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Effect of vitamin A on methotrexate cytotoxicity in L1210 murine leukemia cells in culture*

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Abstract. Vitamin A (VA) protects the small intestine from methotrexate (MTX)-induced damage. However, before VA can be used as a remedy to protect cancer patients from MTX-induced damage to the intestine, it is essential to clarify whether or not it disturbs the antitumor activity of MTX. This study investigated the effect of VA on the antitumor activity of MTX in vitro in L1210 murine leukemia cells. The incorporation of [6-^{3}H]-thymidine and [6-^{3}H]-uridine, [5-^{3}H]-uridine, and [4,5-^{3}H]-leucine into DNA, RNA, and proteins, respectively, was examined to evaluate this effect. The incorporation of thymidine, the uridines, and leucine decreased dose-dependently in MTX-treated L1210 cells and profoundly in the MTX plus VA-treated L1210 cells, since VA itself had a cell-killing activity. Thus, MTX depressed the growth of L1210 cells dose-dependently and this depression was not affected by the presence of VA. The present study proved in L1210 murine leukemia cells in vitro that VA did not disturb the antitumor activity of MTX.

Introduction

Antitumor drugs that inhibit the metabolic pathway, e.g., methotrexate (MTX) and 5-fluorouracil, are known to induce malabsorption and diarrhea [2, 5, 19]. On the occurrence of this side effect, the gastrointestinal tract shows damaged and shortened microvilli of the small intestine and a decrease in its surface area [2]. Furthermore, components of the intestinal membranes such as lipids and proteins decrease [16, 18]. We have previously demonstrated physical changes in small intestinal brush-border membranes of MTX-treated rats [21] and have found that vitamin A (VA) protects the small intestine of rats from such morphological, biochemical, and physical damage [22].

In recent years, various regimens have been tried in cancer chemotherapy to save patients from the side effects induced by antitumor drugs. For example, the high-dose MTX regimen with leucovorin rescue has been extensively applied in patients with osteosarcoma [7, 20]. Our finding that VA protects the small intestine from MTX-induced damage is promising for MTX-based chemotherapy of patients with cancer. However, before the combination of MTX and VA is adopted for clinical use, it is essential to clarify whether or not VA disturbs the antitumor effect of MTX. In the present study, the effect of VA on the antitumor effect of MTX was investigated in vitro in L1210 murine leukemia cells with the aim of validating the use of VA as a remedy to protect the intestine from MTX-induced damage. In addition, the effects of VA on the bone marrow and liver of the rat were examined by analyzing the blood constituents.

Materials and methods

Drugs and chemicals. MTX was purchased from Sigma Chemical Co. (St. Louis, Mo.). VA was obtained from Eastman Kodak Co. (Rochester, N. Y.). RPMI Media 1640 (with L-glutamine), fetal bovine serum, penicillin G, and streptomycin were acquired from Gibco Laboratories (Grand Island, N. Y.). [5-^{3}H]-Uridine (26.3 Ci/mmol, 1.0 mCi/ml), [6-^{3}H]-uridine (26.3 Ci/mmol, 1.0 mCi/ml), [6-^{3}H]-thymidine (15.0 Ci/mmol, 1.0 mCi/ml), [4,5-^{3}H]-leucine (5.0 Ci/mmol, 1.0 mCi/ml), and Omnifluor were supplied by New England Nuclear Corp. (Boston, Mass.). All other reagents were of analytical grade.

Cell culture. L1210 murine leukemia cells were purchased from Dainippon Pharmaceutical Co. (Osaka, Japan). L1210 cells ($1-2\times10^5$ cells/ml) were maintained as stationary suspension cultures in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 50 IU penicillin/ml, and 50 IU streptomycin/ml and were transferred into fresh medium every 3 or 4 days. L1210 cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C. Mycoplasma contamina-

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tion was not detected in the experiments. The cell viability was determined by trypan blue exclusion. The cells used for all experiments in the cytotoxicity study were in the logarithmic growth phase.

Assessment of MTX antitumor activity by enumeration of viable cells. L1210 cells in the logarithmic growth phase (2 days after passage) were diluted to 2.8 or 4.6×10^5 cells/ml as viable cells with RPMI 1640 medium containing 25 m*M* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). In all, 1 ml cell suspension was put into individual wells of a 24-well plate and MTX and/or VA were added to the wells. L1210 cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, and at an appropriate time the number of viable cells was determined by enumerating the cells by trypan-blue dye exclusion.

