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Inhibition of cyclin A gene expression in human B cells by an immunosuppressant mizoribine

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SUMMARY

Mizoribine has been shown to have beneficial effects in the treatment of rheumatoid arthritis and lupus nephritis, in which abnormal B cell functions are involved. Previous studies demonstrated that mizoribine directly suppresses the function of human B cells. The current study explored in detail the mechanism of the suppression of human B cell responses by mizoribine at the molecular level. Highly purified peripheral blood B cells obtained from normal healthy individuals were stimulated with *Staphylococcus aureus* Cowan I (SAC) plus IL-2 in the presence or absence of mizoribine or methotrexate for 48 h to 72 h. The expression of cyclin A mRNA was determined by semiquantitative reverse transcriptase-polymerase chain reaction followed by Southern hybridization. Although at pharmacologically attainable concentrations both mizoribine and methotrexate suppressed the production of IgM of SAC-activated B cells, mizoribine, but not methotrexate, decreased the expression of cyclin A protein as well as mRNA in B cells stimulated with SAC + IL-2. Of note, mizoribine facilitated the degradation of cyclin A mRNA in the presence of actinomycin D, indicating that mizoribine shortens the stability of cyclin A mRNA. The results indicate that mizoribine suppresses the expression of cyclin A mRNA in human B cells by down-regulating its stability, and thus down-regulates their responses.

Keywords human B cell cyclin A mRNA CREB immunosuppressant

INTRODUCTION

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 to prevent rejection of organ allografts in animal models [2–4] as well as in humans [4–7]. It has also been shown that mizoribine prevents the development of nephritis and the expression of anti-DNA antibody in New Zealand B/W F_1 mice [8]. Moreover, mizoribine has now been widely used in Japan in the treatment of patients with rheumatoid arthritis (RA) and lupus nephritis [9,10]. Since abnormalities of B cell functions as well as T cell functions have been considered to be involved in the pathogenesis of RA [11] and systemic lupus erythematosus (SLE) [12], it was possible that mizoribine has direct immunosuppressive influences on B cells. In fact, in murine systems, mizoribine has been found to suppress the production of antibodies against a T cell-independent antigen as well as a T cell-dependent antigen [13].

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In humans, we have previously disclosed that at pharmacologically attainable concentrations (3–10 μg/ml) mizoribine directly suppressed the IgM production of B cells stimulated with *Staphylococcus aureus* Cowan I (SAC) [14]. Mizoribine did not interfere with the initial phase of B cell activation, but rather blocked the transition from G_1 to S phase of the cell cycle. More importantly, mizoribine has been shown to inhibit the expression of cyclin A, which plays an important role in the G_1/S transition in the cell cycle [15–18]. However, little is known about the regulation of the expression of cyclin A gene in human B cells by mizoribine.

The current studies were therefore undertaken to explore in detail the mechanism of the suppression of cyclin A expression in human B cells by mizoribine. Special attention was paid to the effects of mizoribine on the expression of cyclin A mRNA in B cells stimulated with SAC.

The results show that mizoribine decreased cyclin A mRNA levels in SAC-stimulated B cells. Moreover, the data have disclosed that mizoribine decreased the stability of cyclin A mRNA.

MATERIALS AND METHODS

Reagents

Anti-human cyclin A, an IgG1 MoAb directed at human cyclin A

(UBI, Lake Placid, NY) and MOPC 21, and IgG1 control MoAb (Organon-Teknika, West Chester, PA) were used. FITC-conjugated anti-IL-2 receptor (CD25) (mouse IgG1; Becton Dickinson, Mountain View, CA) and FITC-conjugated mouse IgG1 control MoAb (Dako, Glostrup, Denmark) were also used. Formalinized Cowan I strain SA was purchased from Calbiochem-Behring (San Diego, CA) and was used at a concentration of 1/240 000 (v/v). Human rIL-2 (TGP-3) was a gift of Takeda Chemical Industries, Ltd. (Osaka, Japan), and its unit activity was determined by the providers ($4{\cdot}2 \times 10^4$ U/mg of protein). Mizoribine was synthesized and provided by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). Methotrexate and guanosine monophosphate (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 100 μg/ml, L-glutamine 0·3 mg/ml, and 10% fetal calf serum (FCS; Life Technologies) was used for all cultures.

