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Comparison of the Effects of Mizoribine with Those of Azathioprine, 6-Mercaptopurine, and Mycophenolic Acid on T Lymphocyte Proliferation and Purine Ribonucleotide Metabolism

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SUMMARY

The immunosuppressive drug mizoribine has been demonstrated to inhibit T lymphocyte proliferation by depleting these cells of guanine ribonucleotides as a consequence of inhibiting the enzyme inosine monophosphate (IMP) dehydrogenase. Because the immunosuppressive agents azathioprine and 6-mercaptopurine (6MP) are both converted to the IMP analog 6-thio-IMP, we postulated that these drugs might inhibit T cell activation and/or proliferation by a similar mechanism. Incubation of isolated peripheral blood T cells with either mizoribine or the selective IMP dehydrogenase inhibitor mycophenolic acid caused a dose-dependent inhibition of T cell proliferation, which was reversible with the addition of 50 μM guanosine to replete guanine ribonucleotide pools. In contrast, guanosine exacerbated the inhibition of proliferation induced by azathioprine and restored proliferation at IC_{50} concentrations of 6MP by only 10%. Complete restoration of proliferation in the presence of 6MP, but not azathioprine, was achieved with the addition of adenine. The inhibitory effects of azathioprine, as well as those of mizoribine, 6MP, and mycophenolic acid, were identical in cells stimulated with antibody to the T cell receptor and in cells stimulated with phorbol ester and ionomycin. We conclude from these studies that mizoribine selectively inhibits guanine ribonucleotide formation in purified T cells, whereas the effect of 6MP appears to be more dependent on adenine ribonucleotide depletion. Azathioprine, on the other hand, inhibits proliferation by a mechanism independent of purine ribonucleotide depletion. None of these agents inhibits T cell proliferation by interfering with signal transduction mediated by the T cell receptor. Inhibition of guanine ribonucleotide biosynthesis appears to be a novel and perhaps more selective mechanism of inhibiting T cell proliferative responses after T cell activation.

The immunosuppressive drug mizoribine (4-carbamoyl-1-β-D-ribofuranosyl imidazolium-5-olate; bredinin) has been used successfully to prevent rejection of organ allografts in humans and in animal models (1, 2). Mizoribine is an imidazole nucleoside, first isolated from the soil fungus *Eupenicillum brefeldianum* (3). Studies in mizoribine-resistant cell lines led to the hypothesis that mizoribine is phosphorylated by adenosine kinase to its monophosphate, which inhibits IMP dehydrogenase (EC 1.2.1.14), an essential enzyme in the *de novo* synthesis of guanine ribonucleotides from IMP (4, 5) (Fig. 1). We have recently demonstrated that mizoribine inhibits T lymphocyte proliferation in a dose-dependent fashion when cells are stimulated with alloantigen, antibodies to the T cell receptor, or pharmacologic mitogens. In addition, mizoribine causes a dose-dependent decrease in GTP pools, and the addition of guanosine both prevents this GTP depletion and reverses the antiproliferative effects at all but the highest doses of mizoribine (6).

Other immunosuppressive agents, such as azathioprine and 6MP, are also known to inhibit *de novo* purine biosynthesis and IMP metabolism. Because IMP is pivotal to the formation of both adenine and guanine ribonucleotides, the present studies were undertaken to determine whether guanine ribonucleotide depletion alone could account for the immunosuppressive effects of these drugs. In order to determine whether these agents have a common basis for inhibiting T cell proliferation, we have compared their effects on T cell activation as it relates to both adenine and guanine nucleotide pools.

Guanine ribonucleotides have been demonstrated to play an important role in membrane signal transduction, and it has been postulated that depletion of GTP might inhibit T cell activation by decreasing signal transduction via the T cell antigen receptor/CD3 antigen complex. Indirect evidence to support this contention has come from studies demonstrating that T lymphocyte phospholipase C activity can be increased

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ABBREVIATIONS: IMP, inosine monophosphate; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; PMA, phorbol myristate acetate; MPA, mycophenolic acid; 6MP, 6-mercaptopurine; PBS, phosphate-buffered saline; MAb, monoclonal antibody.

