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Cell Kill Kinetics of an Antineoplastic Nucleoside, 1-(2-Deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine

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ABSTRACT. The cytotoxic properties of 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine (DMDC) were compared with those of 1-β-D-arabinofuranosylcytosine (ara-C), using SK-MEL-28(P-36) human melanoma cells. DMDC and ara-C were most cytotoxic to cells in the S phase of the cell cycle. Cell cycle progression in S phase was blocked by both compounds. Treatment with DMDC (1 μg/mL) or ara-C (1 and 30 μg/mL) did not increase cytotoxicity against asynchronous cells when the exposure time was prolonged from 1 to 6 hr, but did increase cytotoxicity thereafter. These findings suggest that cells in S phase are rapidly killed by the treatment but are temporarily prevented or delayed entry into the drug-sensitive S phase. On the other hand, DMDC treatment at a higher concentration (30 μg/mL) increased cytotoxicity in a time-dependent manner. Intracellular DMDC 5′-triphosphate (DMDCTP) increased in proportion to exogenous DMDC concentration, which was not saturated by treatment with a maximum concentration of the compound at 80 μg/mL. In contrast, intracellular ara-C 5′-triphosphate reached peak level when the cells were treated with ara-C at 8 μg/mL. The cytotoxicity of DMDC treatment for 4 hr increased relative to the intracellular DMDCTP accumulated during the period. These findings suggest that in cells treated with DMDC at a high concentration, an effective DMDCTP level is maintained for an extended period after washing out the compound from the medium. Consequently, the cells would be killed in the same way as in the case of extended exposures over 6 hr to DMDC at low concentration or to ara-C, in addition to acute S-phase-specific cytotoxicity. BIOCHEM PHARMACOL 52;8:1279–1285, 1996.

KEY WORDS. DMDC; ara-C; cytotoxicity; cell kill kinetics; cell cycle

DMDC,¶ a synthesized analogue of deoxycytidine [1, 2], has demonstrated antitumor activity against solid tumors in murine models and human cancer xenografts in nude mice as well as against leukemias. This wide spectrum of antitumor activity is in contrast to that of ara-C, which is inactive against solid tumors [1–3]. However, the mechanisms responsible for the difference of antitumor activity between these compounds have not been elucidated.

The cytotoxicity of DMDC and ara-C is reversed by exogenous deoxycytidine [3, 4], and both compounds are inactive against deoxycytidine kinase-deficient cells [4, 5], suggesting that these compounds are activated by a common metabolic pathway that includes phosphorylation by deoxycytidine kinase. DMDCTP and ara-CTP are potent inhibitors of DNA polymerase α from calf thymus in a manner competitive with dCTP, although DMDCTP also inhibits DNA polymerase β and γ [6]. On the other hand, DMDC, unlike ara-C, is hardly inactivated by cytidine/deoxycytidine deaminase prepared from mouse kidney [1] and human KB cells [5]. This feature should be advantageous for the antitumor activity of DMDC. However, it may not be an essential factor in the activity of DMDC against solid tumors, in light of the findings that cyclocytidine, an ara-C derivative resistant to cytidine/deoxycytidine deaminase, has *in vitro* and *in vivo* cytotoxic spectra similar to those of ara-C [3]. A more detailed analysis of the cytotoxic and pharmacokinetic characteristics of DMDC in

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¶ *Abbreviations:* DMDC, 1-(2-deoxy-2-methylene-β-D-*erythro*-pentofuranosyl)cytosine; ara-C, 1-β-D-arabinofuranosylcytosine; DMDCMP, DMDC 5′-monophosphate; DMDCDP, DMDC 5′-diphosphate; DMDCTP, DMDC 5′-triphosphate; ara-CMP, ara-C 5′-monophosphate; ara-CDP, ara-C 5′-diphosphate; ara-CTP, ara-C 5′-triphosphate; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; and TCA, trichloroacetic acid.

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solid tumor cells is required to identify the features that contribute to the unique activity of this compound.

In this study, we compared DMDC with ara-C in terms of cell kill kinetics, the effect on the cell cycle, and intracellular accumulation of active metabolites, using SK-MEL-28(P-36) melanoma cells. These cells have the following advantages: (a) they are derived from a human solid tumor; (b) they are not sensitive to ara-C but are sensitive to DMDC when inoculated in nude mice [3], and this *in vivo* sensitivity is in accord with the *in vitro* sensitivity to these compounds; and (c) they have high cloning efficiency and can be synchronized easily.