Cell preparation

Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult volunteers by centrifugation of heparinized venous blood over sodium diatrizoate–Ficoll gradients (Histopaque; Sigma). PBMC were depleted of monocytes and natural killer (NK) cells by incubation with 5 mM L-leucine methyl ester HCl (Sigma) in serum-free RPMI 1640, as described elsewhere [19]. The treated cell population was washed twice and then incubated with neuraminidase-treated sheep erythrocytes. The rosetting and non-rosetting populations were then separated by centrifugation on sodium diatrizoate–Ficoll gradients. The non-rosetting cells obtained from the interface were again rosetted with neuraminidase-treated sheep erythrocytes 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 OKT3 and OKT11 pan T cell MoAb (American Type Culture Collection, Rockville, MD), followed by analysis by flow cytometry. The cells were additionally characterized as containing $> 90\%$ CD20 (B1; Coulter, Hialeah, FL).

Cell culture techniques for induction of immunoglobulin production

B cells ($2{\cdot}5 \times 10^4$/well) were cultured alone in wells of 96-well U-bottomed microtitre plates (no. 3799; Costar, Cambridge, MA) with SAC + IL-2 (0·1 U/ml) in the presence or the absence of mizoribine. The cells were incubated for 10 days at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After the incubation, the supernatants were harvested and assayed for immunoglobulin contents using ELISA as previously described [20].

Immunofluorescence staining of cyclin A, and analysis by flow cytometry

B cells (1×10^6/ml) were cultured in 17 × 100-mm polypropylene tubes with SAC + IL-2 in the presence or absence of mizoribine for 96 h. After incubation, the cells were washed once with PBS containing 2% normal human 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 with anti-cyclin A MoAb for 30 min at 4°C followed by staining with FITC-conjugated goat anti-mouse immunoglobulin (Cappel, West Chester, PA) and propidium iodide (Sigma). In some experiments B cells (1×10^6/ml) were cultured with SAC + IL-2 in the presence or absence of methotrexate (100 ng/ml) for 24 h or 48 h. After incubation, the cells were stained with FITC-conjugated anti-CD25, or control MoAb in staining buffer. The cells were analysed using an EPICS XL flow cytometer (Coulter), as previously described [14].

Analysis of cyclin A mRNA expression by semiquantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from SAC-stimulated B cells using the acid guanidinium thiocyanate-phenol-chloroform method [21]. cDNA synthesis was conducted using 4 μg of total cellular RNA and avian myeloblastosis virus reverse transcriptase as described elsewhere [22]. Amplification of human cyclin A and β-actin messages was performed in a total volume of 50 μl with 1 μl of the first-strand cDNA as template with oligonucleotide: 5'-CAGAAGAAGCCAGCTGAATC-3′ and 5′-ACTGTTGGAG-CAGCTAAGTC-3′ (sense and antisense for cyclin A) [23]; and 5′-CTTCTACAATGAGCTGCGTG-3′ and 5′-TCATGAGGTA-GTCAGTCAGG-3′ (sense and antisense for β-actin) [24]. The reaction conditions were: after 2 min incubation at 94°C, 31 cycles (cyclin A) or 21 cycles (β-actin) of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, followed by 4 min incubation at 72°C. For negative control, blank samples without cDNA were included in each run. After amplification, the cDNA samples were loaded on 3% agarose gel electrophoresis and were transferred onto nylon membrane filter. The Southern blots were hybridized with ^{32}P-5′-end-labelled human cyclin A oligonucleotide probe (5′-ACCCCCCAGAAGTAGCAGAGTTTGTGTACA-3′) [23] and ^{32}P-multiprime-labelled human β-actin cDNA probe [24]. After hybridization, autoradiography was carried out with exposure at −80°C for 1 day. Band intensities were quantified with the image analyser (ScanJet 3c/ADF; Hewlett Packard, Greenly, CO) followed by analysis with NIH Image v. 1.58. All results were normalized relative to the β-actin band intensity amplified from each cDNA sample.