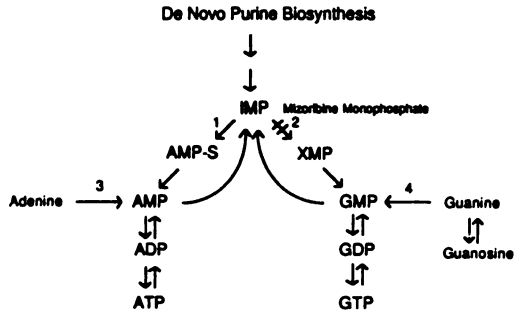

Fig. 1. Schema demonstrating the pathways of *de novo* purine biosynthesis and purine supplementation. *1*, Adenylosuccinate synthetase; *2*, IMP dehydrogenase; *3*, adenine phosphoribosyltransferase; *4*, HGPRT.

with GTP-binding protein activation by guanine ribonucleotides (7) and that anti-CD3 antibodies stimulate GTPase activity in T cell plasma membrane fractions (8). As an additional goal, therefore, we asked whether interference in membrane signal transduction pathways is an important component of the effects of any of these immunosuppressive drugs.

Materials and Methods

MAbs. The fluorescein isothiocyanate-labeled mouse anti-human MAbs 9.6 (anti-CD2IgG2a) and G19–4 (anti-CD3IgG1) were generous gifts of J. Ledbetter (Bristol-Myers Squibb, Seattle, WA). Control fluorescein isothiocyanate-labeled mouse anti-MsIgG2a was purchased from Coulter Immunology (Hialeah, FL).

Reagents. Cell culture reagents, including RPMI 1640, penicillin, streptomycin, and fetal calf serum, were purchased from GIBCO Laboratories (Grand Island, NY). Mizoribine (molecular weight, 259) was a generous gift of Toyo Jozo Co. (Tokyo, Japan). Ionomycin was obtained from Calbiochem Behring Corp. (San Diego, CA). Ionomycin was suspended in dimethylsulfoxide at 1 mg/ml and stored at −20°. PMA, adenine, guanosine, 8-aminoguanosine, azathioprine, MPA, and 6MP were purchased from Sigma Chemical Co. (St. Louis, MO). The PMA was reconstituted as a 1 mg/ml solution in dimethylsulfoxide. Fresh dilutions of PMA (at 5 μg/ml in PBS) were prepared as needed for each experiment. MPA was reconstituted as a 25 mg/ml solution in ethanol, stored at 4°, and further diluted in RPMI 1640. Adenine was dissolved in PBS to supply phosphate for salvage to AMP. The remaining reagents were prepared as needed for individual experiments, in RPMI 1640.

Isolation and characterization of peripheral blood T lymphocytes. All blood donors were healthy volunteers who gave informed consent for venipuncture. Peripheral blood mononuclear cells were isolated from venous blood by density gradient centrifugation, using Ficoll-Hypaque. T cells were purified from the mononuclear cells by negative selection, using a cocktail of MAbs kindly provided by Carl June (Bethesda Naval Medical Research Institute, Bethesda, MD) (6, 9). The selected cells were >95% positive for CD2, compared with control cells stained with the isotype-matched control antibody MsIgG2a.

Cell culture. Cells were cultured at a density of 1×10^6/ml, in RPMI 1640 containing 10^5 units/liter penicillin, 100 μg/liter streptomycin, and 10% fetal calf serum. PMA was used at a final concentration of 3 ng/ml, ionomycin at 125 ng/ml, guanosine at 50 μM, 8-aminoguanosine at 100 μM, and adenine at 100 μM, in 0.1 mM phosphate. Exogenous guanosine was supplied by adding the combination of guanosine and 8-aminoguanosine. Guanosine is converted to guanine by purine nucleoside phosphorylase and then to GMP by HGPRT. The 8-aminoguanosine was added as a weak inhibitor of purine nucleoside phosphorylase, to provide an ongoing source of guanine for stable guanine ribonucleotide pool repletion (10) (Fig. 1). All reagents were added simultaneously with the stimulating agents unless otherwise indicated; adenine was also added at 24-hr intervals. Anti-CD3 antibody was immobilized on goat anti-mouse antibody-coated plastic culture dishes, using a 1 μg/ml solution (11).

