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Inhibition of Expression of Cyclin A in Human B Cells by an Immunosuppressant Mizoribine[1]

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Mizoribine has been used to prevent rejection of organ allografts in humans and in animal models. Recent clinical trials have demonstrated its efficacy in rheumatoid arthritis and lupus nephritis, in which abnormalities of B cell functions are also involved. We therefore examined the effects of mizoribine on the in vitro function of human B cells. IgM production was induced from highly purified B cells obtained from healthy donors by stimulation with *Staphylococcus aureus* Cowan I (SA) plus IL-2. Mizoribine suppressed the production of IgM at its pharmacologically attainable concentrations (1 to 3 μg/ml) in a dose-dependent manner. Mizoribine had to be present within the first 96 h after the initiation of cultures to exert its suppressive effects on B cell responses. Cell cycle analysis disclosed that mizoribine significantly decreased the numbers of B cells in S + G2 + M phases. Mizoribine did not decrease GTP levels in SA-stimulated B cells, whereas mizoribine led to a decrease in GTP levels in activated T cells, which was reversed by addition of GMP. Consistently, the suppressive effects of mizoribine on the IgM production of SA-stimulated B cells was not reversed by the addition of GMP as much as 40 μM, which overcame the inhibitory effects of mizoribine on the proliferation of anti-CD3-stimulated T cells. Although mizoribine did not suppress the expression of CD25 and cdc2 kinase, mizoribine markedly suppressed the expression of cyclin A in SA-activated B cells. These results indicate that mizoribine directly suppresses the function of human B cells without interfering with the initial phase of activation. Moreover, the data demonstrate that mizoribine interferes with the cell cycle progression of activated B cells by suppressing the expression of cyclin A by a mechanism distinct from guanine ribonucleotide depletion. ***The Journal of Immunology,*** **1995, 155: 5175–5183.**

Mizoribine (4-carbamoyl-1-β-D-ribofuranosylimidazolium-5-olate, also known as bredinin) was isolated from the soil fungus *Eupenicillum brefeldianum* (1), and has been shown to be an effective immunosuppressant (2–4). Thus, mizoribine has been used successfully as a primary immunosuppressive drug in human renal transplantation without demonstrable myelosuppression or hepatotoxicity (4–7). Mizoribine is metabolized to its monophosphate form by adenosine kinase (8), and has been postulated to inhibit inosine monophosphate (IMP)[3] dehydrogenase (EC1.2.1.14), which is required for the synthesis of guanine nucleotides from IMP (9). Recent studies have demonstrated that mizoribine suppresses the proliferation of human T cells in a mechanism that is reversed by repletion of intracellular GTP (10). Thus, the inhibition of the T cell proliferation has been shown to result from the block of the transition from G1 to S phase of the cell cycle, which was reversed with GTP repletion (10).

It has been disclosed that mizoribine prevents the development of nephritis and the expression of anti-DNA Ab in New Zealand B/W F_1 mice (11). In addition, recent clinical trials have demonstrated the efficacy of mizoribine in the treatment of patients with rheumatoid arthritis (RA) and lupus nephritis (12, 13). Since abnormalities of B cell functions as well as T cell functions have been considered to be involved in the pathogenesis of RA (14) and SLE (15), it is possible that mizoribine also has direct immunosuppressive influences on B cells. In fact, in murine systems, mizoribine has been found to suppress the production of Abs against a T cell-independent Ag as well as a T cell-dependent Ag (16); however, specific data on the effects of mizoribine on human B cells have not been available.

The current studies were therefore undertaken to explore in detail the immunosuppressive effects of mizoribine on human B cells. We examined the effects of pharmacologically attainable concentrations of mizoribine on the in vitro function of human B cells, using stimulation with *Staphylococcus aureus* (SA) Cowan I. The results indicate that at pharmacologically attainable concentrations mizoribine directly suppresses the IgM production of B cells without interfering with the initial phases of B cell activation. Moreover, the data have demonstrated that mizoribine suppresses the cell cycle progression of SA-activated B cells by inhibiting the expression of cyclin A by a mechanism distinct from depletion of guanine ribonucleotide.

Materials and Methods

Monoclonal antibodies

A variety of T cell-specific mAb were used, including 64.1 (a gift of Dr. Peter E. Lipsky, University of Texas Southwestern Medical Center at Dallas, Dallas, TX), an IgG2a mAb directed at the CD3 molecule on mature T cells, and OKT8 (American Type Culture Collection, Rockville, MD), an IgG2a mAb directed at the CD8 molecule on the suppressor/cytotoxic T cell subset. A variety of miscellaneous mAb was also used,

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[3] Abbreviations used in this paper: IMP, inosine monophosphate; N-SRBC, neuraminidase-treated SRBC; GaMIg, goat anti-mouse Ig; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5 diphenyulbrazolium bromide; TBS, Tris-buffered saline.

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including I_3 (Coulter Immunology, Hialeah, FL), an IgG2a mAb directed at monomorphic HLA-DP, DQ, and DR determinants; anti-Tac, an IgG1 mAb directed at the p55 component of the IL-2R (CD25) (Becton Dickinson, Mountain View, CA); anti-human cyclin A, an IgG1 mAb directed at human cyclin A (UBI, Lake Placid, NY); anti-cdc2Hs, an IgG1 mAb directed against human cdc2 kinase (*Homo sapiens* cdc2) (UBI); and MOPC 21, an IgG1 control mAb (Cappel Laboratories, West Chester, PA).

Reagents

Formalinized Cowan I strain SA was purchased from Calbiochem-Behring (San Diego, CA) and was used at a concentration of 1/60,000 (v/v). Human rIL-2 (TGP-3) was a gift of Takeda Chemical Industries, Ltd. (Osaka, Japan), whose unit activity was determined by the providers (4.2×10^4 U/mg of protein). Mizoribine was synthesized and provided by Asahi Chemical Industry Co. (Tokyo, Japan). Guanosine, 8-aminoguanosine, and GMP were obtained from Sigma Chemical Co. (St. Louis, MO).

Culture medium

RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with penicillin G (100 U/ml), streptomycin (10 μg/ml), L-glutamine (0.3 mg/ml), and 10% FCS (Life Technologies) was used for all cultures.