RESULTS

Effects of mizoribine on the responses of B cells

Initial experiments were conducted to confirm the suppressive effects of mizoribine on B cell responses elicited by stimulation with SAC + IL-2. The effect of methotrexate was also examined for comparison. Human peripheral blood B cells were cultured with SAC + IL-2 for 10 days in the presence of various concentrations of mizoribine and methotrexate. As can be seen in Fig. 1, mizoribine as well as methotrexate suppressed the production of IgM induced by SAC + IL-2 in a dose–response manner. Previous studies showed that mizoribine does not suppress the initial activation of B cells stimulated with SAC + IL-2 [14]. Thus, mizoribine did not inhibit the expression of CD25 on B cells stimulated with SAC + IL-2. Of note, methotrexate at 100 ng/ml, which almost completely inhibited the immunoglobulin production, did not suppress the expression of CD25 on B cells stimulated with SAC + IL-2 (Fig. 2), indicating that methotrexate does not inhibit the initial activation of B cells either. On the other hand, mizoribine at its pharmacologically attainable concentration markedly suppressed not only the progression of B cells from G_1 to the S phase in the cell cycle,

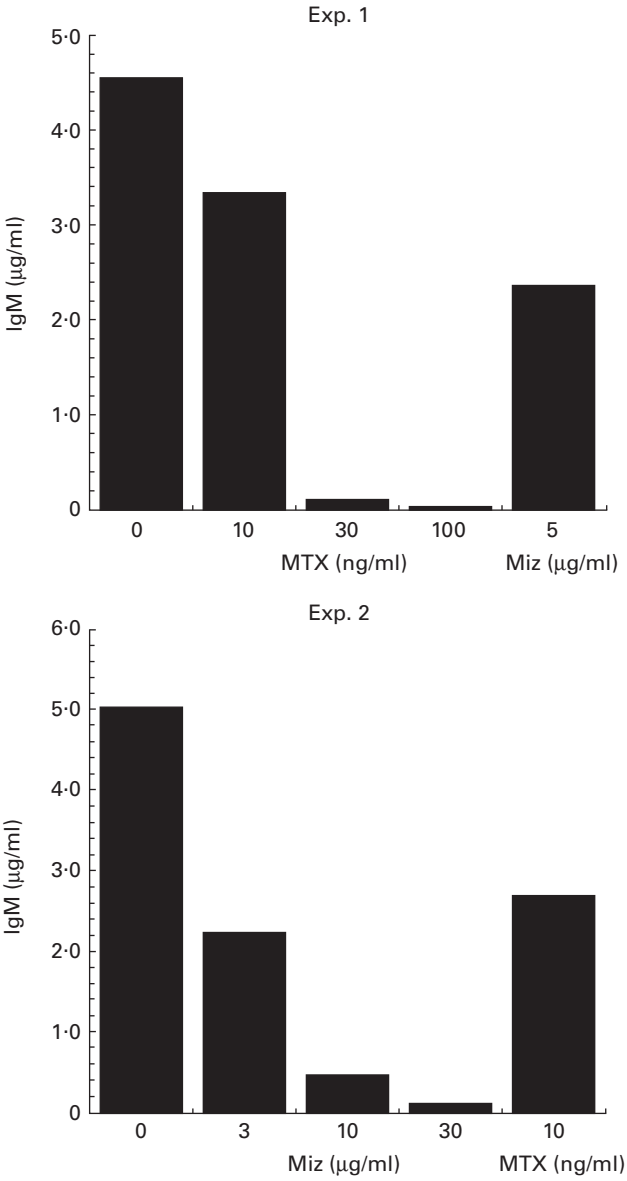

Fig. 1. Mizoribine and methotrexate suppress the production of IgM of human B cells stimulated with *Staphylococcus aureus* Cowan I (SAC) + IL-2. B cells ($2{\cdot}5 \times 10^4$/well) were cultured with SAC + IL-2 (0·1 U/ml) in the presence or absence of various concentrations of mizoribine (Miz) and methotrexate (MTX). After 10 days of culture, the supernatants were harvested and assayed for IgM content by ELISA.

but also the expression of cyclin A in B cells stimulated with SAC + IL-2 for 96 h (Fig. 3), consistent with the previous observation [14]. The data confirm that mizoribine directly suppresses the function of human B cells by inhibiting the expression of cyclin A.

