abbreviations*: MMC, mitomycin C; DNA-ICL, DNA-interstrand cross-link(s); H/PI, Hoechst/propidium iodide; RAGE, renaturing agarose gel electrophoresis; rDNA, ribosomal DNA; SCE, sister chromatid exchanges; and GST/GSH, glutathione-*S*-transferase/glutathione.

‡ Sotiriadis J and Bloom SE, unpublished data.

ceptor and $CD3^+$, $CD4^+/CD8^-$, and Ia antigens [19]. The MSB1 cell line [20] is also a $TCR2^+$ T-cell expressing $CD3^+$, $CD4^+/CD8^-$, and Ia antigens. Both of these T-cell lines were derived from transformation of T-cells with Marek's disease virus, a highly oncogenic herpesvirus. In contrast, RECC-CU91, which also has a T-cell phenotype, was derived from transformation with reticuloendotheliosis virus [21]. These cells express the TCR3 T-cell receptor (TCR3), are $CD3^+/CD4^-$, $CD8^-$, and they overexpress the *rel* oncogene.

All of the cell lines grow optimally and similarly in the same medium formulation, and have doubling times of approximately 12 hr. The cell lines were cultured in RPMI 1640 medium (GIBCO), supplemented with 5% fetal bovine serum, 5% chick serum, penicillin-streptomycin, and L-glutamine. Cells were grown at 39°, 5% CO_2 and 95% humidity.

For studies of inhibition of growth, cells were plated at a concentration of 2×10^5/mL, in a total of 10 mL in 25-cm^2 tissue culture flasks, and allowed to equilibrate for 2 hr. Experimental drugs were added to each flask in 100-μL aliquots to generate the final concentrations for each experiment. Replicate flasks were included for each concentration as well as solvent control treatments. When DMSO was used as the solvent, the final concentration did not exceed 1% in the cultures. At this level, DMSO did not affect either growth or induction of apoptosis in the cultures.

After addition of the drugs, cells were incubated at 39° for 1 hr. After this time, cells were washed with PBS, and replated in fresh medium. For cell counts, aliquots of 250 μL were taken at 0, 24, and 48 hr, diluted into 20 mL of Isoton II diluent (Coulter Electronics, Miami, FL), and counted on a model ZM Coulter Counter (Coulter Electronics). Relative increases in cell numbers, as compared with those of the solvent controls, were determined for each culture after 24 and 48 hr.

Detection of Gene-Specific DNA-ICL

The RAGE assay [11] was used for the detection of MMC- and BMY 25067-induced DNA-ICL in the ribosomal RNA gene cluster. For these experiments, cells were plated at a density of 1×10^6/mL and allowed to equilibrate for 2 hr. Cells were then exposed to the compounds at concentrations of 0, 1.0, or 10 μg/mL for 1 hr at 39°. Immediately following exposure, cells were harvested by centrifugation at 200 *g* for 10 min, washed with PBS, and lysed in 0.2 M NaCl, 10 mM EDTA, 10 mM Tris–HCl (pH 7.8), 2% SDS. Proteinase K was added to a final concentration of 0.5 mg/mL, and the lysates were incubated at 37° overnight. Lysates were then extracted twice with phenol:chloroform, once with chloroform, and precipitated with ethanol. The DNA was dissolved in 10 mM Tris–HCl (pH 7.5), 1 mM EDTA (TE buffer) and treated with RNase A at a final concentration of 10 μg/mL at 37° for 1 hr. The samples were subjected to organic extraction as described above, followed by precipitation with ethanol.

Experiments on persistence of MMC-induced DNA-ICL were performed as above except, after washing with PBS for removal of the drug, the cultures were replated in fresh medium and incubated for various post-treatment times. Cells were then harvested, and DNA was purified as described above.

For the RAGE assay, aliquots of 10 μg of DNA dissolved in TE buffer were digested with the restriction enzyme *Pst*I. Depending on the sample, the DNA was subjected to either control conditions (no denaturing) or heat treated in 0.2 N NaOH at 55° for 10 min. Samples were then subjected to electrophoresis under neutral conditions in a 0.8% agarose gel in Tris–borate buffer (pH 7.5). Following electrophoresis, the gel was immersed in 0.25 M HCl for 10 min, then in 0.5 N NaOH and 1.5 M NaCl for 2 × 20-min periods. DNA was transferred overnight to BRL Photogene Nylon Membrane (Life Technologies Inc., Gaithersburg, MD) using 10× SSC (1× SSC = 0.015 M sodium citrate and 0.15 M NaCl). Following the transfer, the filter was baked for 2 hr at 80°.

Hybridization was performed using the Boehringer Mannheim Genius Chemiluminescent detection system. Plasmid pITS [22], which contains the entire internal transcribed spacer, the coding regions for the entire 5.8S, plus a portion of the 18S and 28S chicken ribosomal RNA, was labeled with digoxigenin-dUTP using the Boehringer Mannheim random primer kit. The membrane was prehybridized for a minimum of 4 hr and then hybridized overnight using the labeled probe described above. Both prehybridization and hybridization were performed at 65°, in 5× SSC, 0.1% *N*-lauroylsarcosine, 0.02% SDS, and 2% (w/v) blocking reagent (Boehringer Mannheim, Indianapolis, IN). Following hybridization, the filter was washed for 3 × 30-min intervals in 0.5× SSC at 65°. Following the washes, the filter was blocked for 3 hr, and subjected to treatment with anti-digoxigenin alkaline phosphatase conjugate for 30 min. Filters were treated with Lumi-phos 530, incubated, and exposed to Kodak XLS-5 film.

Preliminary experiments were performed to ensure linearity of the signal in hybridization experiments. A standard curve was obtained by using known quantities of plasmid DNA or increasing amounts of genomic DNA subjected to hybridization and detection procedures. Densitometry was performed with an LKB model 2222-020 Ultrascan XL laser densitometer.

Detection of Nucleosome Ladders

For the detection of drug-induced nucleosome ladders, cells were treated with various concentrations of the drugs for 1 hr, then washed with PBS and replated in fresh medium. Cells were harvested 8 hr following drug exposure, and DNA was purified as described previously. Aliquots of 10 μg of DNA were subjected to electrophoresis in a 1.5% agarose gel in Tris–borate buffer. The gel was stained with ethidium bromide and photographed using UV-transillumination at 300 nm.