Incorporation of thymidine into L1210 cells. The incorporation of [6-^{3}H]-thymidine or [6-^{3}H]-uridine into the acid insoluble materials was used to measure DNA synthesis according to the method of Mandel et al. [14]. L1210 cells in the logarithmic growth phase were diluted to 3×10^5 cells/ml as viable cells with RPMI 1640 medium containing 25 m*M* HEPES. In all, 1 ml cell suspension was put into individual wells of a 24-well plate and MTX and/or VA were added to the wells. After incubation for 48 h in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, the reaction mixtures were further incubated for 3 h in the presence of [6-^{3}H]-thymidine (2.5 μCi/ml) or [6-^{3}H]-uridine (2.5 μCi/ml). Aliquots (100 μl) of the reaction mixtures were removed from each well at specified times to determine the amount of radioactivity incorporated into DNA.

Immediately after the samples had been taken, the [6-^{3}H]-thymidine or [6-^{3}H]-uridine incorporation was stopped by adding 100 μl of an ice-cold 0.9% NaCl solution to the samples. The samples were filtered on to a wetted Whatman GF/C glass-fiber disk. The cells on the filters were washed with 2 ml of the 0.9% NaCl solution, after which 2 ml of a 0.2 *N* perchloric acid solution was added to precipitate the nucleic acids. The precipitates were washed with 4 ml of the 0.9% NaCl solution, and the filters were placed in scintillation vials and dried at 70 °C for 30 min. Then, 2 ml scintillation fluid containing Omnifluor (1 g) in 1000 ml toluene was added to the dried filters and their radioactivity was counted in an Aloka LSC-903 liquid scintillation counter.

Incorporation of uridine into L1210 cells. The incorporation of [5-^{3}H]-uridine (2.5 μCi/ml) into the acid-insoluble materials was used to measure RNA synthesis according to the methods of Fairchild et al. [6] and Mandel et al. [14]. Incorporation procedures were carried out as described above for the measurement of DNA synthesis.

Incorporation of leucine into L1210 cells. The incorporation of [4,5-^{3}H]-leucine (2.5 μCi/ml) into the acid-insoluble materials was used to measure protein synthesis according to the method of Mandel et al. [14]. The incorporation was studied in leucine-free medium as described above for the measurement of DNA synthesis.

Animals. Male Wistar rats (age, 8–10 weeks; body weights 180–250 g; Japan SLC Inc., Shizuoka, Japan) were used. About 0.5 ml saline solution that contained MTX (15 mg/kg), MTX (15 mg/kg) plus VA (5,000 IU/kg) or VA (5,000 IU/kg), respectively, or 0.5 ml saline solution alone was given p.o. to the rats once daily for 4 days. Rats were fasted overnight prior to each experiment. Blood samples were drawn from the jugular vein just before the rats were treated with MTX and/or VA and on the 5th day after the rats had been treated for 4 days.

Analysis of blood constituents. The numbers of leukocytes and red blood cells, the amount of hemoglobin, and the hematocrit value were determined using a blood-cell counting system consisting of an auto dilutor (Sysmex AD-260, Toa Medical Electronics, Japan), a microcell counter (Sysmex F-500) and a cell monitor (Sysmex CM-5). The activity of glutamic oxaloacetic transaminase (GOT) in the plasma was determined enzymatically by a slight modification of the method of Karmen [8] using reagents of the GOT-TEST Wako (Wako Pure Chemical Industry Ltd., Osaka, Japan). In the present study, statistical differences between group means were analyzed using Student's two-sided *t*-test.

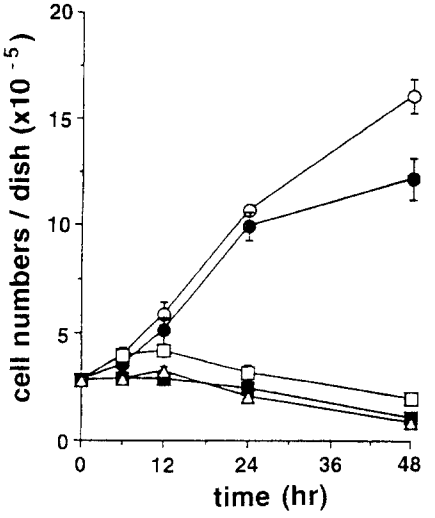

Fig. 1. Effect of MTX exposure on L1210 cells. L1210 cells (2.8×10^5 cells/ml) were incubated for 6, 12, 24, or 48 h in the absence of MTX (○) and in the presence of 0.1 (●), 1 (□), 10 (■), or 100 μ*M* MTX (△). The viable cells were microscopically enumerated. Data represent mean values ± SE for three experiments. The absence of bars indicates that SEs were too small to be expressed