Mizoribine inhibits the expression of cyclin A mRNA in B cells

To explore in detail the mechanism by which mizoribine suppresses the expression of cyclin A in B cells, the next experiments were undertaken, in which the effect of mizoribine on cyclin A mRNA expression in B cells was examined using reverse transcriptase-polymerase chain reaction. As can be seen in Fig. 4, the expression of cyclin A mRNA was already

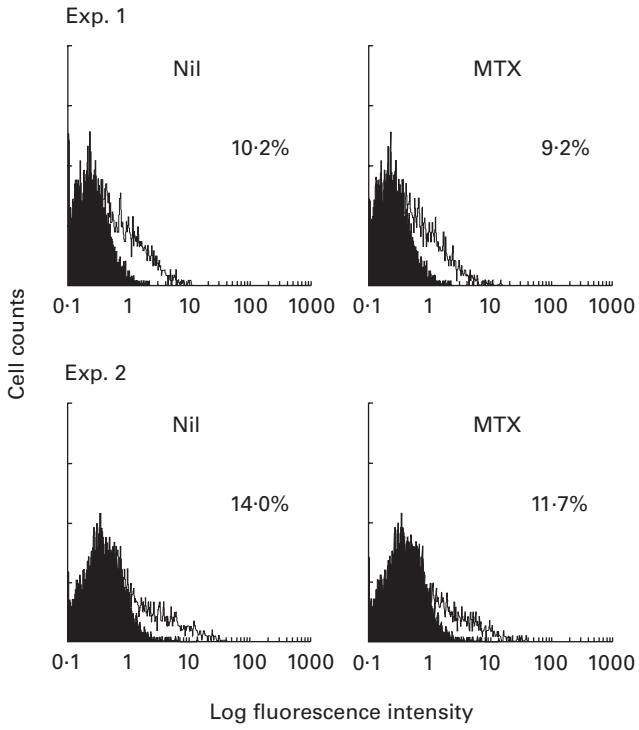

Fig. 2. Methotrexate does not suppress the expression of CD25 of human B cells stimulated with *Staphylococcus aureus* Cowan I (SAC) + IL-2. B cells were cultured with SAC + IL-2 (0·1 U/ml) in the presence or absence of methotrexate (MTX) (100 ng/ml (0·2 μM)). After 24 h (Exp. 1) or 48 h (Exp. 2) of culture, the cells were stained with FITC-conjugated anti-CD25 MoAb or control IgG1 MoAb and then analysed by flow cytometry. The percentages of cells positive for CD25 are shown. Shaded areas indicate the staining with control MoAb.

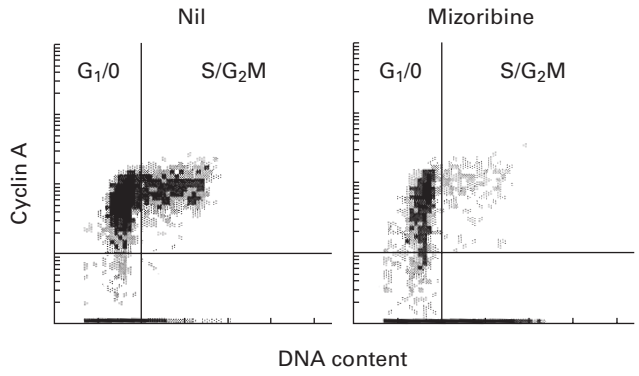

Fig. 3. Mizoribine suppresses the expression of cyclin A and DNA synthesis in B cells. B cells were cultured with *Staphylococcus aureus* Cowan I (SAC) + IL-2 (0·1 U/ml) in the presence or absence of mizoribine (10 μg/ml (40 μM)). After 96 h of culture, the cells were fixed in cold ethanol and were reacted with control MoAb or anti-cyclin A MoAb followed by staining with FITC-conjugated goat anti-mouse immunoglobulin and with propidium iodide (50 μg/ml). The cells were analysed by flow cytometry. The mean fluorescence intensity (MFI) of cyclin A staining in G_1/0 phases and in S/G_2/M phases is: (Nil) 3·44 and 6·74; (mizoribine) 2·15 and 2·98, respectively.