Cell preparation

PBMC were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over sodium diatrizoate-Ficoll gradients (Histopaque, Sigma Chemical Co.). PBMC were depleted of monocytes and NK cells by incubation with 5 mM L-leucine methyl ester HCl (Sigma Chemical Co.) in serum-free RPMI 1640, as described elsewhere (17). The treated cell population was washed twice and then incubated with neuraminidase-treated SRBC (N-SRBC). The rosetting and nonrosetting populations were then separated by centrifugation on sodium diatrizoate-Ficoll gradients. The nonrosetting cells obtained from the interface were again rosetted with N-SRBC and centrifuged on sodium diatrizoate-Ficoll gradients to remove residual T cells. The resultant population of B cells contained <1% T cells as determined by staining with OKT3 and OKT11 pan T cell mAb (American Type Culture Collection, Rockville, MD), followed by analysis by flow cytometry. The cells were additionally characterized as containing >90% CD20 (B1, Coulter Immunology)-positive B cells, <1% CD14 (IOM2, Immunotech, Marseille, France)-positive monocytes, and no CD16 (Leu-11b, Becton Dickinson)-positive NK cells, as determined by flow cytometry.

Purified $CD4^+$ T cell populations were prepared by negative selection, using a panning technique to deplete contaminating $HLA\text{-}DR^+$ cells and $CD8^+$ T cells, as indicated (18). Cells were reacted with saturating concentrations of anti-MHC class II Ag mAb I_3 plus OKT8. After washing, the cells were added to panning dishes coated with goat anti-mouse Ig (GaMIg; Cappel). After a 70-min incubation at 4°C, the nonadherent cells were gently aspirated and were panned a second time on another GaMIg-coated petri dish, after which the nonadherent cells were aspirated. The $CD4^+$ T cell population obtained in this manner contained <2% $CD8^+$ T cells and >96% $CD4^+$ T cells.

Cell culture techniques for induction of Ig production

mAb 64.1 was diluted in RPMI 1640 (2 μg/ml), and 50 μl was placed in each well of 96-well flat-bottom microtiter plates (no. 3596; Costar, Cambridge, MA) and incubated at room temperature for 1 h (18). The wells were then washed once with culture medium to remove nonadherent mAb before the cells were added. Approximately 14 to 20% of the added mAb adhered to the wells (19). Cultures were conducted in duplicate in a total volume of 200 μl. $CD4^+$ T cells that had been treated with mitomycin C (40 μg/ml) before culture (1×10^5/well) were cultured in wells with immobilized anti-CD3 with 2.5×10^4 autologous B cells (18). In some experiments, B cells (5×10^4/well) were cultured alone in wells of 96-well U-bottom microtiter plates (no. 3799; Costar) with SA + IL-2 (0.5 U/ml). The cells were incubated for 10 to 11 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air.

Measurement of IgM

Microtiter plates (Cooke; Dynatech, Alexandria, VA) coated with $F(ab')_2$ fragments of goat anti-human IgM (Cappel) were incubated with cellfree culture supernatants or IgM standards in PBS containing 1% BSA (Miles, Elkhart, IN). Bound IgM was detected with peroxidase-conjugated $F(ab')_2$ fragments of goat anti-human IgM (Cappel) as previously described (20).

Measurement of T cell proliferation

The proliferation of immobilized anti-CD3-stimulated T cells was assessed by the colorimetric assay using MTT-Cell Growth Assay Kit (Chemicon, El Segundo, CA). Briefly, after 72 h of cultures, 100 μl of the supernatants were removed and 10 μl of MTT solution were added to each well. After 3 to 6 h of incubation, 100 μl of 0.04 N HCl in isopropanol were added to each well, followed by vigorous pipetting, and OD 570 was measured in 2-wavelength Microplate Photometer (MTP-120, Corona Electric Co., Ibaraki, Japan). Each point was based on the reading of triplicated wells.

Immunofluorescence staining of cell surface markers, cdc2 kinase, and cyclin A, and analysis by flow cytometry

B cells (1×10^6/ml) were cultured in 17 × 100-mm polypropylene tubes with SA+IL-2 in the presence or absence of mizoribine for various periods. $CD4^+$ T cells (1×10^5/well) that had not been treated with mitomycin C were cultured in wells with immobilized anti-CD3. After the incubation, the cells were washed once with PBS containing 2% normal human AB serum and 0.1% sodium azide (staining buffer) and were fixed with ethanol cooled to −20°C for 5 min. The cells were reacted in suspension by incubating for 30 min at 4°C with anti-cyclin A mAb or anti-cdc2 kinase mAb, after which the cells were washed three times with staining buffer and were stained with FITC-conjugated goat anti-mouse Ig (Cappel), followed by counterstaining with propidium iodide (Sigma Chemical Co.) on some occasions. In some experiments, B cells were stained with FITC-conjugated anti-CD25 mAb. After staining, the cells were washed three times with staining buffer and were fixed with 1% paraformaldehyde in PBS (pH 7.4) for more than 5 min at room temperature. The cells were analyzed using an EPICS Profile or EPICS XL flow cytometer (Coulter) equipped with an argon-ion laser at 488 nm, as previously described (21).

Analysis of DNA content

Following incubation of B cells with SA + IL-2 in the presence or absence of mizoribine, the supernatants were totally removed and replaced with 100 μl PBS containing 0.1% Triton X-100 (Wako Chemical, Osaka, Japan), 0.1% sodium citrate, and 50 μg/ml propidium iodide. After an overnight incubation at 4°C, the red fluorescence was measured on an EPICS Profile flow cytometer. Cellular debris were excluded from analysis by raising the forward scatter threshold, and the DNA content of intact cells was registered on a logarithmic scale.

Cell lysis and immunoprecipitations

Following incubation of B cells with SA + IL-2 in the presence or absence of mizoribine, the similar numbers of viable cells from each preparation were pelleted and lysed at 4°C. The lysis buffer was 50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM NaF, 2 mM EDTA, 1% (v/v) Triton X-100, 1 mM PMSF, 1 mM Na_2MoO_4, 10 μg/ml aprotinin, 10 μM β-methylaspartic acid, 10 μg/ml leupeptin, 0.2 mM sodium orthovanadate, and 1 mM DTT. After lysis, insoluble matters were removed by centrifugation. Cyclin A immunoprecipitates were prepared with 50 μg/ml of anti-cyclin A mAb at 4°C for 2 h with constant mixing, followed by addition of 10 μl protein A-Sepharose (Sigma Chemical Co.) for 1 h at 4°C. The Sepharose beads were washed three times in lysis buffer with 0.5 M LiCl.