Cytological Detection of Apoptosis with the H/PI Assay

The induction of apoptosis in cell cultures was analyzed 24 hr following drug exposure using a double-fluorescence staining technique. The procedure allows simultaneous detection of plasma membrane integrity by dye exclusion and apoptotic phenotypes by observing condensed, segregated chromatin in "live" cells [6]. Briefly, cells were stained in 20 μg/mL PI (emitting red fluorescence) and 100 μg/ml Hoechst 33342 (emitting blue fluorescence) for 15 min, at 37° in the dark. The double fluorescence was detected with a Leitz Aristoplan microscope equipped with an epifluorescence system and a long-pass filter cube A. Dead cells emit red and live cells blue fluorescence. Apoptotic cells have a characteristic phenotype of condensed, segregated chromatin in intact but shrunken cells (fluorescing blue in early stages and red later on). The apoptotic phenotype was easy to detect and discriminate from necrotic cells, which were swollen, had irregular/damaged membranes, and were PI positive. The chromatin was minimally condensed with some accumulation near the nuclear membrane. Typically, 200 cells were scored for each sample and classified as either necrotic, apoptotic, or normal/viable.

Statistical Analysis

Statistical analysis with respect to inhibition of growth and induction of apoptosis was performed using a one-way ANOVA followed by post-hoc testing with either Fisher's Protected Least Significant Difference (Fisher's PLSD) or Sheffe's F-test. Significance levels are indicated in the text. All statistics were performed using the StatView II data analysis program (Abacus Concepts, Inc., Berkeley, CA).

RESULTS

Differential Toxicity of MMC to B- and T-Lymphoma Cells

To study the selective toxicity of MMC towards B-lineage compared with T-lineage lymphoma cells, the responses of six cell lines derived from, and retaining characteristics of, B- and T-lymphocytes *in vivo* were examined. LSCC-cell lines DT40, DT95, and RP9 are B-lymphoma cells, whereas cell lines MDCC-CU159, RECC-CU91, and MDCC-MSB1 are T-lymphoma cells.

Cultures of each cell line were exposed to MMC at concentrations of 0, 0.1, 1.0, and 10 μg/mL for a period of 1 hr, followed by recovery periods in fresh medium of up to 48 hr. Minimal inhibition of growth was detected in two of the T-cell lines, CU159 and MSB1, at 0.1 and 1 μg/mL, with CU159 showing the smallest degree of growth inhibition at all three concentrations (Fig. 1). In contrast, all three B-cell lines, DT40, DT95 and RP9, showed statistically significant decreases in growth (using Scheffe's F-test, $P \leq 0.01$) related to the concentrations of MMC (Fig. 1). Interestingly, the T-cell line CU91 also experienced substantial growth suppression. However, DT40 cells were the most sensitive to MMC-induced growth inhibition, showing a marked growth suppression (≈40% of control) even at the lowest concentration of 0.1 μg/mL. Thus, with the exception of the CU91 cell line, the B-cell lines were par-

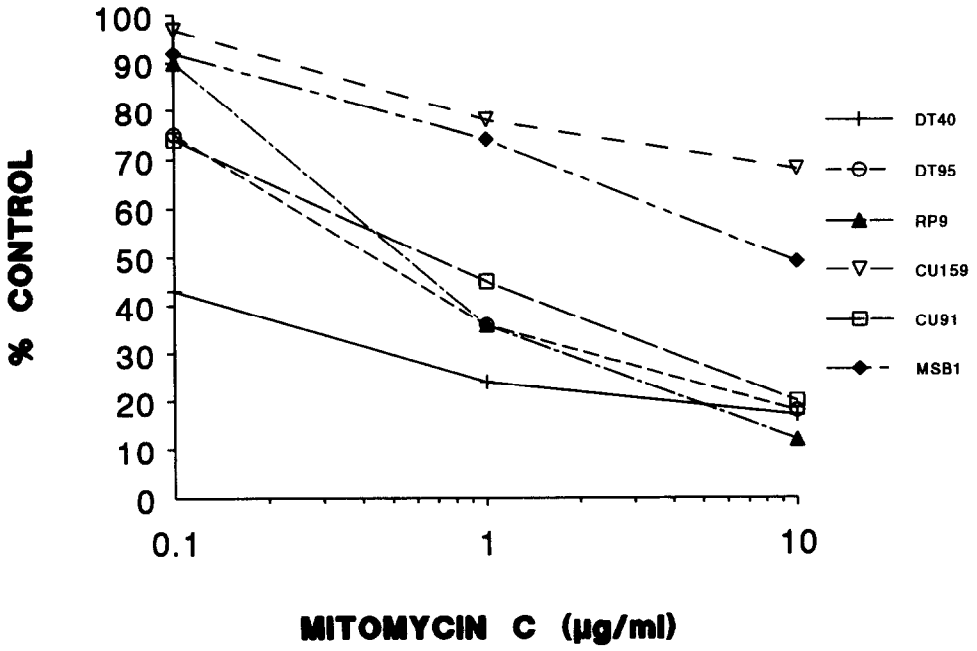

FIG. 1. Inhibition of cell growth by MMC in B- and T-lymphoma cells. Cultures containing 2×10^5 cells/mL of B-lymphoma cell lines DT40, DT95, and RP9 and T-lymphoma cell lines CU159, CU91, and MSB1 were exposed to 0, 0.1, 1.0, or 10 μg/mL of MMC for 1 hr. Cell numbers, expressed as percent of control values, were determined 48 hr following drug exposure. Each point is the average of duplicate cultures for two experiments.

ticularly sensitive and the T-cell lines relatively insensitive to MMC-induced growth inhibition.