Results

Effect of MTX and/or VA exposure on cytotoxicity

L1210 cells were exposed to MTX at concentrations ranging from 0.1 to 100 μ*M* for various incubation periods of up to 48 h, and the cytotoxic effect of MTX was estimated by enumerating the viable cells at designated times (Fig. 1). In the absence of MTX, L1210 cells showed a continuous increase in viable cell numbers during the incubation. In the presence of MTX, the cells showed a behavior different from that observed without MTX. In the presence of 0.1 μ*M* MTX, the numbers of viable cells increased in parallel with those in the cells incubated without MTX for up to 24 h, and then the increase in the cells slowed down at 48 h. In the presence of MTX concentrations higher than 1 μ*M*, the cytotoxic effect remained prominent after an exposure period of 12 h or more.

The effect of VA on L1210 cells was also examined by the same procedures used in the case of MTX (Fig. 2). In L1210 cells exposed to 17.5 μ*M* VA, the viable cell numbers increased with the incubation time, but this increase was smaller than that observed in cells incubated without VA. The viable cell numbers were markedly decreased by the presence of 35 μ*M* VA, and cell viability was almost completely lost at 24 h. Thus, VA affected the growth of L1210 cells in a dose- and time-dependent manner.

Furthermore, the time course of the change in L1210 cell numbers on the exposure of cells to 1 μ*M* MTX and 17.5 μ*M* VA, 24.5 μ*M* VA, or 35 μ*M* VA was examined by the same procedures used in the experiments shown in Figs. 1 and 2. In all cases, almost all of the L1210 cells were dead at 48 h after the start of the incubation (data not shown). On the basis of these results, the effects of MTX and/or VA exposure on the synthesis of DNA,

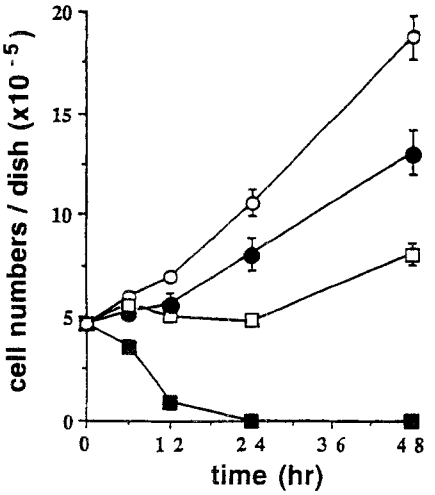

Fig. 2. Effect of VA exposure on L1210 cells. L1210 cells (4.6×10^5 cells/ml) were incubated for 6, 12, 24, or 48 h in the absence of VA (○) and in the presence of 17.5 (●), 24.5 (□), or 35 μ*M* VA (■). The viable cells were microscopically enumerated. Data represent mean values ± SE for three experiments. The absence of bars indicates that SEs were too small to be expressed

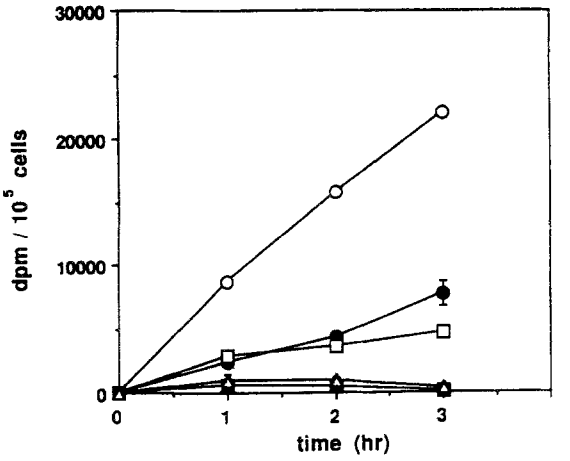

Fig. 4. Effect of MTX and/or VA exposure on [5-^{3}H]-uridine incorporation into RNA in L1210 cells. L1210 cells (3×10^5 cells/ml) were incubated for 48 h in the absence of MTX and VA (○) and in the presence of 1 μ*M* MTX (●), 100 μ*M* MTX (□), 1 μ*M* MTX plus 35 μ*M* VA (■), or 35 μ*M* VA (△). After the incubation, the [5-^{3}H]-uridine incorporation into RNA was determined at 1, 2, and 3 h. Data represent mean values ± SE for three experiments. The absence of bars indicates that SEs were too small to be expressed