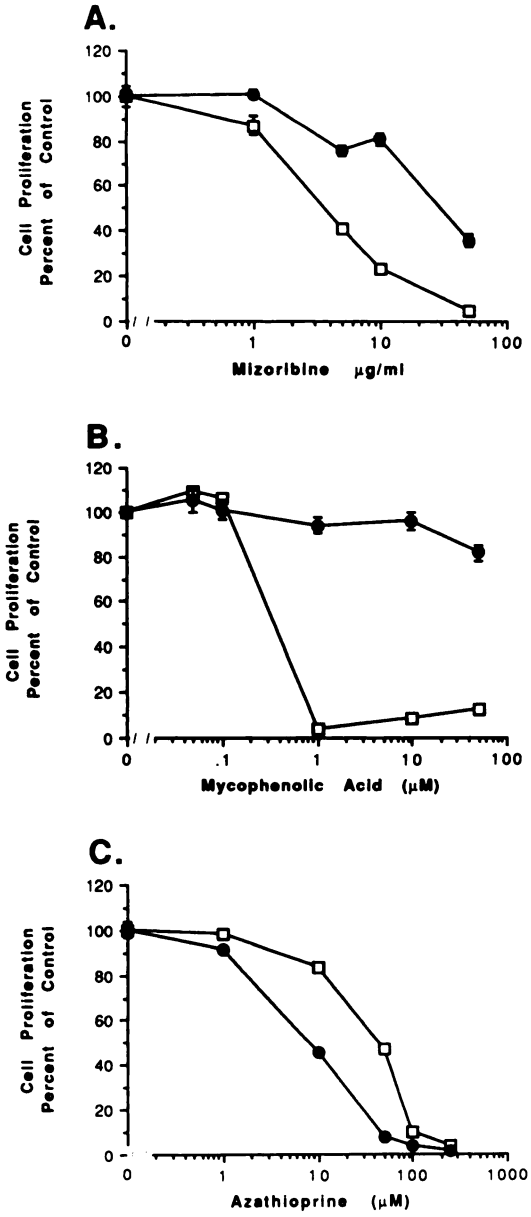

Fig. 2. Effects of guanosine on inhibition of T lymphocyte proliferation induced by mizoribine, MPA, and azathioprine. Purified T cells were stimulated with a combination of 3 ng/ml PMA and 125 ng/ml ionomycin and were incubated with increasing concentrations of drugs alone (□) or in the presence of guanosine (●). Incorporation for control stimulated cells was 6,584 ± 796 cpm in the absence of guanosine and 11,754 ± 1,198 cpm in the presence of guanosine at 72 hr. All values are expressed as a percentage of untreated control and are the mean ± standard error of quadruplicates. Data are representative of seven independent experiments. A, Mizoribine; B, MPA; C, azathioprine.

Proliferation assays. Cells were cultured at 37° and at 5% CO_2 in 96-well round-bottomed microtiter plates, at 5×10^4 cells/well, in a total volume of 0.2 ml. The degree of proliferation was determined by adding 1 μCi of [^{3}H]thymidine (Amersham) to each well for the last 6 hr of a 72-hr culture, after which the plates were harvested with a PHD 200 cell harvesting system (Cambridge Technology, Inc., Cambridge, MA). All assays were performed in quadruplicate.