Differential Inhibition of Cell Growth by BMY 25067 Compared with MMC in B- and T-Lymphoma Cells

The MMC analog BMY 25067 has been reported to be more toxic than its parent form. We wished to determine whether such increased toxicity occurs in the B- and T-cell lines used in the present study, and whether the differential drug resistance between the B and T cells is also exhibited upon treatment with the analog. The two cell lines showing the greatest differential MMC-induced toxicity, the B-cell line DT40 and the T-cell line CU159, were exposed to MMC and BMY 25067 for a period of 1 hr, and then were replated in fresh medium. It was evident that, like MMC, BMY 25067 inhibited the growth of the DT40 cell line preferentially over that of the CU159 cell line (Fig. 2). Both MMC and BMY 25067 inhibited growth of the DT40 cell line starting at 0.01 μg/mL (Fisher's PLSD, $P \leq 0.01$). In contrast, inhibition of growth by BMY 25067 was significant in the CU159 cell line starting at 0.1 μg/mL, whereas inhibition of growth by MMC was significant only at 1 and 10 μg/mL.

A comparison of the potency of the two compounds for inhibiting growth indicated that the aminodisulfide analog was more potent in inhibiting cell growth than MMC in both cell lines. The IC_{50} values for MMC and BMY 25067 were 0.045 and 0.008 μM, respectively, for DT40-lymphoma cells, and 3.3 and 0.3 μM for CU159-lymphoma cells.

It should be noted that the extent of growth inhibition induced by MMC in the experiment shown in Fig. 2 was greater than that observed in the previous experiment (Fig. 1). This enhanced toxicity was consistently obtained when DMSO was used as the solvent, compared to PBS, even though DMSO alone did not have an effect on growth. Since DMSO was the appropriate solvent for BMY 25067, the same solvent was used for MMC whenever a side-by-side comparison of the two compounds was desired. This points out the necessity of using a similar solvent system when directly comparing different compounds.

Determination of Gene-Specific DNA-ICL Induced by MMC and BMY 25067

DNA-ICL are believed to be of primary importance in MMC-induced toxicity. Thus, the increased sensitivity of the B-cell lines to treatment with cross-linking agents may be attributed to an increased level of DNA-ICL induced in the B-cells over the T-cells. Therefore, we measured the extent of MMC- and BMY 25067-induced DNA-ICL in the rDNA of the B- and T-lymphoma cell lines. Ribosomal RNA genes are present in high copy number (~300 per cell) and are transcribed at a high rate in rapidly dividing cells, comprising approximately 50% of total RNA transcription. They are, therefore, an important target gene cluster in which to measure chemically induced DNA damage.

We determined the number of DNA-ICL induced in rDNA by MMC or BMY 25067 using the RAGE assay [11].

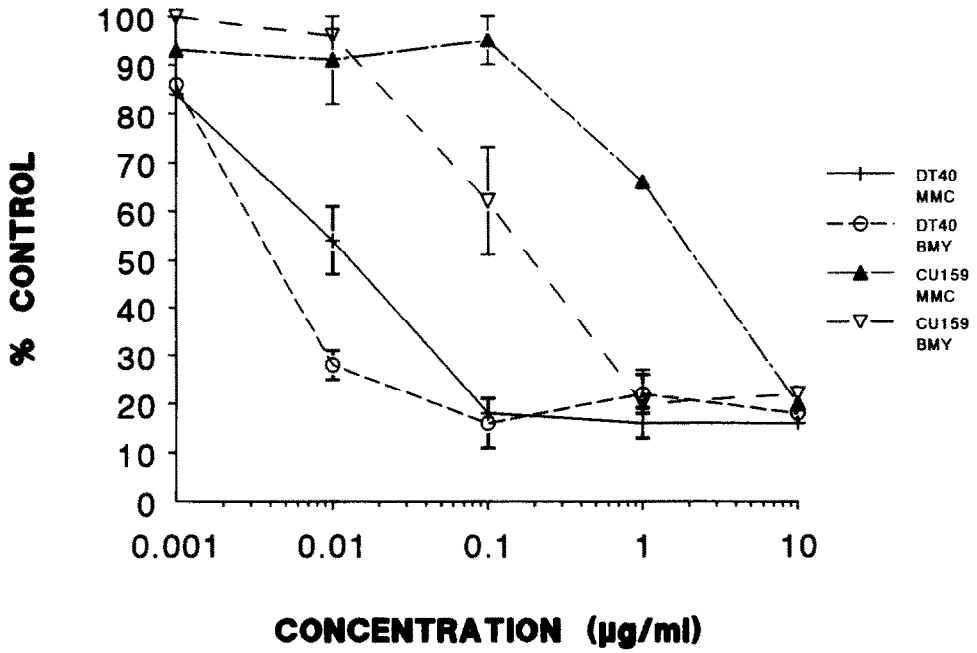

FIG. 2. Inhibition of cell growth by MMC and BMY 25067. Cultures containing 2 × 10^5 cells/mL of the B-lymphoma cell line DT40 and the T-lymphoma cell line CU159 were exposed to 0, 0.001, 0.01, 0.1, 1.0, or 10 μg/mL of MMC or BMY 25067 for 1 hr. Cell numbers, expressed as percent of control values, were determined 24 hr following drug exposure. Each point is the mean ± SEM for three independent experiments.

Briefly, DNA was isolated from control and drug-treated cells, and then digested with restriction enzyme. Next, the restricted DNA was denatured by heat treatment under alkali conditions, after which it was subjected to electrophoresis in a neutral agarose gel. Under these conditions, the cross-linked DNA rapidly renatures, whereas the non-cross-linked DNA remains single-stranded. After transfer to blotting membrane and hybridization with the appropriate probe, the cross-linked and non-cross-linked rDNA can be distinguished by virtue of differences in mobility in the gel. Non-cross-linked DNA (denatured) migrates as single-stranded, while the cross-linked (renatured) DNA migrates as double-stranded. The ratio of single- versus double-stranded DNA in each lane thus reflects the proportion of genes that contain MMC-induced cross-links in each sample. From this ratio, the number of cross-links per kilobase of DNA can be calculated [23].

Under non-denaturing conditions, a fragment of ≈10 kb was generated when chicken rDNA was digested with *Pst*I and hybridized with the pITS probe used in this study (Fig. 3, lanes designated C). Subjecting the DNA of control cultures, which were treated with solvent only, to RAGE conditions resulted in the generation of single-stranded DNA, which migrated at an apparent size of ≈5 kb (Fig. 3, lanes designated 0 μg/mL). Treatment of cell cultures with increasing concentrations of MMC (lanes designated 1.0 and 10 μg/mL) resulted in the appearance of a band migrating as double-stranded, which corresponds to the fraction of the rDNA molecules that were cross-linked by MMC. The intensity of the band increased in a concentration-dependent manner for all of the cell lines.