MATERIALS AND METHODS

Materials

DMDC dihydrate, DMDCMP disodium salt, DMDCDP trisodium salt, DMDCTP trisodium salt, ara-C hydrochloride, ara-CMP disodium salt, ara-CDP trisodium salt, and ara-CTP tetrasodium salt were prepared by the Yamasa Co., Ltd. (Choshi, Japan); [*methylene*-^{14}C]DMDC (777 MBq/mmol) was prepared by Amersham International plc (Buckinghamshire, England); and [5-^{3}H]ara-C (1.15 and 1.22 TBq/mmol), [*methyl*-^{3}H]thymidine (185 GBq/mmol), [5-^{3}H]uridine (962 GBq/mmol), and L-[4,5-^{3}H]leucine (5.66 TBq/mmol) were purchased from Amersham International plc. DMEM was obtained from the Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan); FBS from HyClone Laboratories, Inc. (Logan, UT, U.S.A.); and kanamycin sulfate from Meiji Seika Kaisha, Ltd. (Tokyo, Japan). Trypsin-EDTA solution, a cellular DNA analysis kit, and herring sperm DNA were purchased from Boehringer Mannheim GmbH (Mannheim, Germany). Monosodium phosphate monohydrate was purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). AQUASOL™-2 was obtained from NEN Research Products (Boston, MA, U.S.A.). Acetonitrile (HPLC grade) and all other chemicals (guaranteed reagent grade) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Cell Culture

SK-MEL-28(P-36) melanoma cells [7] were provided by Professor M. Taniguchi (School of Medicine, Chiba University, Chiba, Japan). The cells were grown as monolayers in a medium consisting of 10% heat-inactivated FBS and 90% DMEM supplemented with glucose (3.5 g/L), $NaHCO_3$ (1.8 g/L), and kanamycin sulfate (60 mg/L) at 37° in a humidified atmosphere containing 5% CO_2. The cells were harvested by a short treatment with a solution of 0.05% trypsin plus 0.02% EDTA in PBS, and were routinely subcultured twice a week. In a colony-forming assay, the cells were cultured in the above medium supplemented with 20% FBS.

Colony-Forming Assay

Cells were seeded at a density of 100 cells in 60-mm tissue culture dishes in a volume of 3.0 mL. Most of the cells had attached to the dishes within 4 hr, at which time 1 mL of drug-containing medium was added. The cells were incubated for the desired time period, the dishes were washed twice with 5 mL of PBS, and the cells were cultured in 5 mL of culture medium. Eight to ten days after seeding, the cells were washed with PBS, fixed with 10% formalin, and stained with 0.1% crystal violet. Colonies that consisted of more than 50 cells were counted. In all cases, the cloning efficiency of the control was normalized to 100%, and the cloning efficiency of the treated cells was expressed as a percentage of control survival.

Cell Synchronization

The cells were synchronized by treatment with aphidicolin. This agent is reported to be more useful than hydroxyurea or thymidine in inducing partial synchrony at the G_1-S boundary of the cell cycle [8]. Briefly, exponentially growing cells were cultured with 1 μg/mL of aphidicolin for 24 hr, harvested, and cultured in the conditioned medium. After release from aphidicolin block, the cells progressed through S phase and entered G_2/M phase followed by G_1 phase, in a synchronous fashion. To determine cell cycle phase-dependent cytotoxicity, cells in different phases of the cell cycle were exposed to DMDC or ara-C for 1 hr, and cell survival was determined. The cell-cycle transit of parallel sets of cultures (without either compound) was monitored by flow cytometry.

Flow Cytometry

Cells growing in 60- or 100-mm culture dishes were harvested, washed with PBS, fixed in cold 70% ethanol, and stored at 4° for several days. The fixed cells were washed with PBS, resuspended in PBS at 10^6 cells/mL, treated with RNase, and stained for DNA with propidium iodide, using the cellular DNA analysis kit according to the manufacturer's instructions. The stained cells were kept cool on ice in the dark until analysis. DNA content per cell was assessed by analysis of fluorescence at 620 nm, using an EPICS XL-MCL flow cytometer (Coulter Corp., Hialeah, FL, U.S.A.). Data were collected for 10^4 cells in each sample, and displayed as the total number of cells in each of 1024 channels of increasing fluorescence intensity. The resulting histograms were analyzed for cell cycle distribution, using MULTICYCLE software (Coulter Corp.). Subcellular debris and cell clumps were eliminated by gating list mode data on the peak signal versus the integral signal.