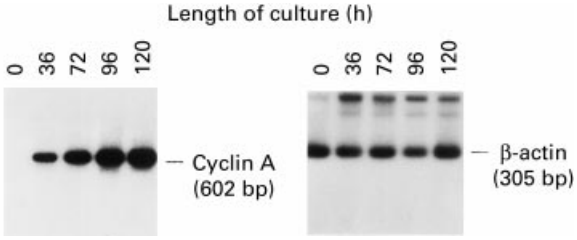

Fig. 4. Time kinetics of the expression of cyclin A mRNA in *Staphylococcus aureus* Cowan I (SAC)-activated B cells. B cells were stimulated with SAC + IL-2 (0·1 U/ml). After various lengths of incubation, B cells were harvested and assayed for cyclin A mRNA expression with reverse transcriptase-polymerase chain reaction followed by Southern hybridization. Representative of two independent experiments.

observed after 36 h of stimulation of B cells with SAC + IL-2 and reached maximal level after 72 h of stimulation. Mizoribine 5 μg/ml significantly suppressed the expression of cyclin A mRNA in B cells stimulated with SAC + IL-2 for 48 h to 72 h (Fig. 5). By contrast, methotrexate 100 ng/ml, which also markedly inhibited immunoglobulin production of B cells, did not suppress the expression of cyclin A mRNA in B cells (Fig. 6). The results indicate that the suppression of cyclin A mRNA expression in B cells is a characteristic feature of mizoribine, but is not due to a non-specific suppression of B cells.

We have previously shown that the suppressive effects of mizoribine on cyclin A protein expression in B cells were not reversed by the addition of as much as 15 μg/ml (40 μM) of GMP [14]. Consistently, the suppressive effects of mizoribine on cyclin A mRNA expression in B cells were not reversed by the addition of as much as 40 μM of GMP (Fig. 7). The results therefore

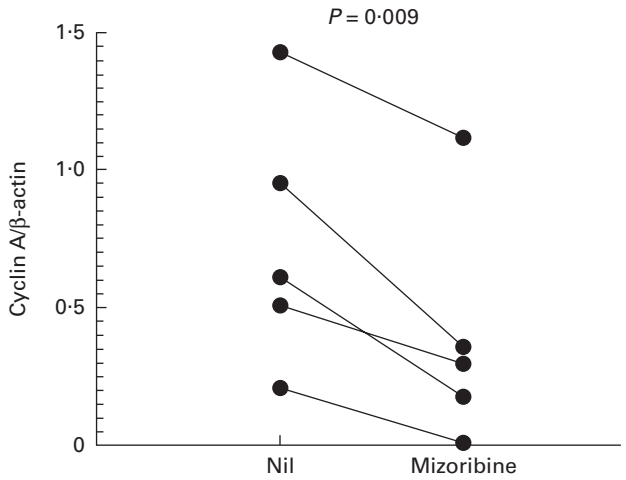

Fig. 5. Mizoribine suppresses the expression of cyclin A mRNA. B cells were cultured with *Staphylococcus aureus* Cowan I (SAC) + IL-2 (0·1 U/ml) in the presence or absence of mizoribine (5 μg/ml (20 μM)). After 48 h or 72 h of incubation, B cells were harvested and assayed for the expression of cyclin A mRNA and β-actin mRNA with reverse transcriptase-polymerase chain reaction followed by Southern hybridization. The ratio of cyclin A mRNA/β-actin mRNA was calculated after analysis with densitometer. Statistical significance in six independent experiments was evaluated by paired sample *t*-test.

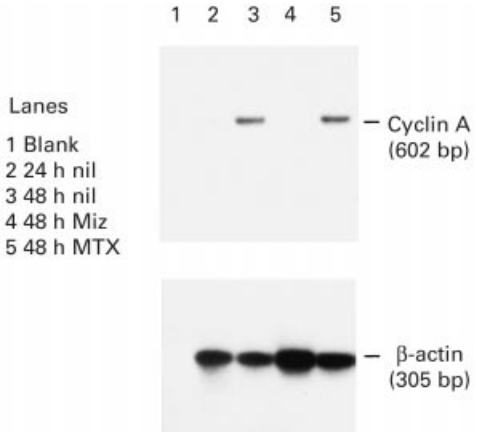

Fig. 6. Differential effects of mizoribine and methotrexate on the expression of cyclin A mRNA. B cells were cultured with *Staphylococcus aureus* Cowan I (SAC) + IL-2 (0·1 U/ml) in the presence or absence of mizoribine (Miz) (5 μg/ml (20 μM)) or methotrexate (MTX) (100 ng/ml (0·2 μM)). After 24 h or 48 h of incubation, B cells were harvested and assayed for the expression of cyclin A mRNA with reverse transcriptase-polymerase chain reaction followed by Southern hybridization. Representative of two independent experiments.

confirm that mizoribine suppresses the expression of cyclin A at the mRNA level in a mechanism distinct from guanine ribonucleotide depletion.