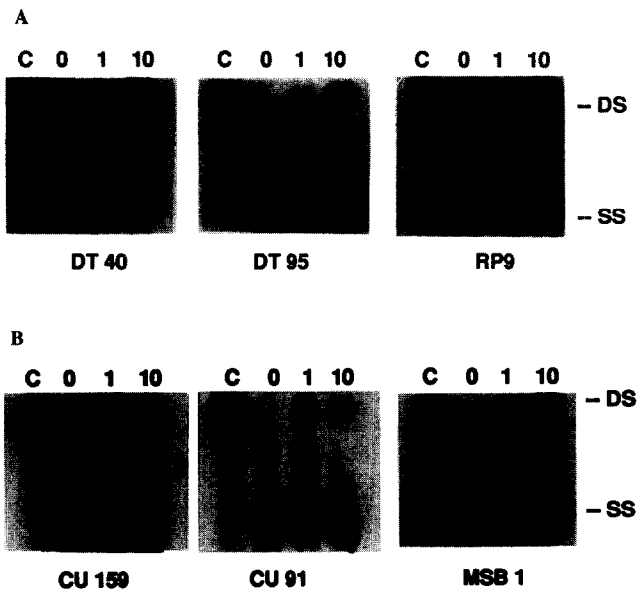

FIG. 3. RAGE blot for detection of MMC-induced DNA-ICL in rDNA. Cultures of B-cell lines DT40, DT95, and RP9 (A) and T-cell lines CU159, CU91, and MSB1 (B) were exposed to 0, 1.0, or 10 μg/mL of MMC for 1 hr. Following exposure, DNA was purified, digested with *Pst*I, and subjected to RAGE blotting analysis. Samples for each cell line are: control DNA (lane C), not subjected to denaturation, and DNA from cells treated with 0, 1.0, or 10 μg/mL MMC, subjected to denaturation (lanes 0, 1, and 10). Positions of the double-stranded (DS) and single-stranded (SS) 10 kb rDNA fragments that hybridize to the pITS probe are indicated.

After quantitation of the RAGE blots by densitometry, the percent of the rDNA cross-linked was determined for each sample (Table 1). Although a concentration-dependent increase in the level of DNA-ICL was obtained for all of the cell lines, no significant differences in the levels of DNA-ICL were detected among the cell lines at 1.0 and 10 μg/mL MMC. Calculation of the number of DNA-ICL per kb of DNA indicated that a level of 20% DNA-ICL was the equivalent of one DNA-ICL/40 kb, while a level of 50% DNA-ICL was the equivalent of one DNA-ICL/15 kb.

We used the RAGE analysis to determine if the increased toxicity of the aminodisulfide analog was reflected by a corresponding increase in the number of cross-links induced in rDNA as compared to MMC, and to compare cross-linking levels by the analog in B-versus T-cells (Fig. 4). Increasing signals of double-stranded DNA fragments, corresponding to cross-linked rDNA molecules, were observed over the three concentrations (0.1, 1.0, and 10 μg/mL) of MMC and analog for both cell lines. However, even by visual inspection of the RAGE blot, it is apparent that treatment of cells with the analog BMY 25067 resulted in increased levels of cross-links compared with MMC.

The results of quantitation of the RAGE blot (Fig. 4) by densitometry are shown in Table 2. At both concentrations, BMY 25067 induced approximately 2-fold more gene-specific DNA-ICL than MMC. However, the extent of DNA-ICL formation in the two cell lines was similar.

To determine if T-cells were generally more efficient than B-cells in the removal of DNA cross-links, we measured the persistence of DNA-ICL in the rDNA following exposure to MMC. Table 3 summarizes the percent of rDNA cross-linked in the CU159 and DT40 cell lines immediately following a 1-hr exposure of cells to MMC (day 0) and 1 and 2 days after drug treatment. For the CU159

TABLE 1. Percent of rDNA cross-linked by MMC in B- and T-cell lines*

Cell line	Percent of rDNA cross-linked	
	1 μg/mL MMC	10 μg/mL MMC
B-Cell lines		
LSCC-DT40	19 ± 1	52 ± 1
LSCC-DT95	16 ± 3	45 ± 2
LSCC-RP9	29 ± 4	54 ± 3
T-Cell lines		
MDCC-CU159	21 ± 4	45 ± 4
MDCC-MSB1	20 ± 1	54 ± 5
RECC-CU91	21 ± 3	47 ± 1

* The percent of rDNA cross-linked represents the mean ± SEM of 3 independent determinations, using the RAGE assay as shown in Fig. 3. Values were determined based on densitometry scans of double-stranded versus total (single- plus double-stranded DNA) for each treatment. No significant differences were found among the cell lines at either concentration. Values for all cell lines were significantly different between the 1 and 10 μg/mL concentrations ($P \leq 0.01$).

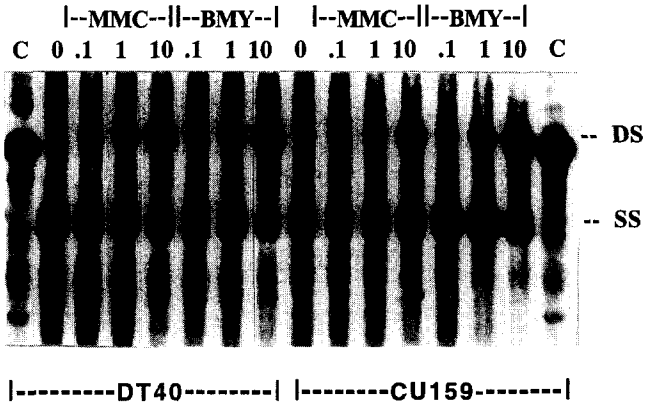

FIG. 4. Differential induction of DNA-ICL in rDNA by MMC and BMY 25067. Cultures of the B-lymphoma cell line DT40 and the T-lymphoma cell line CU159 were exposed to 0, 0.1, 1.0, or 10 μg/mL MMC or BMY for 1 hr. Following exposures, DNA was purified, digested with *PstI*, and subjected to RAGE blotting analysis. Samples are: control DNA (lane C) not subjected to denaturation, and DNA from cells treated with 0, 0.1, 1.0, or 10 μg/mL MMC or BMY and subjected to denaturation. Positions of the double-stranded (DS) and single-stranded (SS) 10 kb rDNA fragments that hybridize to the pITS probe are indicated.