SDS-PAGE and immunoblotting

After preparation as described above, the samples were boiled for 5 min in SDS gel sample buffer containing 50 mM DTT before analysis. Gels to be analyzed by immunoblotting were transferred to nitrocellulose membranes, which were then blocked for 2 h at room temperature with Block Ace (Dainippon Pharmaceutical, Osaka, Japan). Blots were probed with anti-human cyclin A mAb (Santa Cruz Biotechnology, Santa Cruz, CA) at 10 μg/ml in TBS with 0.05% (v/v) Tween 20 for 2 h at room temperature, and developed with peroxidase-conjugated $F(ab')_2$ fragments of GaMIg (Cappel) diluted by 1/10,000 in TBS with 0.05% (v/v) Tween 20 for 1 h at room temperature. Visualization was performed with an ECL system (Amersham).

Measurement of GTP

GTP pools in T cells and B cells were quantified using HPLC on a Shodex AXpak WA-624 column (Shimadzu, Kyoto, Japan). A total of 5 to 10 × 10^6 cells were harvested, counted, and washed twice. Cell viability always exceeded 95% as determined by trypan blue exclusion. The cells were pelleted and were extracted with ice-cold 0.4 N perchloric acid, followed by neutralization with 1 N KOH. The volume of sample analyzed was adjusted for cell count, and 100 μl of the supernatant were injected for each analysis. GTP was separated in the mobile phase consisting of 80% (v/v) 0.5 M NaH_2PO_4 and 20% (v/v) CH_3CN at a flow rate of 1.2 ml/min. The

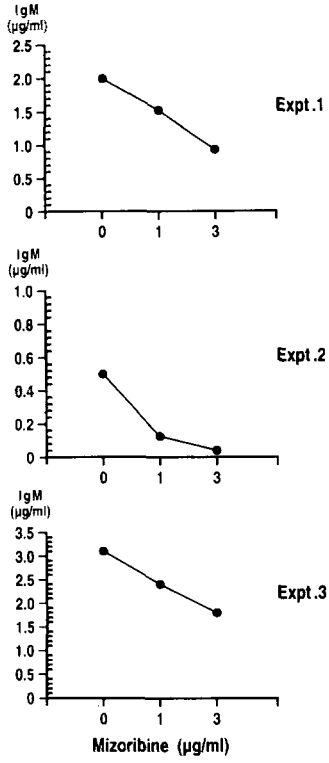

FIGURE 1. Mizoribine suppresses the IgM production of human B cells stimulated with SA + IL-2. Highly purified B cells (5×10^4/well) were stimulated with SA + IL-2 (0.5 U/ml) in the presence or absence of various concentrations of mizoribine. After 10 days of cultures, the supernatants were harvested and assayed for IgM contents by ELISA.

Table I. *T cell derived cytokines do not restore the suppressive effects of mizoribine on human B cells*[a]

Addition	T Cell Cytokines: IgM Production (μg/ml)		
	Nil	IL-2	IL-2 + T-sup
Nil	8.072	9.384	9.469
Mizoribine	0.773	1.173	1.276

[a] B cells (2.5×10^4/well) were cultured with mitomycin C-treated $CD4^+$ T cells (2×10^5/well) in wells with immobilized anti-CD3 (64.1, 100 ng/well) in the presence or absence of mizoribine (3 μg/ml). IL-2 (0.5 U/ml) or factors generated from immobilized anti-CD3-activated $CD4^+$ T cells (T-sup, 10% in final concentration) was added where indicated. After 10 days of incubation, the supernatants were harvested and assayed for IgM contents by ELISA.

GTP levels in the samples were calculated by comparing areas of peaks absorbing at 260 nm to the areas generated by known amounts of GTP.

Results

Pharmacologic effects of mizoribine on the production of IgM

Initial experiments were conducted to test the influences of pharmacologically attainable concentrations of mizoribine on the production of IgM by B cells. IgM production was induced from highly purified B cells by stimulation with SA + IL-2. As can be seen in Figure 1, mizoribine suppressed the production of IgM induced by SA + IL-2 at concentrations of 1 to 3 μg/ml (pharmacologically attainable concentrations of mizoribine were approximately 3 μg/ml) (10, 22). As shown in Table I, although mizoribine also suppressed the IgM production induced by immobilized anti-CD3-activated $CD4^+$ T cells, addition of IL-2 and factors generated from immobilized anti-CD3-activated $CD4^+$ T cells did not restore the suppressive effects of mizoribine on the IgM production. Moreover, mizoribine (3 μg/ml) did not inhibit the expression of CD40 ligand on immobilized anti-CD3 stimulated $CD4^+$ T cells (data not shown). Taken together, these results confirm that mizoribine directly inhibits the functions of B cells.

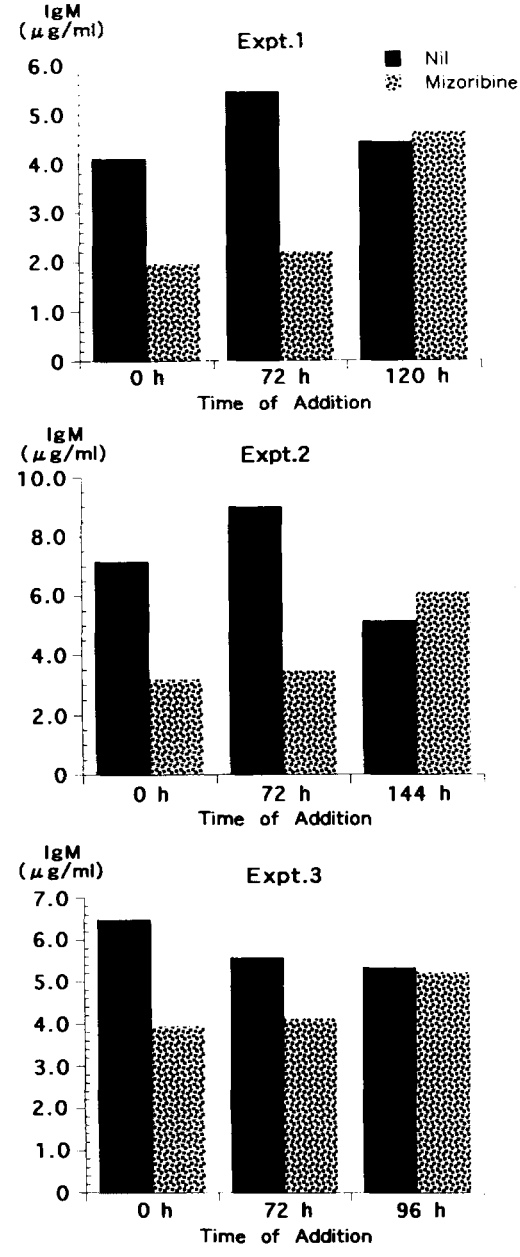

FIGURE 2. Mizoribine is required to be present within 96 h after the initiation of cultures to exert its suppressive effects on the differentiation of B cells. B cells (5×10^4/well) were cultured with SA + IL-2 (0.5 U/ml). Mizoribine (3 μg/ml) or culture medium was added at various periods after the initiation of cultures. After 10 days of total incubation, the supernatants were harvested and assayed for IgM contents by ELISA.