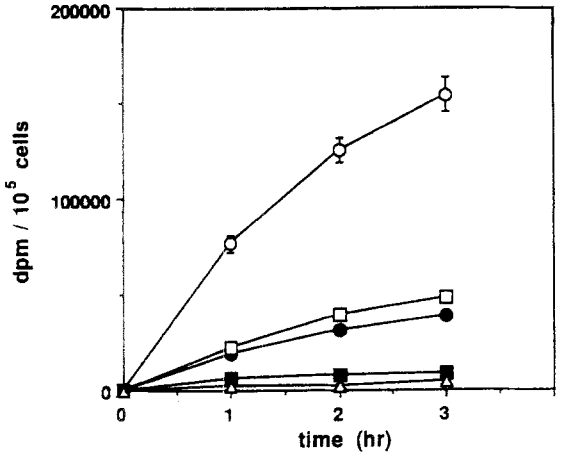

Fig. 3. Effect of MTX and/or VA exposure on [6-^{3}H]-thymidine incorporation into DNA in L1210 cells. L1210 cells (3×10^5 cells/ml) were incubated for 48 h in the absence of MTX and VA (○) and in the presence of 1 μ*M* MTX (●), 100 μ*M* MTX (□), 1 μ*M* MTX plus 35 μ*M* VA (■), or 35 μ*M* VA (△). After the incubation, the [6-^{3}H]-thymidine incorporation into DNA was determined at 1, 2, and 3 h. Data represent mean values ± SE for nine experiments. The absence of bars indicates that SEs were too small to be expressed

RNA, and proteins in L1210 cells were investigated using an MTX concentration of 1 or 100 μ*M* and a 48-h exposure period.

Effect of MTX and/or VA exposure on DNA synthesis

After L1210 cells had been exposed to MTX and/or VA for 48 h, the incorporation of [6-^{3}H]-thymidine into DNA was examined over 3 h (Fig. 3). The [6-^{3}H]-thymidine incorporation in L1210 cells incubated in the absence of MTX and VA (control) increased continuously. The incorporation in L1210 cells incubated with 1 or 100 μ*M* MTX increased in a similar manner, but this increase was much lower than that observed in the control; that is, the DNA synthesis in these cells was inhibited to approximately 30% of the control value. The incorporation in L1210 cells incubated with 1 μ*M* MTX plus 35 μ*M* VA or with 35 μ*M* VA alone was only slight, indicating that DNA synthesis was inhibited almost completely. Furthermore, the incorporation of [6-^{3}H]-uridine into DNA was also investigated in L1210 cells exposed to MTX and/or VA for 48 h, the results of which supported those obtained using [6-^{3}H]-thymidine (data not shown).

Effect of MTX and/or VA exposure on RNA synthesis

After L1210 cells had been exposed to MTX and/or VA for 48 h, the incorporation of [5-^{3}H]-uridine into RNA was examined over 3 h (Fig. 4). The [5-^{3}H]-uridine incorporation in L1210 cells incubated in the absence of MTX and VA (control) increased continuously. The incorporation in L1210 cells incubated with 1 or 100 μ*M* MTX increased in a similar manner, but this increase was much lower than that observed in the control; that is, the RNA synthesis in these cells was inhibited to approximately 30% of the control value. The incorporation in L1210 cells incubated with 1 μ*M* MTX plus 35 μ*M* VA or with 35 μ*M* VA alone was only slight, indicating that RNA synthesis was almost completely inhibited.