Mizoribine reduces the stability of cyclin A mRNA in B cells

The next experiments were undertaken to determine whether the inhibition of cyclin A mRNA expression by mizoribine resulted from interference with the stability of cyclin A mRNA. Highly purified B cells were cultured with SAC + IL-2 for 48 h, after which B cells were further incubated with actinomycin D in the presence or absence of mizoribine for various periods of time. As can be seen in Fig. 8a, the expression of cyclin A mRNA appeared to be decreased faster in the presence of mizoribine. In fact, mizoribine significantly reduced the ratio of cyclin A mRNA/β-actin mRNA in the presence of actinomycin D, where

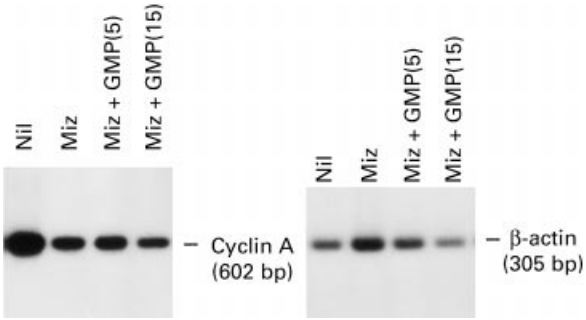

Fig. 7. Inhibition of the expression of cyclin A mRNA in *Staphylococcus aureus* Cowan I (SAC)-activated B cells by mizoribine is not reversed by supplemental GMP. B cells were cultured with SAC + IL-2 (0·1 U/ml) in the presence or absence of mizoribine (5 μg/ml (20 μM)) and GMP (5 μg/ml (13 μM) or 15 μg/ml (40 μM)). After 72 h of incubation, B cells were harvested and assayed for cyclin A mRNA expression with reverse transcriptase-polymerase chain reaction followed by Southern hybridization. Representative of two independent experiments.

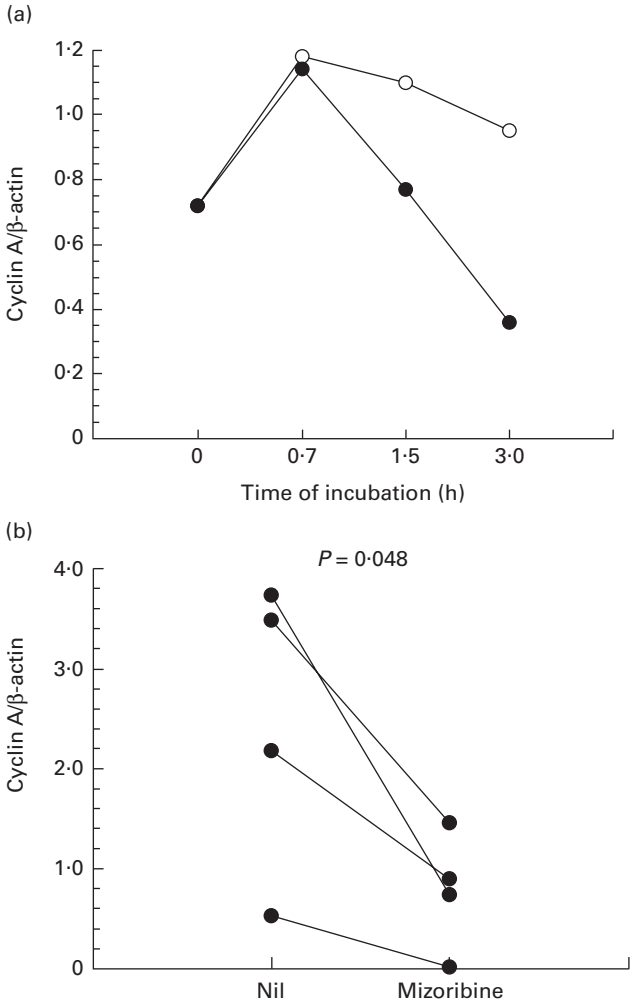

Fig. 8. Mizoribine reduces the stability of cyclin A mRNA in B cells. B cells were cultured with *Staphylococcus aureus* Cowan I (SAC) + IL-2 (0·1 U/ml) for 48 h, after which actinomycin D (50 μg/ml) was added with (●) or without mizoribine (○) (5 μg/ml (20 μM)). After various lengths of incubation, the cells were harvested and assayed for the expression of cyclin A mRNA and β-actin mRNA with reverse transcriptase-polymerase chain reaction followed by Southern hybridization. The ratio of cyclin A mRNA/β-actin mRNA was calculated after analysis with densitometer. (a) A typical time kinetics of the ratio of cyclin A mRNA/β-actin mRNA in the presence of actinomycin D. (b) Summary of the ratio of cyclin A mRNA/β-actin mRNA after 3–4 h from the addition of actinomycin D. Statistical significance in four independent experiments was evaluated by paired sample *t*-test.