To explore the stages of B cell activation on which mizoribine exerts its suppressive effects, experiments were next undertaken in which mizoribine was added at different time points after the initiation of cultures. As shown in Figure 2, the suppressive effects of mizoribine on B cell responses were comparable when it was added within 72 h of cultures. By contrast, mizoribine was no more

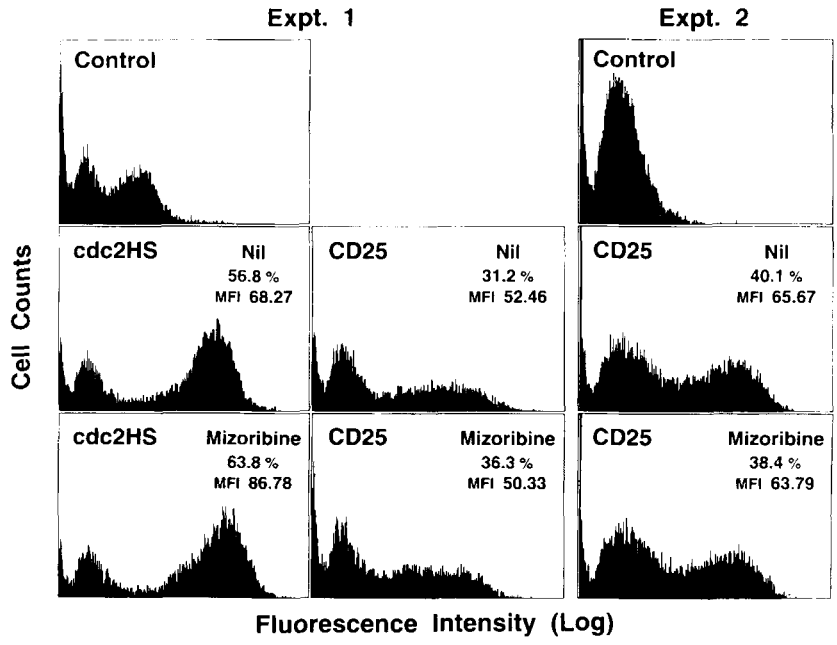

FIGURE 3. Mizoribine does not suppress the expression of IL-2R (CD25) or cdc2 kinase on SA-activated B cells. B cells were cultured with SA + IL-2 (0.5 U/ml) in the presence or absence of mizoribine (3 μg/ml). After 72 h of cultures, the cells were fixed in cold ethanol and were reacted with anti-cdc2 kinase mAb (cdc2HS) or anti-CD25 mAb followed by staining with FITC-conjugated GaMIg (Expt. 1). Alternatively, after 72 h of incubation, unfixed B cells were stained with FITC-conjugated anti-CD25 mAb (Expt. 2). The cells were then analyzed on flow cytometry. The percentages of cells positive for cdc2 kinase and CD25 are shown. MFI, mean fluorescence intensity.

suppressive when it was added after 96 h from the initiation of cultures. These results indicate that mizoribine does not exert its suppressive effects on the IgM production after the differentiation of B cells into Ig-secreting cells starts to take place.

Mizoribine does not inhibit the expression of IL-2R and cdc2 kinase in B cells

To further specify the stages of B cell activation on which mizoribine exerts its suppressive effects, we next examined the influences of mizoribine on the expression of IL-2R and cdc2 kinase in B cells, the cellular events characteristics of G1 phase in the cell cycle (23). B cells were stimulated with SA + IL-2 for 72 h in the presence or absence of mizoribine, and the expression of the α-chain of IL-2R (CD25) and cdc2 kinase was assessed by flow cytometry. As shown in Figure 3, mizoribine did not significantly affect the expression of IL-2R and that of cdc 2 kinase in B cells activated by SA + IL-2. In addition, mizoribine did not inhibit either the expression of transferrin receptor (CD71) on SA-stimulated B cells or the production of IL-6 by SA-activated B cells (data not shown). These results confirm that mizoribine does not block the early events in B cell activation.

Mizoribine interferes with the cell cycle progression of B cells

Since it was shown that mizoribine did not suppress the expression of cdc 2 kinase, which is expressed at the R (restriction) point in the G1 phase of the cell cycle (23), experiments were undertaken in which the effects of mizoribine on the cell cycle progression of SA-activated B cells were examined. B cells were stimulated with SA + IL-2 in the presence or absence of mizoribine for 72 h, after which B cells were stained with propidium iodide and analyzed on flow cytometry. As can be seen in Figure 4, cultures with mizoribine contained significantly decreased numbers of cells in S + G2 + M phases compared with those without mizoribine. These results therefore indicate that mizoribine interferes with the cell cycle progression of B cells through the G1 and S phases, although the block between G1 and S phases was not complete.