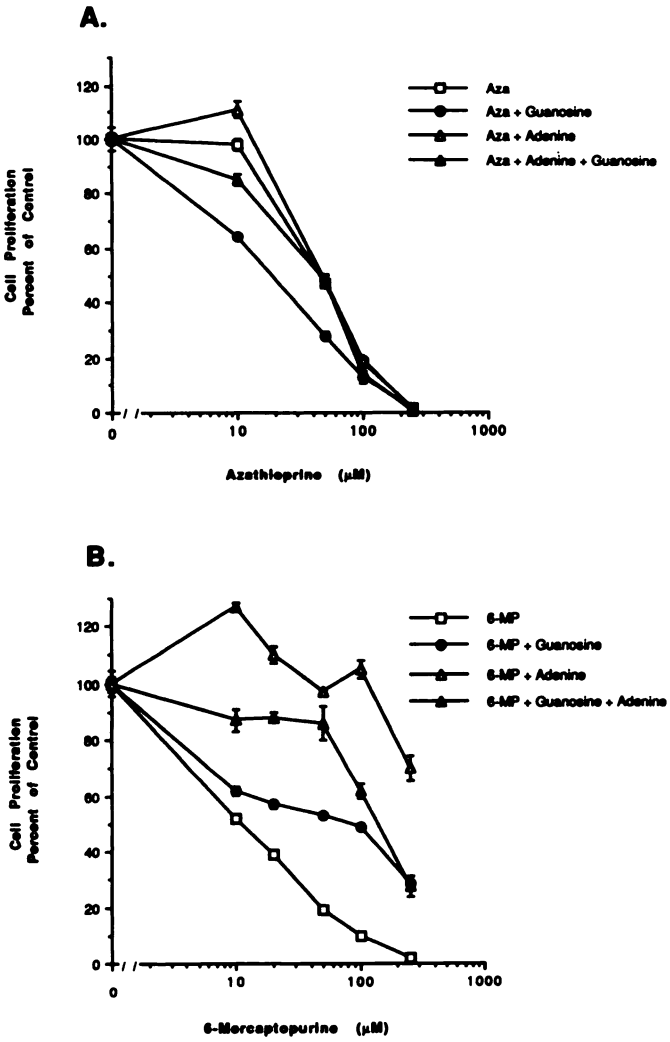

Fig. 3. Effects of exogenous purines on inhibition of T cell proliferation induced by azathioprine (*Aza*) (A) and 6MP (B). Cells were cultured as in Fig. 2, with varying concentrations of drug. Stimulated cells in the absence of drug incorporated 25,446 ± 4,152 cpm without additives, 41,704 ± 3,444 cpm in the presence of adenine, 35,904 ± 1,792 cpm in the presence of guanosine, and 45,270 ± 1,454 cpm in the presence of both adenine and guanosine. Corresponding values for B were 27,003 ± 2,312 cpm, 14,179 ± 2,318 cpm, 58,991 ± 6,154 cpm, and 27,185 ± 2,241 cpm, respectively. All values are expressed as a percentage of untreated control and are the mean ± standard error of quadruplicates. Data are representative of three and two independent experiments for azathioprine and 6MP, respectively.

Measurement of ATP and GTP. Nucleotide pools were quantitated by high performance liquid chromatography, using a Partisil-10 SAX anion exchange column (Whatman, Clifton, NJ), as previously described (6). Total intracellular nucleotide levels were calculated by comparing areas of peaks absorbing at 254 nm with the areas generated by nanomole amounts of pure standards, using a 3390A Hewlett Packard integrator. Pool values were compared with control values by using a Student's *t* test.

Results

Differential effects of exogenous guanosine on inhibition of T cell proliferation induced by different immunosuppressive agents. T cells were purified as described above and stimulated to proliferate with PMA and ionomycin. Fig. 2A demonstrates that the addition of increasing concentrations of mizoribine to these cultures results in a dose-related inhibition of proliferation, with an IC_{50} value of approximately 5 μg/ml. The addition of guanosine to identical cultures shifts the IC_{50} to approximately 50 μg/ml. The growth-inhibitory effect of MPA, a selective inhibitor of IMP dehydrogenase, is also reversed by exogenous guanosine (Fig. 2B). In marked contrast, however, guanosine enhances the antiproliferative effect of azathioprine on stimulated peripheral blood T cells (Fig. 2C). These data confirm our previous studies, which showed that the immunosuppressive effect of mizoribine on T cells is mediated by guanine ribonucleotide depletion (6), and suggest that MPA has similar effects. However, the effect of azathioprine on T lymphocytes is clearly not mediated by this mechanism.

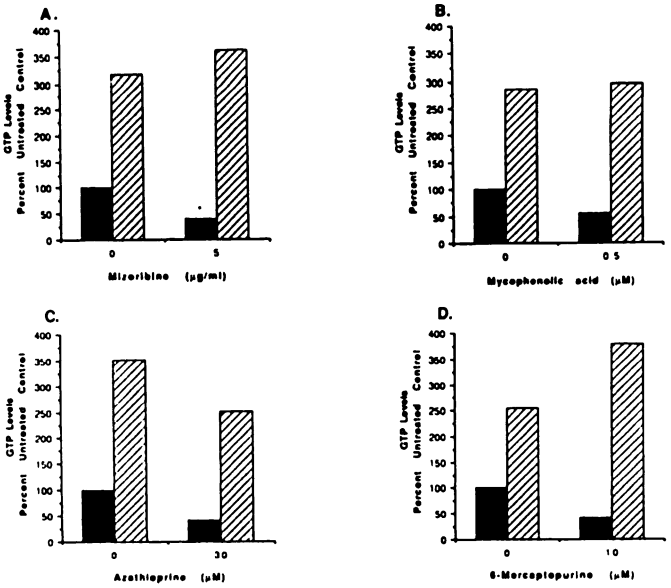

Fig. 4. Alterations in GTP pools induced by mizoribine (*A*), MPA (*B*), azathioprine (*C*), and 6MP (*D*). Purified T cells were stimulated with a combination of 3 ng/ml PMA and 125 ng/ml ionomycin in the presence of the drugs at approximate IC_{50} concentrations. ■, control cultures; ▨, cultures in the presence of guanosine. All values are expressed as a percentage of values obtained from untreated control cultures. The GTP content was 0.35 ± 0.06 nmol/10^6 untreated cells and 1.27 ± 0.62 nmol/10^6 cells in the presence of guanosine. *, $p < 0.05$; †, $p < 0.01$.