Determination of Macromolecular Synthesis

Cells were seeded in 24-well plates at 6×10^4 cells/well. After overnight culture, the cells were exposed to DMDC or ara-C for 4 hr. Two hours before the end of the exposure period, radioactive precursors of nucleic acids and protein were added individually at the following concentrations: [*methyl*-^{3}H]thymidine, 18.5 kBq/mL; [5-^{3}H]uridine, 92.5

kBq/mL; and L-[4,5-^{3}H]leucine, 185 kBq/mL. After exposure, the cells were washed three times with PBS and dissolved in 0.1% SDS solution containing 50 μg/mL of herring sperm DNA as a carrier. For determination of the radioactivity in total nucleic acids and protein, the SDS solution was mixed with an equivalent volume of cold 10% TCA. The resultant precipitate was collected by filtration on glass fiber filters (GF/C, Whatman International Ltd., Maidstone, England) and washed three times with cold 5% TCA and then with 80% ethanol. The filters were dried, placed in a toluene-based scintillation fluid, and assayed for radioactivity with an Aloka liquid scintillation system (LSC-700). For determination of the radioactivity in the alkali-stable fraction, the SDS solution was mixed with 0.3 N NaOH, incubated overnight at 37°, neutralized with HCl, and mixed with an equivalent volume of cold 10% TCA. The resultant precipitate was collected and assayed for radioactivity as described above. The radioactivity of [^{3}H]thymidine incorporated into DNA was assayed by determining the activity in the alkali-stable, TCA-precipitable fraction; the radioactivity of [^{3}H]uridine incorporated into RNA was assayed by subtracting the alkali-stable, TCA-precipitable count from the total TCA-precipitable count; the radioactivity of [^{3}H]leucine incorporated into protein was assayed by determining the activity in the total TCA-precipitable fraction. The procedures for the determination of macromolecular synthesis described above are modifications of those described by Hershko *et al.* [9].

Determination of Intracellular Levels of Phosphorylated Metabolites

Cells growing exponentially in 60-mm culture dishes were exposed to various concentrations of [^{14}C]DMDC or [^{3}H]ara-C for 4 hr. The cells were then washed twice with PBS, harvested, collected by centrifugation, and suspended in deionized water. The suspension was mixed with an equivalent volume of acetonitrile and stored at −80° until used. Insoluble material in the solution was removed by centrifugation, and the supernatant was then lyophilized and dissolved in deionized water containing nonradioactive standards (authentic samples of DMDC, ara-C, and their 5′-mono-, 5′-di-, and 5′-triphosphates) just before analysis. DMDC, ara-C, and their phosphorylated metabolites in the cell extract were analyzed by HPLC, using an LC-10A system (Shimadzu, Kyoto, Japan) equipped with a TSK gel DEAE-2SW column (250 mm × 4.6 mm, TOSOH, Tokyo, Japan). For the separation of DMDC and its phosphorylated metabolites, the HPLC system was run isocratically for 10 min at a flow rate of 0.8 mL/min with a mixture (65:35) of buffer A (4 mM NaH_2PO_4, pH 3.7) and buffer B (700 mM NaH_2PO_4, pH 4.0). For the separation of ara-C and its phosphorylated metabolites, the HPLC system was run isocratically for 10 min with a mixture (90:10) of buffer A and buffer B; the percentage of buffer B was then increased linearly to 60% over the next 50 min. Fractions of 1–2 mL were collected, mixed with scintillation solution (AQUASOL™-2), and assayed for radioactivity. The amount of the compound or its metabolite in each fraction was calculated on the basis of the radioactivity, and the value was normalized by the number of the cells harvested. By monitoring the absorbance at 275 nm, each radiolabeled compound and its metabolite were identified by the retention time of the peak of the authentic sample that was co-eluted.

RESULTS

Effects on Macromolecular Synthesis

The effects of DMDC and ara-C on DNA, RNA, and protein syntheses in the cells are shown in Fig. 1. DMDC at 1 μg/mL and ara-C at 1–100 μg/mL inhibited DNA synthesis almost completely, without simultaneously inhibiting RNA and protein syntheses. DMDC at 10 and 100 μg/mL, however, slightly but significantly inhibited RNA and protein syntheses, in addition to completely inhibiting DNA synthesis.