the *de novo* transcription was abrogated (Fig. 8b). The results indicate that mizoribine specifically inhibits the stability of cyclin A mRNA in B cells. It is therefore suggested that mizoribine might inhibit the expression of cyclin A mRNA by decreasing its stability.

DISCUSSION

It is well known that methotrexate, one of the most potent disease-modifying anti-rheumatic drugs which has been used all over the world [25], inhibits the differentiation of B cells [26]. Moreover, we have found that methotrexate inhibits the cell cycle progression without interfering with the initial phase of B cell activation, as evidenced by the inability of methotrexate to suppress the expression of CD25 on B cells. In this regard, the suppressive effects of mizoribine on the function of B cells were comparable to those of methotrexate [14]. However, the results of the present study have disclosed that methotrexate does not suppress the expression of cyclin A mRNA in B cells, although mizoribine suppresses it. The data therefore remove the possibility that the suppression of cyclin A mRNA expression might be a non-specific event associated with the suppression of the cell-cycle progression after the G_1 phase. Moreover, it is also suggested that the inhibition of the enzymes, such as dihydrofolate reductase and 5-aminoimidazole-4-carboxamidoribonucleotide transformylase, the activity of which methotrexate inhibits [27], might not result in the suppression of the expression of cyclin A mRNA. These observations therefore suggest the possibility of the design of immunosuppressive drug combinations that take advantage of non-overlapping mechanisms of actions, thus obtaining synergistic immunosuppression with minimizing toxicity.

Although 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 [28] or GMP [14], the suppressive effects of mizoribine on B cell differentiation can not be reversed with supplemental GMP [14]. In fact, it has been shown that mizoribine does not result in decreased levels of GTP in SAC-stimulated B cells [14]. Moreover, it has also been demonstrated that inhibition of the expression of cyclin A in B cells can not be overcome with supplemental GMP, which is presumably related to the fact that the concentrations of GMP in B cells are much lower than that in T cells, and are not effected by mizoribine [14]. Consistently, the data in the current study revealed that supplemental GMP did not reverse the mizoribine-mediated suppression of the expression of cyclin A mRNA in B cells. The results therefore lead to the conclusion that mizoribine suppresses the expression of cyclin A in B cells at the mRNA level by a mechanism distinct from guanine ribonucleotide depletion.

As a first step towards elucidating the mechanism of mizoribine-mediated inhibition of the expression of cyclin A mRNA in B cells, the effects of mizoribine on the stability of cyclin A mRNA were examined under the influence of actinomycin D. The results clearly demonstrated that mizoribine significantly inhibited the stability of cyclin A mRNA. Since addition of cyclohexamide did not restore the degradation of cyclin A mRNA by mizoribine (data not shown), it is unlikely that the down-regulatory effects of mizoribine on the stability of cyclin A mRNA involve *de novo* protein synthesis. Rather, it appears likely that mizoribine itself is involved in the degradation of cyclin A mRNA, although further studies are required to delineate this point.

Recent studies have characterized several regulatory elements and cognate DNA-binding proteins which are crucial for cyclin A gene transcription [29,30]. Thus, it has been found that the ATF site and its cognate DNA binding proteins (ATF-1 and CREB) are critical in mediating high-level cyclin A gene expression [29,30]. In fact, it has been demonstrated that homocysteine increased cyclin A promoter activity through induction of ATF-binding proteins, including ATF-1 and CREB, in rat aortic smooth muscle cells [31]. Although the current study did not explore the effects of mizoribine on the transcription of cyclin A genes, further studies designed to explore the effects of mizoribine on the activation of

ATF-binding proteins, which play an important role in the transcription of cyclin A genes, would be important for a complete understanding of the mechanism of action of mizoribine.

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