Effect of MTX and/or VA exposure on protein synthesis

After L1210 cells had been exposed to MTX and/or VA for 48 h, the incorporation of [4,5-^{3}H]-leucine into cellular proteins was examined over 3 h (Fig. 5). The [4,5-^{3}H]-

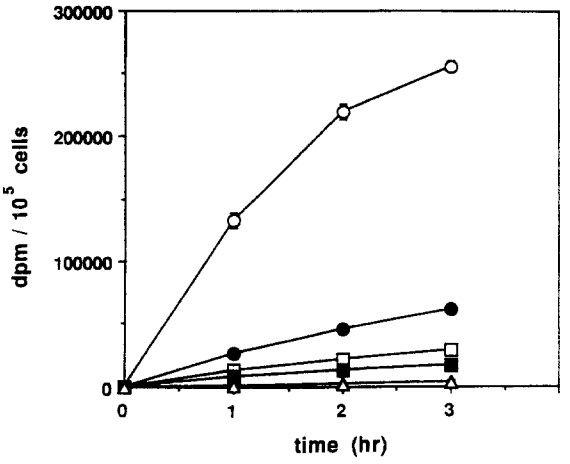

Fig. 5. Effect of MTX and/or VA exposure on [4,5-^{3}H]-leucine incorporation into proteins in L1210 cells. L1210 cells (3×10^5 cells/ml) were incubated for 48 h in the absence of MTX and VA (○) and in the presence of 1 μ*M* MTX (●), 100 μ*M* MTX (□), 1 μ*M* MTX plus 35 μ*M* VA (■), or 35 μ*M* VA (△). After the incubation, the [4,5-^{3}H]-leucine incorporation into proteins was determined at 1, 2, and 3 h. Data represent mean values ± SE for three experiments. The absence of bars indicates that SEs were too small to be expressed

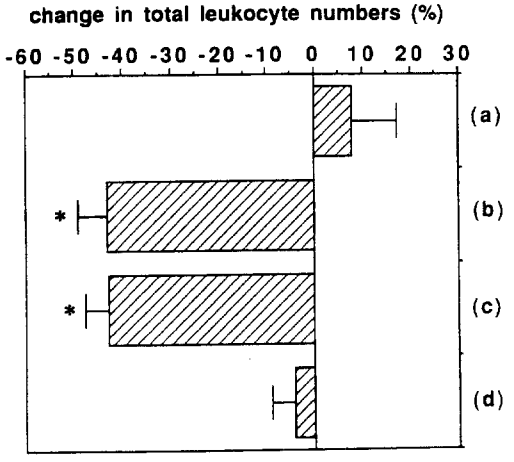

Fig. 6. Change in total leukocyte numbers in the blood of treated rats. *a*, Control (saline solution) rats; *b*, MTX (15 mg/kg)-treated rats; *c*, MTX (15 mg/kg) plus VA (5,000 IU/kg)-treated rats; *d*, VA (5,000 IU/kg)-treated rats. The change was expressed as the percentage (%) of total leukocyte numbers after the treatment relative to that before the treatment. Data represent mean values ± SE for 6 rats. * Significantly different from control rats ($P < 0.01$)

leucine incorporation in L1210 cells incubated in the absence of MTX and VA (control) increased continuously. The incorporation in L1210 cells incubated with 1 or 100 μ*M* MTX was much lower than that observed in the control; that is, the protein synthesis in these cells was inhibited to approximately 10%–25% of the control value. The incorporation in L1210 cells incubated with 1 μ*M* MTX plus 35 μ*M* VA or with 35 μ*M* VA alone was only slight, suggesting that protein synthesis was almost completely inhibited.

Effect of MTX and/or VA exposure on the bone marrow and liver

The rats were treated p.o. with MTX (15 mg/kg), MTX (15 mg/kg) plus VA (5,000 IU/kg), VA (5,000 IU/kg), or saline solution alone once daily for 4 days. The treatment indicated the protective effect of VA against MTX-induced damage to the intestine [9]. The constituents of blood from the treated rats were analyzed. The total number of leukocytes in the blood of rats treated with either MTX or MTX plus VA decreased significantly in comparison with the pretreatment values, but no significant difference was found between rats treated with MTX and those treated with MTX plus VA (Fig. 6). Treatment with VA alone did not affect the total number of leukocytes. Neither the number of red blood cells nor the amount of hemoglobin nor the hematocrit value was significantly affected by treatment with MTX and/or VA, and the plasma GOT activity was not affected either (data not shown).

Discussion

To examine whether or not VA, which protects the small intestine from MTX-induced cellular damage, would lessen the antitumor activity of MTX, we investigated the effect of VA on the antitumor activity of MTX using L1210 cells.