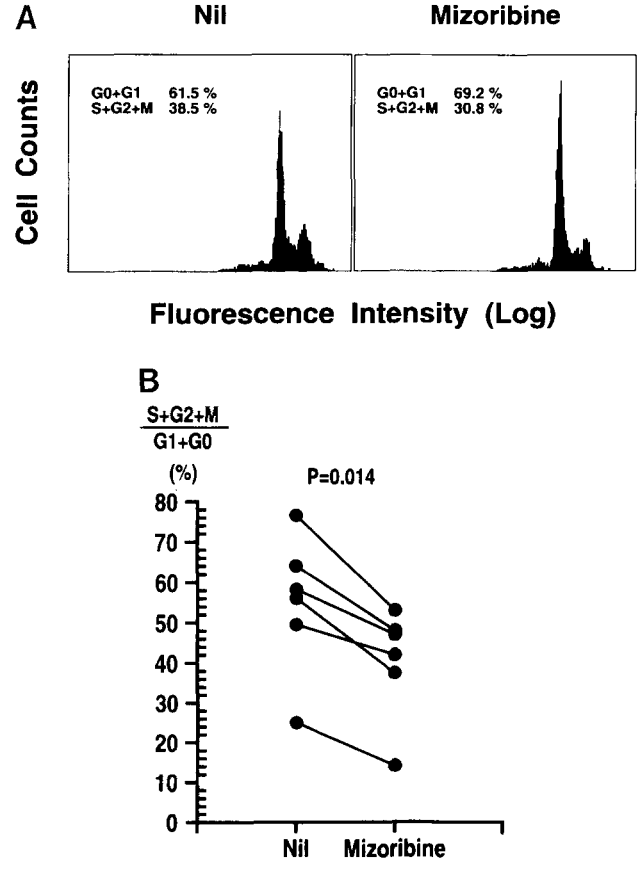

FIGURE 4. Mizoribine suppresses the progression of B cells from the G1 to the S phase in the cell cycle. B cells (5×10^4/well) were cultured with SA + IL-2 (0.5 U/ml) in the presence or absence of mizoribine (3 μg/ml). After 72 h of cultures, B cells were stained with propidium iodide and analyzed on flow cytometry. A representative histogram (*A*) and a summary of six independent experiments (*B*) are presented. Statistical analysis was performed using Wilcoxon's signed rank test.

Table II. *Differential influences of supplemental guanine metabolites on the suppressive effects of mizoribine on the functions of human B cells and T cells*[a]

Expt. No.	Addition (μg/ml)	IgM Production (μg/ml)		T Cell Proliferation (OD 570, Mean ± SEM)	
		Nil	Mizoribine	Nil	Mizoribine
1	Nil	3.75	1.46	0.521 ± 0.018	0.434 ± 0.016[b]
	8-AG/G (0.15)	2.70	1.44		
	8-AG/G (1.5)	2.13	1.41		
	8-AG/G (15)	0.83	0.84	0.450 ± 0.015	0.451 ± 0.017
2	Nil	1.38	0.38	0.544 ± 0.040	0.383 ± 0.044[b]
	GMP (5)	1.06	0.15	0.523 ± 0.014	0.506 ± 0.022
	GMP (15)	1.58	0.41	0.534 ± 0.045	0.534 ± 0.037
	GMP (50)	0.06	0.02	0.223 ± 0.011	0.207 ± 0.008
3	Nil	6.48	2.05	1.067 ± 0.020	0.831 ± 0.029[c]
	GMP (5)	7.80	2.53	1.056 ± 0.046	0.849 ± 0.023[b]
	GMP (15)	8.31	2.73	1.050 ± 0.031	1.033 ± 0.037
	GMP (50)	1.16	0.83	0.759 ± 0.030	0.711 ± 0.024

[a] B cells (5×10^4/well) were cultured with SA + IL-2 or T cells (1×10^5/well) were cultured in wells with immobilized anti-CD3 (64.1, 100 ng/well) in the presence or absence of mizoribine (Expts. 1 and 2, 3 μg/ml; Expts. 3, 5 μg/ml). Guanosine (G), 8-aminoguanosine (8-AG) (30 μg/ml), or GMP was added where indicated. After 10 days of cultures, the supernatants were harvested and assayed for IgM contents by ELISA. After 3 days of cultures, the T cell proliferation was assessed by the colorimetric assay as described in *Materials and Methods*.

[b] Significant at $p < 0.05$.

[c] Significant at $p < 0.01$ as determined by Student's *t*-test compared with cultures without mizoribine.

Exogenous GMP does not restore the suppressive effects of mizoribine on the function of B cells

Mizoribine has been postulated to be an inhibitor of IMP dehydrogenase, which catalyzes the conversion of IMP to GMP (8). In fact, previous studies have shown that mizoribine led to a decrease in intracellular GTP levels (10). In addition, repletion of GTP has been demonstrated to reverse the antiproliferative effects of mizoribine on T cells (10). Previous studies have demonstrated that addition of the combination of guanosine and 8-aminoguanosine results in a sustained rise in intracellular concentration of GTP in cultured T cells (10). In the present study, addition of guanosine and 8-aminoguanosine almost completely restored the proliferation of immobilized anti-CD3-stimulated T cells (Table II). However, addition of guanosine and 8-aminoguanosine suppressed the IgM production in a dose-dependent manner without the presence of mizoribine (Table II). On the other hand, addition of GMP as much as 40 μM (15 μg/ml) also completely restored the proliferation of anti-CD3-stimulated T cells, as is consistent with a previous report (24). By contrast, addition of GMP as much as 40 μM, which was not itself suppressive for the IgM production, did not reverse the suppressive effects of mizoribine (3 μg/ml) on the IgM production of B cells stimulated with SA + IL-2. These results therefore strongly suggest that mizoribine might suppress the Ig production of B cells in a mechanism distinct from guanine ribonucleotide depletion.

To further confirm that the suppressive effects of mizoribine on the function of B cells are distinct from guanine ribonucleotide depletion, we directly measured the GTP levels in B cells and T cells. In general, the GTP levels in SA-stimulated B cells were much lower than in T cells stimulated with PMA or immobilized anti-CD3. In fact, in one experiment, the GTP levels in B cells were too low to be detected (Table III). Mizoribine led to a decrease in intracellular GTP levels in activated T cells, which was repleted by exogenous GMP as much as 40 μM, as is consistent with a previous report (24). By contrast, mizoribine did not significantly decrease intracellular GTP levels in SA-stimulated B cells. These results confirm that mizoribine suppresses the B cell functions in a mechanism distinct from GTP depletion. Moreover, the data suggest that the metabolism of guanine ribonucleotide in B cells might be differently regulated from that in T cells.

Table III. *Differential effects of mizoribine on the intracellular GTP levels in human T cells and B cells*[a]

Expt. No.	Addition	GTP Levels (pmol/10^7 Cells)	
		T cells	B cells
1	Nil	1124.76	178.26
	Mizoribine	858.16	169.72
	GMP	1345.47	188.16
	GMP + mizoribine	1234.46	164.95
2	Nil		137.65
	Mizoribine		133.39
	GMP + mizoribine		110.16
3	Nil		86.68
	Mizoribine		81.49
	GMP + mizoribine		75.38
4	Nil	1268.94	ND[b]
	Mizoribine	290.64	ND

[a] T cells (5×10^6/ml) were stimulated in a 6-ml tube with PMA (1 ng/ml) (Expt. 1) or T cells (2×10^5/well) were cultured in wells with immobilized anti-CD3 (64.1, 100 ng/ml) (Expt. 4) for 72 h. B cells (1×10^6/ml) were stimulated with SA + IL-2 (0.5 U/ml) in a 6-ml tube for 96 h (Expts. 1 to 3) or for 72 h (Expt. 4). Mizoribine (Expts. 1 to 3: 3 μg/ml; Expt. 4: 5 μg/ml); and GMP (40 μM (15 μg/ml)) were added where indicated. After the incubation, the cells were harvested and the intracellular GTP levels were measured as described in *Materials and Methods*.