Cytotoxicity against Asynchronous Cells

Figure 2 shows the concentration–survival curves for SK-MEL-28(P-36) melanoma cells exposed to DMDC or ara-C for 1 or 4 hr. The survival rate decreased logarithmically as the concentration of the compounds increased up to 10 μg/mL. With a 1-hr exposure, the concentration–survival curves reached a constant saturation value at concentra-

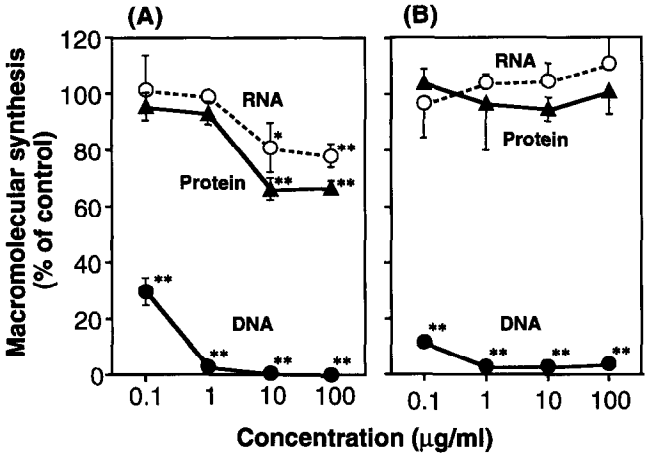

FIG. 1. Effects of DMDC and ara-C on DNA, RNA, and protein syntheses in SK-MEL-28(P-36) melanoma cells. Cells were treated with DMDC (A) or ara-C (B) for 4 hr; radioactive precursors ([^{3}H]thymidine, [^{3}H]uridine, and [^{3}H]leucine) were added 2 hr before harvesting the cells, and radioactivity was determined in DNA, RNA, and protein fractions. The control values for the DNA, RNA, and protein fractions were: 23,230 ± 4,260, 46,280 ± 4,330, and 3,140 ± 360 dpm, respectively. Values are means ± SD of six determinations for control and three determinations for the test sample. Key: (*) $P \leq 0.05$ versus control, and (**) $P \leq 0.01$ versus control (Student's unrelated *t*-test).

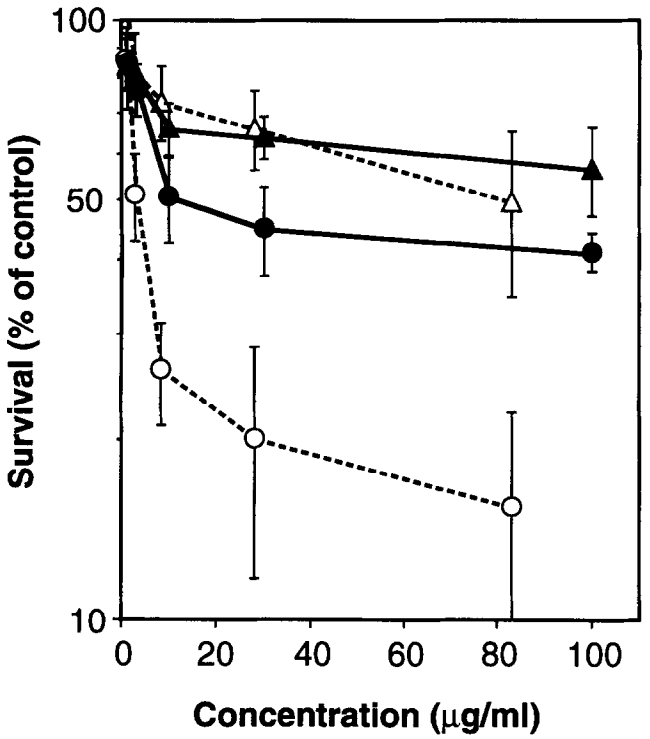

FIG. 2. Survival of SK-MEL-28(P-36) melanoma cells after exposure to DMDC or ara-C. Cells were exposed to DMDC for 1 hr (●) or 4 hr (○), or to ara-C for 1 hr (▲) or 4 hr (△). Survival of the cells was determined by a colony-forming assay. Cloning efficiency of control cells was 90%. Values are means ± SD of five determinations.

tions of more than 10 μg/mL (40–50% survival for DMDC, 60–70% survival for ara-C), indicating that these compounds kill the cells in a specific phase or phases of the cell cycle. Upon a 4-hr exposure, the concentration–survival curve of ara-C was similar to that of a 1-hr exposure, whereas DMDC decreased the survival rate concentration-dependently to 10–20%. The time–courses of DMDC and ara-C cytotoxicity are shown in Fig. 3. After treatment with DMDC at 1 μg/mL or ara-C at 1 or 30 μg/mL, there was an almost immediate (within 1 hr) drop in the survival rate to 65–80%. These levels were maintained for up to 6 hr, and decreased thereafter according to the period of exposure. A similar plateau in the survival curve was reported previously in mouse L-cells [10] and in L1210 cells [11] when exposed to ara-C. After treatment with DMDC at 30 μg/mL, however, there was no such plateau in the survival curve, and the survival rate decreased exponentially in a time-dependent manner to 0% upon 24-hr exposure.