Fairchild et al. [6] examined the effect of MTX exposure on L1210 cytotoxicity and reported that since a 12-h period was equivalent to one cell cycle, more cells were exposed to MTX in the cytotoxic S phase during a 12-h exposure period than during a 6-h period and that a 24-h exposure resulted in a greater increase in MTX-induced cell killing than did a 12-h exposure. The results shown in Fig. 1 for MTX were coincident with the findings of Fairchild et al. [6]. The effects of exposure to VA alone or to MTX plus VA on L1210 cells were examined at VA concentrations of 17.5, 24.5, and 35 μ*M* by the same procedures used in the experiments shown in Fig. 1. As a result, VA did not inhibit the cell-killing activity of MTX but rather enhanced it because of the cytotoxic effect of VA itself.

MTX is reported to deplete cellular reduced folate pools by inhibiting dihydrofolate reductase, resulting in the inhibition of de novo thymidylate and purine synthesis [15, 23], which in turn results in inhibition of the synthesis of both DNA and RNA [4]. As illustrated in Figs. 3 and 4, the incorporation of both [6-^{3}H]-thymidine into DNA and [5-^{3}H]-uridine into RNA was depressed markedly by the exposure of L1210 cells to MTX. It is noteworthy that exposure of L1210 cells to MTX plus VA depressed the incorporation of both [6-^{3}H]-thymidine into DNA and [5-^{3}H]-uridine into RNA more profoundly than did exposure of the cells to MTX alone. Interestingly, such a strong depression in the incorporation of these two radiotracers was also observed following exposure of the cells to VA alone. This finding indicates the cytotoxic effect of VA against L1210 cells. [6-^{3}H]-Uridine incorporation is reported to enable the evaluation of DNA synthesis [6]. In

the present study, the incorporation of [6-^{3}H]-uridine into DNA in L1210 cells exposed to MTX and/or VA showed a depression similar to those seen using [6-^{3}H]-thymidine (data not shown). This observation further supports the present finding. The effect of MTX and/or VA exposure on the protein synthesis in L1210 cells was investigated (Fig. 5). The incorporation of [4,5-^{3}H]-leucine into cellular proteins in L1210 cells exposed to MTX and/or VA was depressed as well.

The cytotoxic effects of MTX have been recognized as being caused by inhibition of the de novo synthesis of purines, thymidylate, and certain amino acids [4]. Allegra et al. [1] reported that the inhibition of de novo purine synthesis was an important factor in the MTX-induced cell killing of human MCF-7 breast cancer cells. We have recently shown that MTX administration to rats depresses the de novo synthesis of purines in the crypt cells of small intestines in vivo and that VA coadministration prevents this inhibition of de novo purine synthesis [9]. VA is an important factor in epithelial differentiation and is likely involved in protein synthesis [3, 11, 24]. On the other hand, retinoids are known to have antitumor activity [12, 17, 24]. Retinoic acid and retinyl acetate have been reported to inhibit the growth of L1210 cells [12, 13]. As shown in the present study, VA itself induced an inhibition of the growth of L1210 cells accompanied by an inhibition of the synthesis of DNA, RNA, and proteins. MTX produced an inhibition of the growth of L1210 cells accompanied by an inhibition of the synthesis of DNA, RNA, and proteins, and the presence of VA in the cultures of L1210 cells with MTX apparently enhanced this inhibition. This observation indicates that the concurrent use of VA with MTX does not lessen the antitumor activity of MTX itself. It can be said that the presence of VA enhances the cell-killing activity of MTX rather than inhibiting it.

VA protects the small intestine from the damage known to be a serious side effect of MTX [9, 22]. The suppression of bone marrow is another serious side effect of this drug [4]. Whether or not VA would protect the bone marrow of rats was examined under the same experimental conditions under which we found its intestinal protective capacity. However, under these conditions, VA did not protect the bone marrow from MTX-induced depression (Fig. 6). This may be due to the observation that VA is a factor in epithelial differentiation [11, 24]. MTX is also reported to induce chronic liver injury [10], but the plasma GOT activity was not affected in the present study, suggesting that neither MTX nor VA caused liver injury during the treatment of rats with MTX and/or VA. Thus, the protective activity of VA described herein appears to be focused on MTX-induced damage to the intestine.

In summary, the present study using the in vitro culture of L1210 murine leukemia cells reveals that VA does not inhibit the antitumor activity of MTX. VA may be useful as a remedy to protect the intestine from MTX-induced damage.

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