Effects of exogenous purines on azathioprine- and 6MP-induced inhibition of T cell proliferation. Because azathioprine and 6MP are known inhibitors of *de novo* purine biosynthesis that could deplete adenine as well as guanine ribonucleotide pools, we examined the ability of adenine, guanosine, or both compounds in combination to reverse the drug-induced inhibition of T cell proliferation. T cells were incubated with increasing concentrations of azathioprine or 6MP in the presence or absence of guanosine and/or adenine in PBS. The addition of adenine alone has no effect on azathioprine-induced inhibition of T cell proliferation (Fig. 3A). If adenine is added to T cells treated with azathioprine and guanosine, the enhanced inhibition of proliferation induced by guanosine is reversed, but proliferation remains inhibited at the level caused by drug alone.

6MP inhibits T cell proliferation with an IC_{50} of 15 μM. The addition of guanosine shifts this IC_{50} to 100 μM (Fig. 3B). The inhibitory effect of 6MP at concentrations up to 100 μM is completely reversed with adenine alone. The combination of adenine and guanosine completely reverses the inhibition

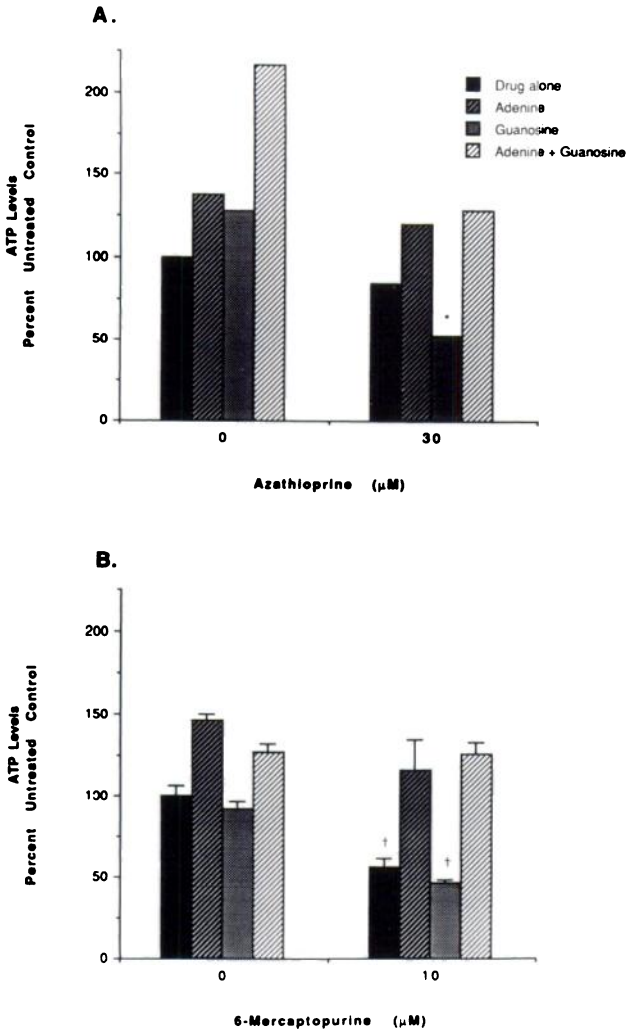

Fig. 5. Effects of exogenous purines on alterations in ATP pools induced by azathioprine (*A*) and 6MP (*B*). Cells were cultured as in Fig. 3, in the absence or presence of IC_{50} concentrations of drugs. Cultures were supplemented with adenine and/or guanosine. All values are expressed as a percentage of values from untreated control cultures. The untreated base-line ATP value was 1.12 ± 0.15 nmol/10^6 cells and that in the presence of adenine was 1.61 ± 0.08 nmol/10^6 cells. *, $p < 0.05$; †, $p < 0.01$.

caused by 6MP at concentrations of 6MP up to 50 μM but is less effective than adenine alone. This evidence suggests that 6MP is inhibitory primarily by depleting the cell of adenine ribonucleotides. In addition, this experiment provides direct evidence that azathioprine has antiproliferative activity not attributable to its conversion to 6MP, because the effects of 6MP are reversed by purine supplementation, whereas those of azathioprine are not.