Cytotoxicity against Synchronous Cells

The survival of cells exposed to DMDC or ara-C at 30 μg/mL for 1 hr in different phases of the cell cycle is shown in Fig. 4. It was quite clear that the cells in S phase were the most sensitive to these compounds. In addition, the cytotoxicity of DMDC against the cells in this phase was greater than that of ara-C.

Effects on Cell Cycle Progression

Figure 5 shows the DNA histograms of the cells treated continuously with DMDC and ara-C up to 48 hr. The treatment with DMDC at 1 μg/mL and higher decreased the peak of the G_2/M phase and increased the number of cells in the G_1/S phase. When the concentration was decreased to 0.3 μg/mL, the cells accumulated in S phase. Similar results were obtained with ara-C treatment. The effect of DMDC on cell cycle progression would be expected from analogy with that of ara-C [12, 13] as follows: at 0.3 μg/mL, progression of the cells through S phase should be slowed by inhibiting DNA synthesis; at higher concentration, the inhibition is so great that cells initially in G_1 phase should be blocked immediately upon entrance into S phase, and most of the cells initially in S phase should be arrested in this phase.

Accumulation of Phosphorylated Metabolites

Figure 6A shows intracellular accumulation of the metabolites of [^{14}C]DMDC when the cells were treated with

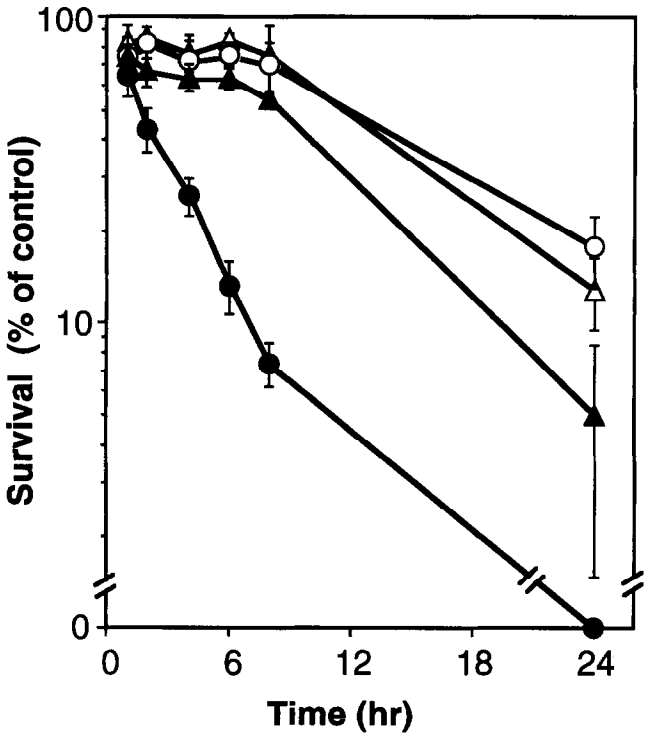

FIG. 3. Survival of SK-MEL-28(P-36) melanoma cells after exposure to DMDC or ara-C as a function of time. Cells were exposed to DMDC at 1 μg/mL (○) or 30 μg/mL (●) or to ara-C at 1 μg/mL (△) or 30 μg/mL (▲). Survival of the cells was determined by a colony-forming assay. Cloning efficiency of control cells was 76%. Values are means ± SD of five determinations.