cell line, the ratio of the percent of rDNA cross-linked for each time point to that of the percent of rDNA cross-linked on day 0 parallels closely the relative increase in cell numbers for the cultures. Therefore, the reduction in the percent of rDNA cross-linked can be attributed to the effects of dilution due to DNA replication. At the lowest concentration that cross-linked DNA can reasonably be measured (1 μg/mL), it was not possible to obtain intact DNA from the 48-hr cultures because of the extreme toxicity of MMC towards the DT40 cells. Thus, only the cross-link data corresponding to the days 0 and 1 time points are shown. As was the case with the T-cell line, there was no evidence for a significant reduction in the number of cross-links in the DT40 cell line over 24 hr.

Preferential Induction of Apoptosis in B- Compared with T-Cell Lines

Apoptosis is a process in which, upon exposure to specific cellular signals, the cell undergoes a series of physiological and morphological changes culminating in cell death. We examined the hypothesis that the preferential toxicity of MMC and its analog towards B-cells is due to their high sensitivity to drug-induced apoptosis. We studied apoptosis in two ways: detecting the presence of condensed, segregated chromatin by fluorescence microscopy after staining with Hoechst 33342 and propidium iodide (Figs. 5 and 6) and the formation of the characteristic nucleosomal ladder (Fig. 7).

MMC induced apoptosis in each of the six cell lines in a concentration-dependent manner (Fig. 6). In addition, the level of induction of apoptosis was statistically greater (Scheffe's F-test, $P \leq 0.01$) for all of the B-cell lines as compared with the T-cell lines. This parallels the results

TABLE 2. Percent of rDNA cross-linked by MMC and its aminodisulfide analog, BMY 25067*

Cell line	Treatment	Percent of rDNA cross-linked 1 μg/mL	10 μg/mL
LSCC-DT40	MMC	15	42
	BMY	27	70
MDCC-CU159	MMC	10	45
	BMY	21	89

* The percent of rDNA cross-linked was determined by quantitation of the blot shown in Fig. 4. Values were determined from densitometry scans of double-stranded versus total (single- plus double-stranded DNA) for each treatment.

TABLE 3. Persistence of interstrand cross-links in the rDNA of B and T cell lines*

Days of culture post-treatment	No. of viable cells/mL ($\times 10^6$)	% rDNA cross-linked	Factor reduction
MDCC-CU159 Cell line			
Day 0	1.0	15.0	1.0
Day 1	1.6	9.3	1.6
Day 2	2.9	6.5	2.3
LSCC-DT40 Cell line			
Day 0	0.4	19.9	1.0
Day 1	0.5	16.7	1.2
Day 2	ND†	ND	

* Cells were exposed to 10 μg/mL MMC for 1 hr and then allowed to recover in drug-free medium.

† ND = not determined due to extensive drug-induced cell damage and degradation of DNA.

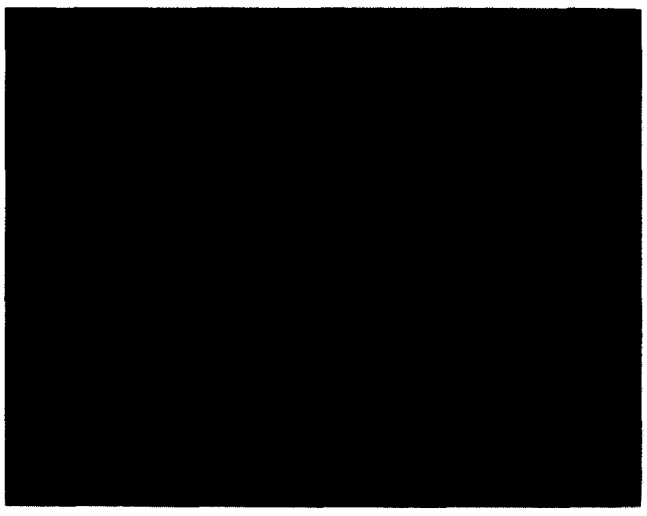

FIG. 5. Cytological detection of apoptosis in lymphoblastoid cells. Cells were stained in Hoechst 33342 and propidium iodide, and then were examined by simultaneous epifluorescence and transmitted light microscopy. Photomicrograph shows one DT40 B-cell undergoing apoptosis, with characteristic segregated, fragmented chromatin. Other cells in the field are viable DT40 cells. Original magnification: ×1875.