Effects of immunosuppressive agents upon purine nucleotide pools in purified T cells. In order to determine the relative magnitudes of perturbations of purine ribonucleotide pools induced by these immunosuppressive agents, cells were stimulated with PMA and ionomycin and ATP and GTP levels were measured concomitantly with DNA synthesis at 72 hr. At the approximate IC_{50} concentrations of each drug, mizoribine, MPA, azathioprine, and 6MP all cause decreases in GTP pools, ranging from 40 to 60% of control values (Fig. 4). GTP pools are restored to values above control levels by the addition of guanosine in each case. With mizoribine and MPA, ATP pools remain at control levels at these concentrations (data not shown). However, azathioprine and 6MP cause 20–40% decreases in ATP levels at their respective IC_{50} concentrations (Fig. 5). These effects on ATP pools are prevented by the addition of 10 μM adenine in the presence of phosphate. The combination of adenine and guanosine completely prevents the perturbation of both ATP and GTP pools caused by azathioprine and 6MP. It is of note that the addition of guanosine to azathioprine-treated cultures causes a depletion of ATP that is 35% greater than that induced by azathioprine alone (Fig. 5A). Enhanced depletion of ATP is also observed at azathioprine concentrations of 10 μM and 50 μM (data not shown). This depletion may explain the greater inhibition of proliferation caused by guanosine (Figs. 2C and 3A), an effect that is reversible by adenine.

Time course of drug-induced inhibition of T cell proliferation. In order to determine whether depletion of guanine ribonucleotides induced by these agents affects signal transduction at the membrane level, we stimulated T lymphocytes with antibodies to the T cell receptor (CD3), rather than PMA and ionomycin, and added the drugs at different time points either before or after the stimulation of T cells. If the depletion of guanine ribonucleotides inhibits initial events in the signal transduction pathway, addition of drugs at later time points should result in a decrease in antiproliferative activity. Fig. 6 shows the effect of the various drugs added 48 or 66 hr after the stimulation of T cells with anti-CD3 antibody. Mizoribine (Fig. 6A), MPA (Fig. 6B), and 6MP (Fig. 6D) cause similar degrees of inhibition of proliferation whether the drug is added at the time of stimulation or 48 or 66 hr after stimulation. Identical results are obtained in cells stimulated with PMA and ionomycin. The degrees of GTP depletion induced by each of these three drugs at 72 hr are equivalent whether the drug is added at 0, 48, or 66 hr (data not shown). This result provides direct evidence that GTP depletion is not affecting early events in T cell activation. In marked contrast, azathioprine must be added no later than 24 hr after the initiation of culture to produce inhibition of proliferation (Fig. 6C). This effect is also seen in cells stimulated with a combination of PMA and ionomycin (data not shown). These observations provide additional evidence that azathioprine inhibits T lymphocyte proliferation by a mechanism completely independent of its depletion of purine nucleotides and strongly suggest that its effect is not due to interference with the T cell receptor-mediated signal transduction pathway.

Discussion

Previous studies on the immunosuppressive drug mizoribine have documented that it inhibits T lymphocyte proliferation by depleting these cells selectively of guanine ribonucleotides (6), and we hypothesized that the T cell-suppressive effects of azathioprine and 6MP might be mediated by the same mechanism. This supposition was supported by the recent studies that document that both MPA, a highly specific inhibitor of IMP dehydrogenase, and its morpholinoethyl ester derivative are effective in prolonging the survival of heart allografts in mice and thus have potential as immunosuppressive agents (12–14). Early work had shown that MPA was an effective drug in the treatment of psoriasis, but clinical studies on its immunosuppressive potential have been limited. MPA has been