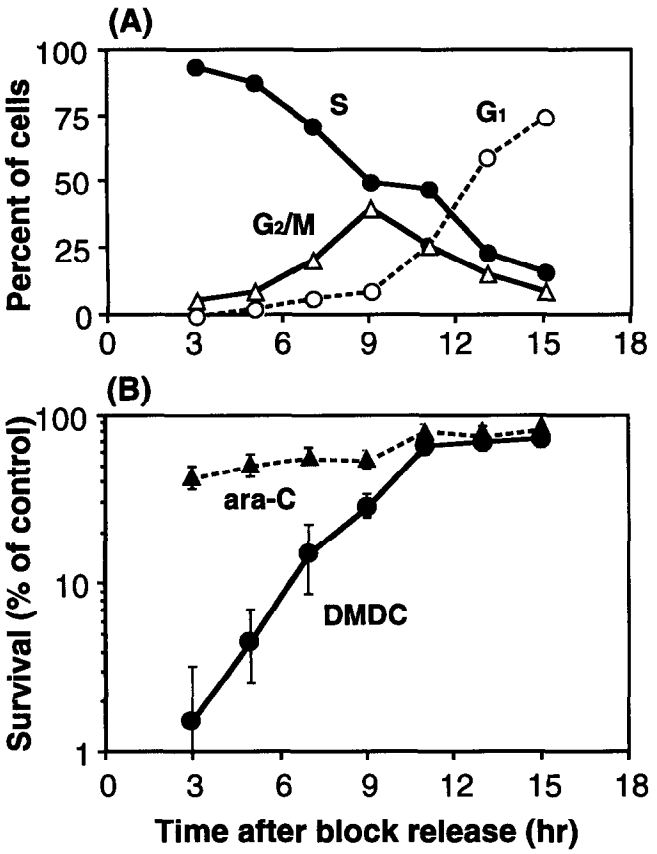

FIG. 4. Survival of SK-MEL-28(P-36) melanoma cells exposed to DMDC or ara-C in different phases of the cell cycle. After release from aphidicolin block, the cells are plated for flow cytometry (1.0×10^6 cells/dish) and for colony-forming assay (100 cells/dish). Dual-parameter flow cytometry (A) and colony-forming assay (B) were carried out in parallel at the times indicated. The cells were exposed to 30 μg/mL of DMDC or ara-C for 1 hr in the colony-forming assay; cloning efficiency of control cells of each time point ranged between 48 and 62%. The times shown are halfway points during the period of exposure. Survival data are means ± SD of five determinations.

[^{14}C]DMDC for 4 hr. The major metabolite of [^{14}C]DMDC in the cells was [^{14}C]DMDCTP (60–70% of total radioactivity in the cells), whereas unchanged [^{14}C]DMDC, [^{14}C]DMDCMP, and [^{14}C]DMDCDP were only minor components. Intracellular accumulation of [^{14}C]DMDCTP increased in proportion to exogenous [^{14}C]DMDC concentration, which was not saturated by the treatment with a maximum concentration of the drug at 80 μg/mL. In contrast, intracellular [^{3}H]ara-CTP reached peak level when the cells were treated with [^{3}H]ara-C at 8 μg/mL (Fig. 6B), and the intracellular level was much lower than that of [^{14}]DMDCTP when the cells were treated with [^{14}]DMDC at 3 μg/mL and higher (Fig. 6A). Similar patterns of accumulation of the metabolites were observed in T24 human bladder carcinoma cells that are also not sensitive to ara-C but are sensitive to DMDC (data not shown). The relationship between cell survival and intracellular levels of the 5′-triphosphates of the compounds (Fig. 7) demonstrates

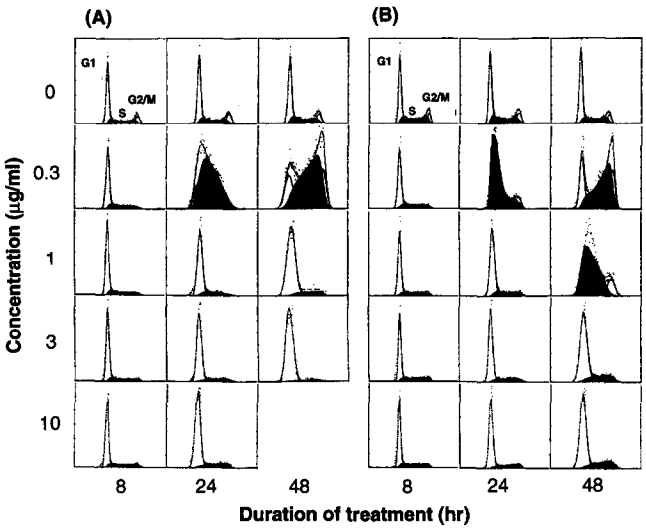

FIG. 5. DNA histograms of SK-MEL-28(P-36) melanoma cells exposed to DMDC (A) or ara-C (B) for up to 48 hr. The x- and y-axes show DNA fluorescence and relative cell number, respectively. Dots represent the data points; the two Gaussian distributions on the left and right represent the distribution of G_1- and G_2/M-phase cells, respectively; the central distribution, painted in black, corresponds to S-phase cells. A histogram of the sample treated with 10 μg/mL of DMDC for 48 hr was not obtained, because only few viable cells were collected.

that the cytotoxicity increased relative to the intracellular level of DMDCTP.