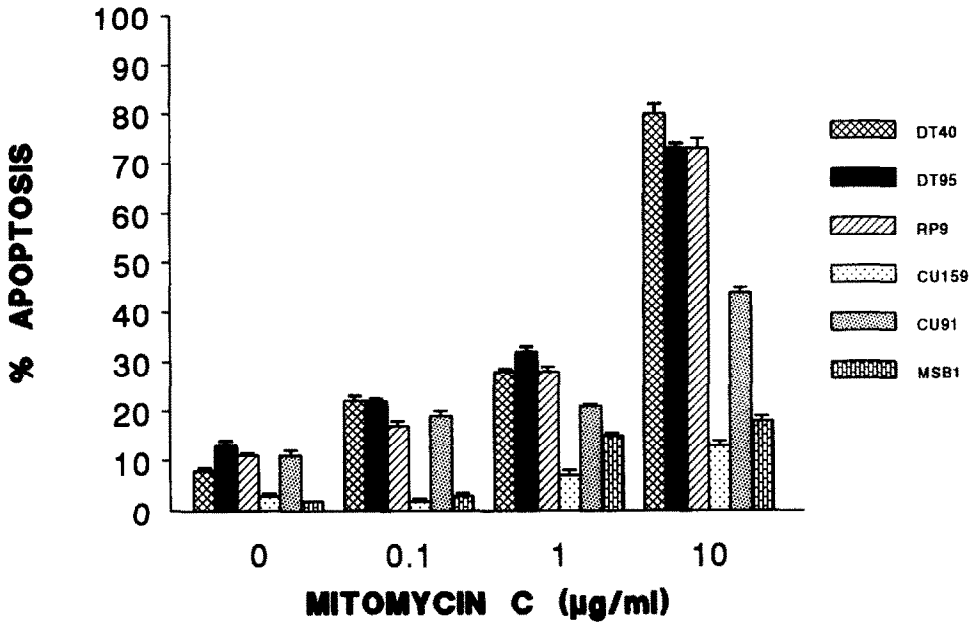

FIG. 6. Induction of apoptosis by MMC in B- and T-cell lines. Cultures containing 2 × 10^5 cells/mL of B-lymphoma cell lines DT40, DT95, and RP9 and T-lymphoma cell lines CU159, CU91, and MSB1 were exposed to 0, 0.1, 1.0, or 10 µg/mL of MMC for 1 hr. The percent of apoptotic cells in each culture was determined 24 hr following exposure using the Hoechst 33342/propidium iodide fluorescence assay. Values represent the means ± SEM for duplicate cultures for two experiments.

found in the initial studies of cell growth. Thus, the preferential increased toxicity of MMC to the B-cell lines can be attributed, at least in part, to the induction of apoptosis in these cells. Similarly, the one T-cell line that showed increased sensitivity to MMC-induced growth inhibition, CU91, also showed a corresponding increase in the induction of apoptosis.

When DNA isolated from cells that are undergoing apoptosis is subjected to gel electrophoresis, a characteristic nucleosomal ladder is usually detected. Concentration-dependent increases in the intensity of the nucleosome ladder in the DT40 cell line with both MMC and BMY 25067 were observed as soon as 8 hr after drug exposure (Fig. 7). However, the intensity of the nucleosomal ladder was markedly greater in the analog-treated samples than in the MMC-treated samples. Similarly, the nucleosome ladder was visible to a greater extent in the CU159 cultures that had been treated with BMY 25067 as compared with MMC. With both compounds, the laddering was greater in the DT40 cell line than in the CU159 cell line.

Quantitation of the induction of apoptosis by MMC and BMY 25067 using the H/PI technique is shown in Fig. 8. In this experiment, some induction of necrosis was detected (open areas), which was not detected in the previous experiment, shown in Fig. 6. As in the previous comparison between the two compounds, the solvent used here was DMSO. Although no increase in the induction of necrosis was found using the solvent alone, increased levels of necrosis were found in the presence of solvent plus drug. Regardless, there was a clear, concentration-related increase

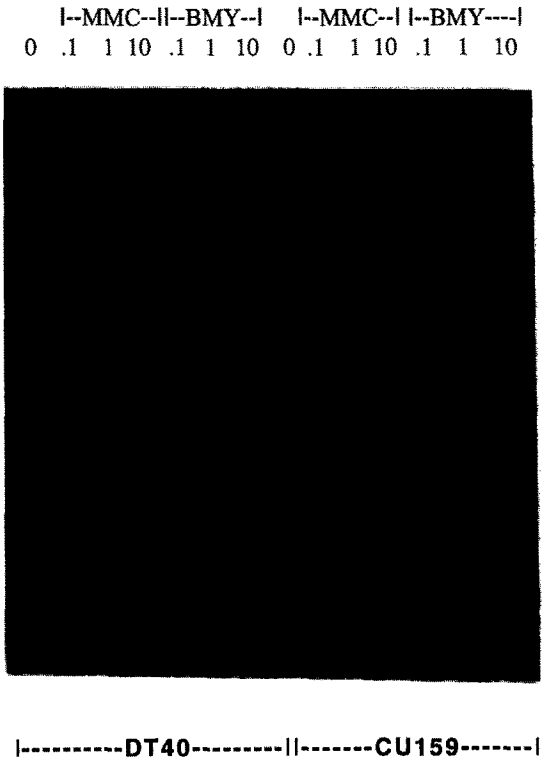

FIG. 7. Induction of DNA nucleosome ladders following exposure to MMC or BMY 25067. Cultures of the B-lymphoma cell line DT40 and the T-lymphoma cell line CU159 were exposed to 0, 0.1, 1.0, or 10 µg/mL of MMC or BMY for 1 hr. Total genomic DNA was purified 8 hr following exposure, and subjected to electrophoresis on a 1.5% agarose gel.