[b] ND, not detectable (under detection limit).

Mizoribine inhibits the expression of cyclin A in B cells

It was shown that mizoribine did not block B cells to pass the R point in the G1 phase, but inhibited the cell cycle progression after late G1 phase of such B cells. Cyclin A has been shown to play a critical role in the progression of the cell cycle, especially from G1 to S phase, in eukaryotes (25–28). We next examined whether mizoribine might inhibit the expression of cyclin A in B cells. B cells were stimulated with SA + IL-2 for 72 h or 96 h in the presence or absence of mizoribine, and the expression of cyclin A

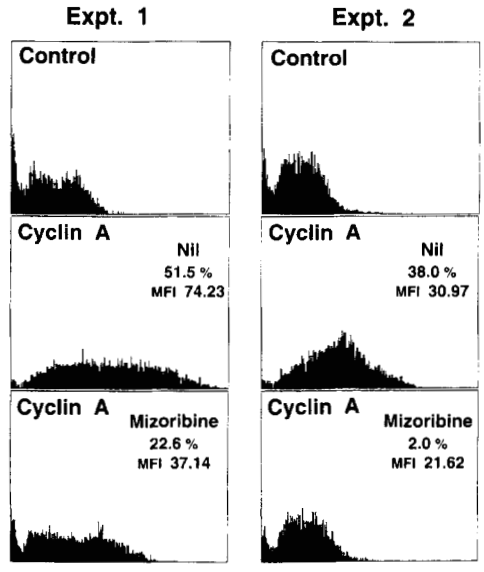

FIGURE 5. Mizoribine suppresses the expression of cyclin A in B cells. B cells were cultured with SA + IL-2 (0.5 U/ml) in the presence or absence of mizoribine (3 μg/ml). After 96 h (Expt. 1) or 72 h (Expt. 2) of cultures, the cells were fixed in cold ethanol and were reacted with control mAb or anti-cyclin A mAb followed by staining with FITC-conjugated GaMIg. The cells were analyzed on flow cytometry. The horizontal and vertical axes denote fluorescence intensity (log) and cell counts, respectively. The percentages of cells positive for cyclin A are shown. (MFI, mean fluorescence intensity.)

was assessed by flow cytometry. As shown in Figure 5, mizoribine inhibited the expression of cyclin A in B cells. Of note, supplemental GMP (40 μM) did not reverse the inhibition of the cyclin A expression in B cells (Fig. 6*A*). In addition, the results in the double staining using propidium iodide disclosed that mizoribine suppressed the cyclin A expression not only in B cells in G1/0 phases but in those in S/G2/M phases (Fig. 6*B*), suggesting that the suppressive effects of mizoribine might be sustained in B cells in S/G2/M phases.

To further confirm that mizoribine inhibits the expression of cyclin A in B cells, analysis with immunoprecipitation followed by immunoblotting was performed. As shown in Figure 7, mizoribine inhibited the expression of cyclin A in B cells in a dose-dependent manner. Of interest, SA-981, a derivative of bucillamine, which has been shown to inhibit the function of B cells (29, 30), did not suppress the expression of cyclin A in B cells, suggesting that the inhibition of the expression of cyclin A in B cells might not be a nonspecific feature associated with the inhibition of the B cell maturation.

Final experiments were undertaken to examine whether mizoribine might suppress the cyclin A expression in T cells. As shown in Table IV, mizoribine suppressed the expression of cyclin A in anti-CD3-stimulated T cells as well as the T cell proliferation in a dose-dependent manner. Moreover, the suppressive effects of mizoribine on the cyclin A expression in anti-CD3 stimulated T cells were significantly reversed by addition of GMP in accordance with the restoration of the T cell proliferation. These results therefore indicate that mizoribine suppresses the cyclin A expression in T cells as well as that in B cells. More importantly, the data argue that the mechanism of suppression of the cyclin A expression is different between T cells and B cells with regard to the dependency on the guanine ribonucleotide depletion.

Discussion

Mizoribine, an imidazole nucleoside, was first isolated from a soil fungus in 1974, and has proved to have beneficial effects in both clinical and experimental transplantation (4–7). Previous studies, in fact, demonstrated that mizoribine blocks the proliferation of T cells to a variety of stimuli (10). On the other hand, mizoribine has been shown to have beneficial effects in the treatment of RA and lupus nephritis in several clinical trials (12, 13). Since abnormal B cell functions have been implicated in the pathogenesis of RA as well as lupus nephritis (14, 15), it is possible that mizoribine might modulate the functions of B cells. However, little is known of its effects on human B cell functions. The results in the current studies have clearly demonstrated that mizoribine directly inhibits the differentiation of human B cells without interfering with the initial phases of B cell activation. Thus, mizoribine did not inhibit the events occurring in the initial phases of G1, including the B cell production of IL-6 and the expression of the α-chain of the IL-2R (CD25) as well as the transferrin receptor (CD71)

The cell cycle analysis by staining with propidium iodide revealed that mizoribine decreased the numbers of B cells in S + G2 + M phases. In addition, it was found that mizoribine did not inhibit the expression of cdc2 kinase in B cells. Since the mRNA for cdc2 kinase has been shown to be expressed at the R point in G1 (23), these results indicate that mizoribine blocks the cell cycle progression of B cells after G1 phase distal to the R point. In this regard, the effects of mizoribine on B cells were comparable with those on T cells (10). On the other hand, previous studies have demonstrated that the suppressive influences of mizoribine on T cell proliferation depend on the GTP depletion and can be reversed with GTP repletion by supplemental guanosine and 8-aminoguanosine (10). By contrast, the data in the present study demonstrated that mizoribine does not result in a decrease in GTP levels in SA-stimulated B cells. Consistently, the suppressive effects of mizoribine on B cell differentiation could not be reversed with supplementation of GMP, which also repleted the GTP pools in T cells and overcame the inhibitory effects of mizoribine on the T cell proliferation. It should be noted that GTP levels in B cells were much lower than those in T cells even in the presence of exogenous GMP. In fact, the GTP levels in B cells were too low to be detected with HPLC assay in some experiments. These results suggest that the metabolism of guanine ribonucleotide in B cells might be different from that in T cells. Thus, it is likely that GMP synthesis in T cells might be more dependent on IMP dehydrogenase than in B cells, whereas GMP synthesis in B cells might be dependent more on other pathways, as is suggested from the proposed purine metabolic pathways (31). More importantly, our data indicate that mizoribine blocks the differentiation of B cells by a mechanism distinct from guanine ribonucleotide depletion.