DISCUSSION

DMDC and ara-C were most cytotoxic to SK-MEL-28(P-36) melanoma cells in S phase, and these compounds pre-

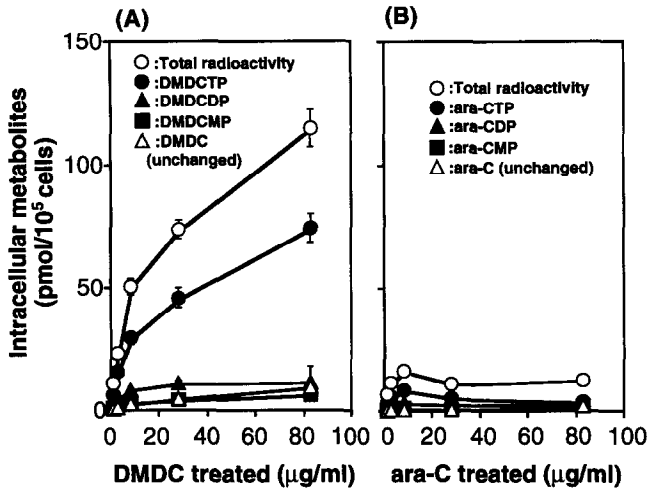

FIG. 6. Accumulation of intracellular 5′-phosphorylated metabolites of DMDC and ara-C in SK-MEL-28(P-36) melanoma cells as a function of the exogenous concentration of the compounds. The cells were incubated with [^{14}]DMDC (A) or [^{3}H]ara-C (B) for 4 hr. Intracellular levels of the unchanged form and of the 5′-mono-, 5′-di-, and 5′-triphosphates of the compounds were determined as described in Materials and Methods. Values are means ± SD of three determinations.

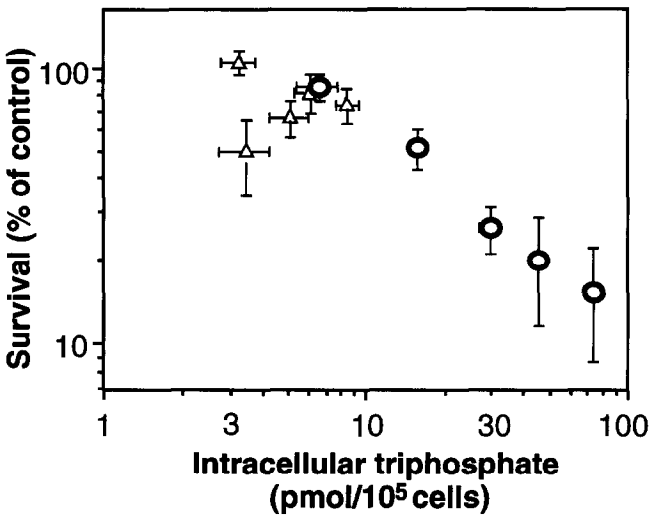

FIG. 7. Relationship between survival of SK-MEL-28(P-36) melanoma cells and the intracellular level of DMDCTP or ara-CTP after treatment with DMDC or ara-C for 4 hr. The cell survival data are from Fig. 2, and the data for intracellular DMDCTP and ara-CTP are from Fig. 6. Key: (○) DMDC-treated; and (△) ara-C-treated.

vented the cells from progressing in S phase. The cytotoxic effects of DMDC at 1 μg/mL and of ara-C at 1 and 30 μg/mL did not increase with prolongation of exposure from 1 to 6 hr, but did increase thereafter. These data are consistent with results reported for ara-C [10, 11, 14, 15].

The cellular cytokinetic effects of ara-C have been well characterized [10, 11, 15], and the time–course of the cytotoxicity of ara-C is explained as follows: ara-C kills cells in S phase immediately; then there follows a plateau lasting for several hours which apparently is due to a block in the entry of cells into the drug-sensitive S phase. Thus, ara-C seems to have the ability to protect, at least partially, against its own cytotoxicity (self-limiting effect). After the plateau in survival, there is a time-dependent increase in cytotoxicity caused by "unbalanced growth." Unbalanced growth is a phenomenon associated with the prolonged suppression of macromolecular synthesis. In the case of ara-C, inhibition of DNA synthesis, without concomitant inhibition of RNA and protein syntheses, results in a marked increase in cell volume, and subsequent cell death [15, 16]. The time–course of cytotoxicity of DMDC at 1 μg/mL (Fig. 3) can be explained in the same way as that of ara-C mentioned above because the effects on macromolecular synthesis, cell volume (data not shown), cell survival, and cell cycle progression were the same as those of ara-C.

On the other hand, DMDC at a higher concentration (30 μg/mL) killed the cells in a time-dependent manner. Why are the cell kill kinetics of DMDC different between high and low concentrations?