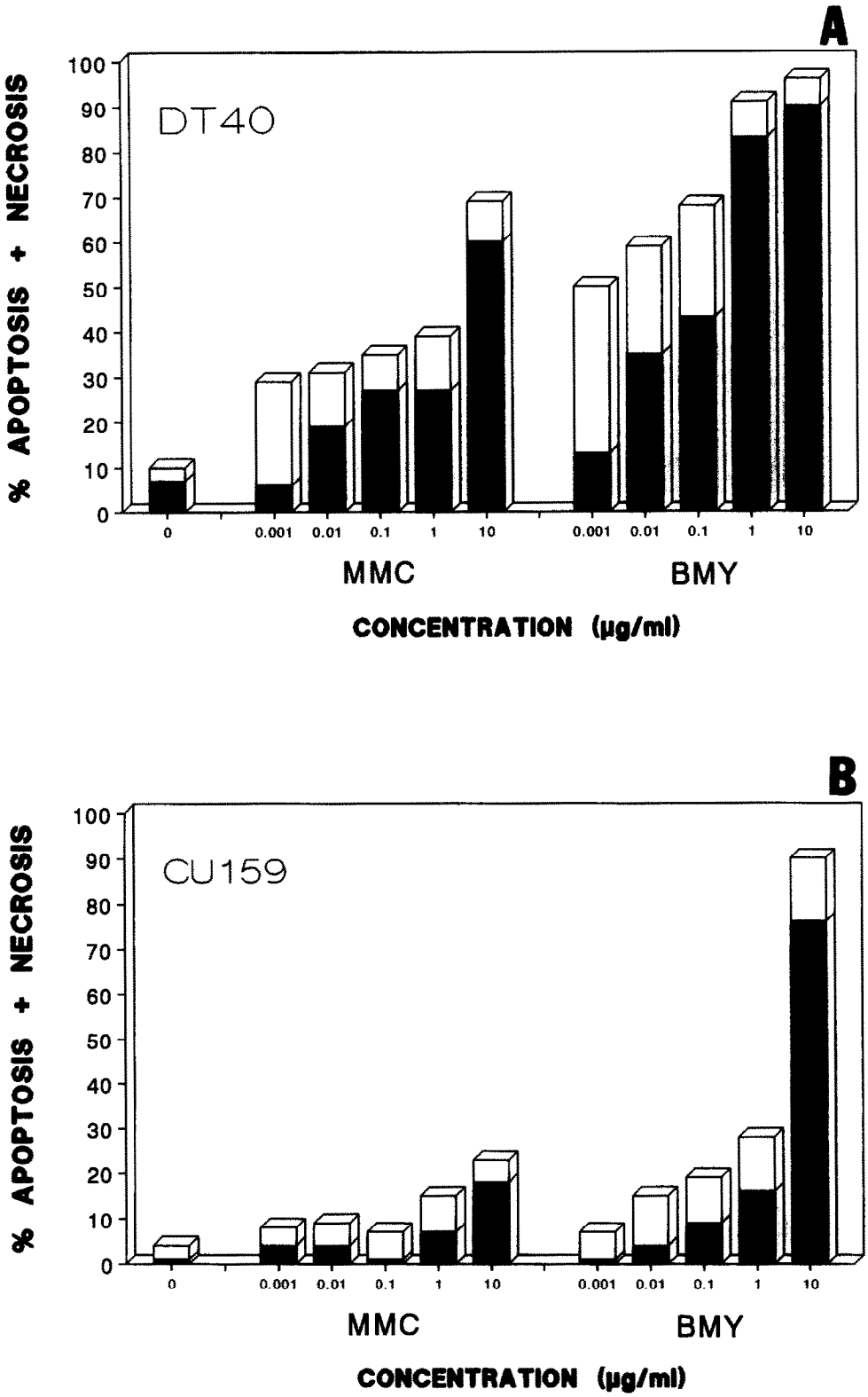

FIG. 8. Induction of apoptosis by MMC and BMY 25067. Cultures of the B-lymphoma cell line DT40 (A) and the T-lymphoma cell line CU159 (B) were exposed to 0, 0.001, 0.01, 0.1, 1.0, or 10 µg/mL MMC or BMY for 1 hr. The percent of apoptotic (shaded areas) and necrotic (open areas) cells was determined 24 hr following exposure using the Hoechst 33342/propidium iodide fluorescence assay. Values represent means from three independent experiments.

in the percent of apoptotic cells induced by the two compounds in both cell lines (Fig. 8, shaded areas). In addition, the level of apoptosis detected using the H/PI assay correlated with the nucleosome ladder formation depicted in Fig. 7. For DT40, a statistically significant increase in induction of apoptosis was detected starting at 0.01 μg/mL for both compounds, whereas for the CU159 cell line, a statistically significant increase was detected starting at 1 μg/mL for MMC and 0.1 μg/mL for BMY 25067 (using Fisher's PLSD, $P \leq 0.05$). These data parallel closely the inhibition of growth previously detected using these compounds. However, although the inhibition of growth 24 hr following exposure by MMC or BMY 25067 was similar in the DT40 cells, induction of apoptosis by the analog was statistically greater at all of the concentrations. This difference was reflected in the long-term survival of the cultures in that DT40 cells treated with 0.01 μg/mL MMC eventually recovered and continued to grow, whereas those treated with a similar concentration of BMY 25067 did not. CU159 cell cultures showed recovery and long-term survival at all but the highest (10 μg/mL) concentration of MMC; however, they only showed recovery at concentrations of BMY 25067 at 0.1 μg/mL and below.

DISCUSSION

Using the chicken B-lymphoma cell lines DT40, DT95, and RP9, and the T-lymphoma cell lines CU159, CU91, and MSB1, we were able to demonstrate high sensitivity of B-lymphoma cells and relative resistance of T-lymphoma cells to cytotoxicity induced by MMC and its analog. This differential sensitivity parallels that seen in differentiating lymphocytes *in vivo* in the chicken embryo [7]. Thus, the mechanism(s) underlying differential toxic responses is due at least in part, to intrinsic differences in the B- versus the T-lineage lymphocytes.

The MMC analog BMY 25067 is reported to show increased toxicity to a variety of cells over the parent compound [15]. Depending on the study and the cell lines examined, this enhanced toxicity has been attributed to increased levels of DNA cross-linking by the compound [15], to increased levels of free radical formation [24], and/or to mitochondrial toxicity involving oxidative damage [25]. Of particular relevance in the current study is the fact that BMY 25067-induced toxicity is not modulated by GST/GSH detoxification pathways [15]. This contrasts with the parent compound MMC, where GST/GSH has been invoked to play a major role in detoxification of the reactive species [15, 16, 26, 27].

In the present study, we found that compared with MMC, BMY 25067 was a more potent inducer of apoptosis for B- and T-lymphoma cells. Thus, detoxification by GST/GSH is likely to operate in avian lymphoma cells exposed to MMC, and inability to detoxify BMY 25067 by this pathway may account for its increased potency. We do not yet know the levels of GST/GSH in the cell lines used in the present study. However, the increase in the level of gene-specific DNA-ICL induced by BMY 25067 as compared with MMC further suggests that lack of detoxification by GST/GSH results in increased levels of the reactive species, producing more DNA damage. Thus, enhanced genotoxic activity of the analog is likely to be, at least in part, responsible for its increased toxicity in our experimental system. However, we cannot exclude the possibility that mitochondrial toxicity, as reported by others, may also contribute to enhanced toxicity of the analog over that of MMC.

Interestingly, we did find differential toxicity of BMY 25067 towards B-lymphoma compared with T-lymphoma cells. If reduced capacity to detoxify compounds via GST/GSH were entirely responsible for the preferential sensitivity of B-lymphoma cells over that of T-lymphoma cells, then the analog, which is not detoxified by this pathway, should not show preferential toxicity to B-cells. Since we did observe differential toxicity with the analog, we conclude that detoxification by GST/GSH probably does not play a major role in the preferential toxicity of MMC towards B-lymphoma cells.