Cyclin A has been shown to play a critical role in the regulation of the cell cycle progression, especially through the G1 and S phases (25–28). In the current studies, mizoribine at pharmacologically attainable concentrations inhibited the expression of cyclin A in B cells without decreasing GTP levels in B cells. Moreover, the inhibition of the expression of cyclin A in B cells was not overcome with supplemental GMP, confirming that mizoribine suppresses the expression of cyclin A in B cells by a mechanism distinct from guanine ribonucleotide depletion. Of note, mizoribine at low concentrations (3 μg/ml) suppressed the expression of cyclin A in T cells in a fashion that was reversed by GTP repletion with supplemental GMP. Taken together, these results indicate that at pharmacologically attainable concentrations mizoribine inhibits

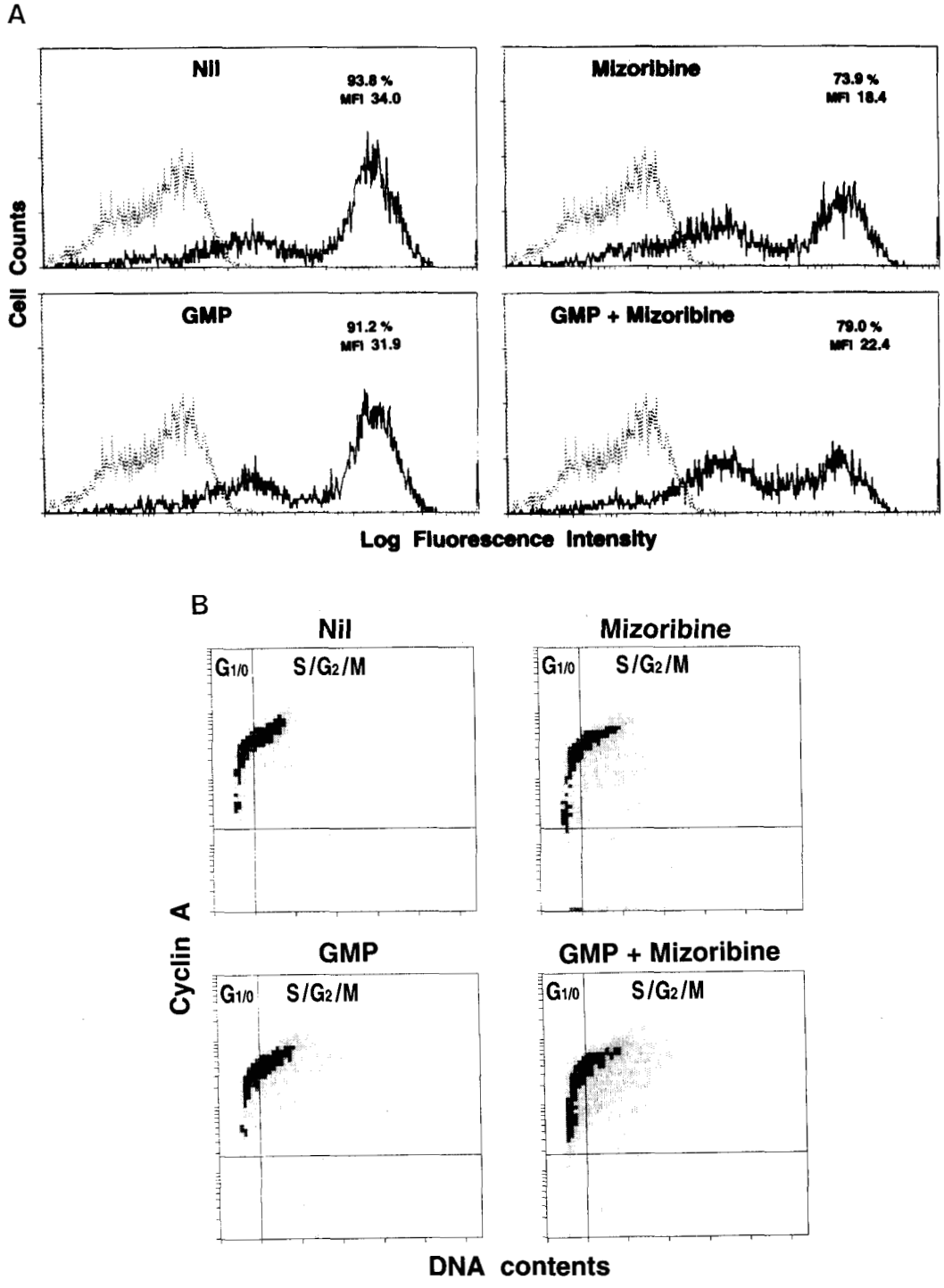

FIGURE 6. Mizoribine suppresses the expression of cyclin A in B cells: effects of supplemental GMP. B cells were cultured with SA + IL-2 (0.5 U/ml) in the presence or absence of mizoribine (3 μg/ml) and GMP (40 μM (15 μg/ml)). After 96 h of cultures, the cells were fixed in cold ethanol and were reacted with control mAb or anti-cyclin A mAb followed by staining with FITC-conjugated GaMIg (*A*). The cells were further stained with propidium iodide (50 μg/ml) (*B*). The cells were analyzed on flow cytometry. The mean fluorescence intensity (MFI) of cyclin A staining in G1/0 phases and in S/G2/M phases is: (Nil) 20.3 and 40.9; (mizoribine) 14.5 and 30.4; (GMP) 23.1 and 46.2; (GMP + mizoribine) 16.3 and 36.9, respectively.

B cell functions by suppressing the expression of cyclin A in a mechanism distinct from guanine ribonucleotide depletion, whereas mizoribine also suppresses the expression of cyclin A in T cells by a mechanism dependent on guanine ribonucleotide depletion. Thus, the data argue that B cells and T cells might be also different in the mechanism for the mizoribine mediated-suppression of cyclin A expression.