In the case of ara-C, it is possible to relate the cytokinetic response to the pharmacokinetics of ara-CTP in a quantitative fashion according to Pallavicini [15]: cells are not released from the block of cell cycle progression until the intracellular ara-CTP concentration falls below an inhibiting threshold, and hence the initial level of ara-CTP is a determinant of the duration of the block of cell cycle progression after ara-C treatment. In our preliminary experiment, the rates of decay of intracellular DMDCTP and ara-CTP in SK-MEL-28(P-36) melanoma cells after treatment with the drugs for 4 hr were similar and independent of the initial level (data not shown). Therefore, the intracellular level of DMDCTP and ara-CTP would also be a determinant of the duration of the cell cycle-phase block in SK-MEL-28(P-36) melanoma cells after treatment with the drugs. A high level of DMDCTP was accumulated upon treatment with DMDC at a high concentration, as shown in Fig. 6, and an effective DMDCTP level was maintained for an extended period even if the cells were cultured further after washing the drug out of the medium. Accordingly, the cytotoxicity of DMDC at high concentration would be expected to result not only from acute S-phase-specific toxicity but also from unbalanced growth, which is caused by the block of cell cycle progression for an extended period. The assumption mentioned above enables us to explain the time-dependent cytotoxicity of DMDC at high concentration (Fig. 3) and the correlation between accumulation of DMDCTP and cytotoxicity (Fig. 7).

Another possibility may be considered: DMDC at high concentration may kill cells positioned at very early S phase, cells that DMDC at low concentration and ara-C do not kill. This assumption makes it easy to understand the time-dependent cytotoxicity of DMDC at high concentration as shown in Fig. 3, because the fraction of the cells accumulating at this phase will increase time dependently. Smets and Homan-Blok [17] reported that in stationary L1210 cells there is a cell cycle component termed S1, positioned around the G_1-S boundary (very early S phase), which is refractory to ara-C at low concentration but sensitive to the drug at high concentration. In the very early S phase of SK-MEL-28(P-36) melanoma cells, some crucial DNA replication events may take place, and these may be sensitive targets for DMDCTP at high concentration. Inhibition of RNA synthesis by DMDC treatment at high concentration (Fig. 1) may also contribute to the cytotoxicity of the agent, considering that actinomycin-D and nogalamycin (inhibitors of RNA synthesis) are most cytotoxic at the G_1-S boundary [14], and that camptothecin (an inhibitor of DNA and RNA syntheses) kills non-S-phase cells [14] or late G_1-phase cells [18] in addition to S-phase cells.

DMDC was more cytotoxic than ara-C against cells in the S phase, as shown in Fig. 4. It has been found that DMDCTP is incorporated into DNA chains [6] in a manner similar to that of ara-CTP. It was also found that DMDCTP inhibits both DNA polymerase β and DNA polymerase α, while ara-CTP inhibits only DNA polymerase α [6]. Enzymes including DNA polymerase β are thought to mediate repair synthesis of DNA. These data suggest that DNA containing ara-C may be partially repaired, and some cells may escape the toxicity of the drug; however, DNA containing DMDC may be hardly repaired.

Intracellular accumulation of DMDCTP was proportional to the concentration of the agent used in the treatment, whereas that of ara-CTP was not (Fig. 6). Increasing the extracellular ara-C concentration to more than 8 μg/mL failed to increase ara-CTP accumulation, suggesting saturability in ara-C metabolism, a phenomenon not seen with DMDC. Feedback inhibition of ara-C phosphorylation by an ara-CTP pool accumulating in the cells is likely responsible for the plateau in ara-CTP accumulation in the cells, as have been reported in human leukemia cells [19]. In our preliminary experiment, ara-CTP tended to inhibit deoxycytidine kinase activity more strongly than did DMDCTP. Deamination seems to make little contribution to the saturation of ara-C metabolism, since tetrahydrouridine, a potent deaminase inhibitor, had no effect on the profile of accumulation of phosphorylated ara-C (data not shown). The dependency of cellular uptake on nucleoside transporter(s) also may be a determinant of the rate of accumulation of these compounds. The lipophilicity (partition coefficient between *n*-octanol and phosphate buffer, pH 7.4) of DMDC was 3-fold greater than that of ara-C (data not shown), a factor that may contribute to the cellular penetration of DMDC by passive diffusion which is not influenced by the capacity of the transporter(s).

In contrast to ara-C, DMDC exhibits a wide spectrum of antitumor activity. Differences in metabolism between DMDC and ara-C would explain not only their different cell kill kinetics *in vitro*, but also their different antitumor activities *in vivo*. Pharmacokinetic studies of DMDC and ara-C in tumor tissues *in vivo* are needed to confirm our conclusion.

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