To determine the actual levels of DNA damage induced by the two compounds, we chose to analyze the levels of gene-specific DNA-ICL in rDNA. This is a large gene complex, with ≈300 copies of the tandemly repeated genes comprising ≈6 megabases of DNA [28]. The rRNA genes, which are transcribed at extremely high rates in rapidly growing cells, are also highly accessible to compounds that interact with DNA. For some genotoxic compounds, such as aflatoxin B_1 [29], the primary mode of induction of cell death is due to interaction with rDNA, resulting in the concurrent inhibition of rRNA synthesis.

The high copy number of the rDNA also makes it amenable to the RAGE assay for gene-specific detection of chemically induced cross-links [23, 30, 31]. Using this assay, we found that, although the levels of DNA-ICL increased in a concentration-dependent manner, equivalent amounts of MMC-induced DNA-ICL were present in the B- and T-lymphoma cell lines. In addition, we have preliminary results that indicate equivalent amounts of DNA-ICL induction by both MMC and its analogue in B- and T-cell lines using a single-copy gene, the c-*myc* oncogene.* These findings argue against some of the other possibilities for differential toxicity including differences in drug uptake or metabolic activation, since differences in any of these processes are expected to be reflected in different levels of cross-linking in the B- and T-lymphoma cells. In addition, while BMY 25067 produced increased levels of DNA-ICL, and a concurrent increase in toxicity in both cell types, the levels of DNA-ICL between B- and T-lymphoma cells were the same. Like MMC, BMY 25067 was also differentially toxic to the B-lymphoma cells. Thus, the enhanced sensi-

* Muscarella DE and Bloom SE, unpublished data.

tivity of B-lymphoma cells to MMC and its analog reflects cellular processes downstream of the initial DNA damage.

The ability to remove or repair lesions in DNA may be an important factor contributing to drug sensitivity of cells. Depending on the compound used and the source of the cells, varying degrees of repair of lymphocyte DNA have been reported [32, 33]. We found no evidence for significant removal of cross-link lesions in the rDNA of either B- or T-lymphoma cell lines. Although it has been reported that repair of rDNA is slower when compared to genes that are transcribed by RNA polymerase II [34], this lack of repair in rDNA varies depending on the cell line [35] as well as the compound used [31]. For example, although pyrimidine dimers and DNA-ICL induced by nitrogen mustard are repaired less efficiently in the rDNA compared with the dihydrofolate reductase gene in Chinese hamster ovary (CHO) cells, DNA-ICL induced by cisplatin are similarly repaired [31]. In addition, other studies demonstrated some repair of cross-links induced in rDNA by MMC [23, 30]. We cannot exclude the possibility that enhanced transcription-coupled repair of genes transcribed by RNA polymerase II may occur in T-cells, or that differential effects of the compounds on transcription itself may underlie the differential induction of apoptosis between B- and T-lymphoma cell lines. However, our finding of persistence of MMC-induced cross-links in the present study is in agreement with an analysis of the persistence of SCE in these cells, where elevated SCE levels were maintained for several cell cycles following exposure to MMC [36].

We found a significant inhibition of growth, along with a concurrent increase in apoptosis and a marked reduction in long-term survival, for the B-cell lines exposed to MMC and BMY 25067. However, the T-cell lines CU159 and MSB1 were able to grow in the presence of equivalent levels of DNA damage, resulting in long-term survival of the cultures. A block of DNA replication in the B-cell line DT40 upon exposure to MMC has also been demonstrated by flow cytometry, whereas the CU159 cell line showed only a slowing of cells in S-phase and continued replication over successive cell cycles [6]. It is possible that the T-lymphoma cells have an enhanced replicative bypass capacity leading to increased DNA damage tolerance and reduced ability to engage in apoptosis. Replicative bypass of DNA monoadducts [35] and, although less efficiently, interstrand cross-links has been observed in human cell lines as well as rodent cell lines [23]. Enhanced replication bypass has been suggested as the primary mechanism for cisplatin and doxorubicin resistance in human tumor cell lines [37]. It may also be operative in human ovarian cancer cell lines in which cells that are highly resistant to cisplatin show levels of DNA adducts that exceed levels in the parental cell line from which they were derived [38].

Inhibition of cell growth induced by MMC and BMY 25067 can be attributed to the combined effects of induction of apoptosis and cell cycle inhibition by these compounds. In the present study, induction of apoptosis was detected cytologically by the production of segregated chromatin masses in cells, and by the generation of a characteristic nucleosomal ladder. These data are in agreement with the preferential induction of apoptosis in B-lymphoma cells detected *in vivo* in the developing chick embryo following MMC exposure [6]. Thus, the sensitivity of B-lineage cells to chemically induced apoptosis seen *in vivo* is retained in the transformed cells.

In developing lymphocytes, apoptosis plays a major role in a variety of processes, particularly in the negative selection of auto-reactive cells. It is now known that a variety of genes, including *c-myc*, *bcl2*, *rel*, and *p53* are involved in regulating the apoptotic response in lymphocytes upon exposure to chemicals or growth factors [39]. In addition, most of these genes are also involved in normal lymphocyte growth and differentiation. One or more of these genes may also play a role in determining the relative sensitivity of the B- and T-lymphoma cell lines to chemically induced apoptosis. For example, in some cases expression of *myc* is known to predispose cells to apoptosis. All three B-lymphoma cell lines examined here overexpress the *myc* oncogene due to insertional activation by ALV. In contrast, overexpression of *bcl2* inhibits the induction of apoptosis. However, the level of expression of this oncogene in the cell lines is not yet known. The one T-cell line, CU91, that shows somewhat elevated levels of apoptosis following drug treatment is the only T-cell line in this study that is transformed by REV, and overexpresses the *rel* oncogene. Overexpression of *rel* may account for the increased apoptotic response of these cells, since it is known to play a role in apoptosis induction during development of the chick embryo. Thus, these cell lines provide a useful system to study the intrinsic properties of B- and T-lineage lymphocytes that determine their propensity to undergo apoptosis, as well as the additional role that key oncogenes, tumor suppresser genes, and other regulating systems may play in modulating drug-induced apoptosis in tumor cells. Additionally, this system will be useful for studying acquired resistance mechanisms related to retroviral herpes virus induced transformation.

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