Previous studies have reported that mizoribine at high concentrations (50 μg/ml) suppresses T cell proliferation even with GTP repletion, suggesting that mizoribine might have an additional mechanism of action on T cell proliferation distinct from GTP depletion (10). Consistently, we have found that high concentration of mizoribine (50 μg/ml) inhibited the cyclin A expression in

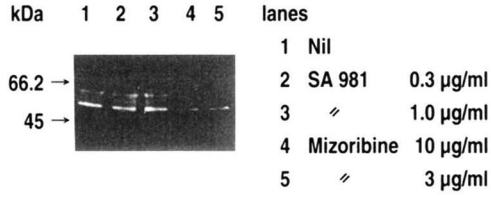

FIGURE 7. Mizoribine, but not an intramolecular disulfide form of bucillamine, suppresses the expression of cyclin A in B cells. B cells were cultured with SA + IL-2 (0.5 U/ml) in the presence or absence of mizoribine (3 μg/ml or 10 μg/ml) or an intramolecular disulfide form of bucillamine (SA-981) (0.3 μg/ml or 1 μg/ml). After 96 h of cultures, the cells were harvested, pelleted and lysed at 4°C in the lysis buffer. Cyclin A immunoprecipitates were prepared, and were analyzed with SDS-PAGE followed by Western blotting using anti-cyclin A mAb. The data are representative of three independent experiments.

T cells without affecting cell viability in a manner that was not reversed by GTP repletion with supplemental GMP (40 μM) or combination of guanosine (15 μg/ml) and 8-aminoguanosine (30 μg/ml) (data not shown). On the other hand, the current studies revealed that GTP levels in B cells were much lower than T cells even in the presence of exogenous GMP. Moreover, mizoribine did not result in GTP depletion in B cells, but in T cells. Taken together, it is suggested that the inhibition of cyclin A expression by mizoribine might be correlated with the intracellular GTP levels irrespective of the cell types. It should be noted that there exists structural homology between mizoribine and guanine nucleoside (1). It is therefore likely that the suppression of cyclin A expression by mizoribine might be antagonized by some guanine metabolites, whose levels might be correlated with intracellular GTP levels, although further studies are required to identify such metabolites as well as to delineate the precise sequelae of the inhibition of cyclin A expression by mizoribine.

Recent studies have disclosed that the down-regulation of cyclin D3 expression and cyclin D3-dependent kinase activity leads to growth arrest in the G1 phase of the cell cycle in T cells (32). In the present study, mizoribine only incompletely suppressed the G1-S transition of activated B cells, but did not result in G1 arrest of such B cells. In this regard, cyclin D3 is considered to play a more critical role in the G1-S transition in the cell cycle than cyclin A, and the inhibition of cyclin A expression might not be sufficient for the complete block of G1-S transition. Of note, mizoribine also suppressed the cyclin A expression in such B cells that have entered the S phase. It is therefore likely that mizoribine might interfere with the cell-cycle progression of activated B cells throughout the course after R point in the G1 phase.

Mizoribine has been admitted as an antirheumatic drug in Japan (13). It has been also shown that bucillamine, *N*-(2-mercapto-2-methylpropionyl)-L-cysteine, one of the most potent disease-modifying antirheumatic drugs, inhibits the differentiation of B cells (29, 30). We have also found that SA-981, an intramolecular disulfide of bucillamine, inhibited the cell cycle progression without affecting the IL-6 production, or the expression of CD25 and cdc2 kinase (S. Hirohata, T. Yanagida, and P. E. Lipsky, unpublished observations). In this regard, the suppressive effects of mizoribine on the function of B cells were comparable with those of bucillamine. However, the results in the present study have disclosed that SA-981 does not suppress the expression of cyclin A in B cells. The data therefore obviate the possibility that the suppression of the cyclin A expression might be a nonspecific event associated with the suppression of the cell-cycle progression after the G1 phase. Of note, previous studies revealed that SA-981 did not inhibit the function of T cells (30), whereas mizoribine has been shown to suppress T cell proliferation (10). These observations therefore suggest the possibility of the design of immunosuppressive drug combinations that take advantage of nonoverlapping mechanisms of actions, thus obtaining synergistic immunosuppression with minimizing toxicity.

In summary, the current studies have shown that mizoribine inhibits B cell differentiation without interfering with proximal events in B cell activation. The mechanism of action of pharmacologically attainable concentrations of mizoribine on B cells is totally different from that on T cells, which is a result from guanine ribonucleotide depletion. Thus, mizoribine at pharmacologically attainable concentrations has been demonstrated to suppress the expression of cyclin A in B cells in a mechanism distinct from guanine ribonucleotide depletion, whereas mizoribine inhibits the cyclin A expression in T cells in a mechanism that is reversed by guanine ribonucleotide repletion. Our data therefore suggest that B cells and T cells might be different in the regulation of guanine ribonucleotide metabolism as well as in the mechanism for the

Table IV. *Comparable inhibitory effects of mizoribine on the proliferation and on the expression of cyclin A of immobilized anti-CD3 stimulated T cells*[a]

Expt. No.	Addition		T Cell Proliferation	Cyclin A Expression	
	GMP	Mizoribine (μg/ml)	OD_{570} mean ± SEM	% Positive	Mean fluorescence intensity
1	(−)	0	0.713 ± 0.010	75.3	113.1
	(−)	3	0.634 ± 0.003[b]	43.5	40.4
	(−)	10	0.465 ± 0.016[b]	37.4	26.4
	(+)	0	0.713 ± 0.030	71.1	145.0
	(+)	3	0.694 ± 0.015	65.9	119.0
	(+)	10	0.644 ± 0.024	52.5	83.0
2	(−)	0	0.318 ± 0.008	69.4	95.4
	(−)	3	0.270 ± 0.005[b]	68.3	119.8
	(−)	10	0.180 ± 0.006[b]	45.9	102.7
	(+)	0	0.300 ± 0.007	71.3	108.9
	(+)	3	0.291 ± 0.002	73.9	104.8
	(+)	10	0.228 ± 0.016[b]	67.8	123.6

[a] T cells (1×10^5/well) were cultured in wells with immobilized anti-CD3 (64.1, 100 ng/well) in the presence or absence of mizoribine and GMP (40 μM (15 μg/ml)). After 72 h of cultures, the T cell proliferation was assessed by the colorimetric assay and the expression of cyclin A was determined by flow cytometry as described in *Materials and Methods*. Results of the cyclin A expression are indicated by the percentages of cells positive for cyclin A and the mean fluorescence intensity values.

[b] Significant at $p < 0.01$ as determined by Student's *t*-test compared with cultures without mizoribine.

mizoribine-mediated inhibition of cyclin A expression. Further studies designed to explore the precise sequelae of inhibition of the cyclin A expression by mizoribine and its relevance with intracellular guanine ribonucleotide metabolism would be important for a complete understanding of the regulatory mechanism of the cyclin A in human lymphocytes.

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