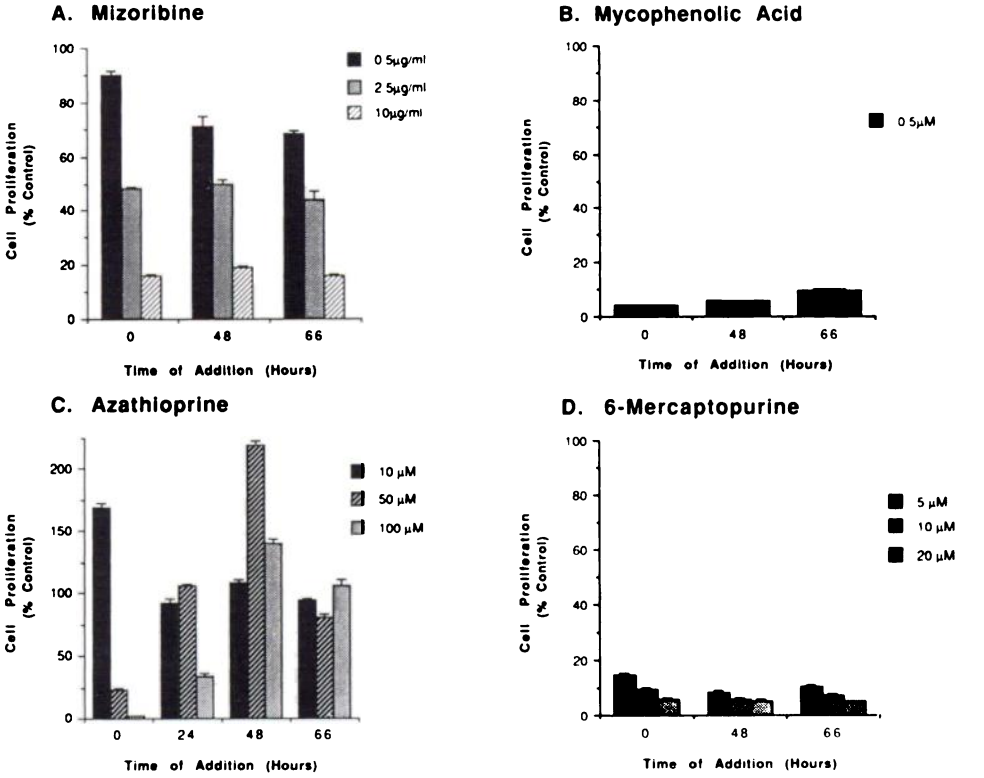

Fig. 6. Effects of time of addition of drugs on T lymphocyte proliferation induced by anti-CD3 antibody. Cultures were established with varying drug concentrations and 1 μg/ml anti-CD3 immobilized in tissue culture wells. Drugs were added at 24, 48, and 66 hr of culture. All values are expressed as a percentage of untreated control proliferation. Unstimulated cells incorporated 758 ± 492 cpm of thymidine at 72 hr, and anti-CD3-stimulated cells incorporated 77,412 ± 14,772 cpm. A, Mizoribine (2.5 μg/ml); B, MPA (0.1 μM); C, azathioprine (38 μM); D, 6MP (1.0 μM).

clearly demonstrated to deplete selectively guanine, as opposed to adenine, ribonucleotide pools (15), lending further credence to the hypothesis that guanine ribonucleotides could be essential for the T cell activation and/or proliferative response.

Previous studies with 6MP have clearly documented that its activity is mediated by conversion to 6-thioinosinic acid, an intermediate that has been shown to inhibit a variety of enzymes in the purine synthetic and salvage pathways, including phosphoribosylpyrophosphate amidotransferase and adenylosuccinate synthetase, as well as IMP dehydrogenase (16) (Fig. 1). Azathioprine is metabolized to 6MP and an electrophilic methylnitroimidazole moiety (17). Whether its immunosuppressive effects are mediated by 6MP, the imidazole moiety, or a combination of the two has been the subject of some debate and remains an unresolved question (17–20). We have demonstrated that 6MP depletes both ATP and GTP pools and that its antiproliferative effect can be almost completely reversed by the addition of adenine in phosphate buffer, suggesting that adenine nucleotide depletion plays a primary role in its activity. A previous study by Al-Safi and Maddocks (19) on a mixed lymphocyte population demonstrated almost complete reversal of 6MP inhibitory effects by adenosine, inosine, and hypoxanthine, all of which would be expected to feed into the IMP pool by the eventual salvage of hypoxanthine. Adenine alone had a minimal effect, most likely due to the absence of phosphate, which is required for adenine phosphoribosyltransferase activity. It is thus likely that the adenine added was ineffective in repleting ATP, because ATP pools were not quantitated in the study.

Azathioprine also depletes both adenine and guanine ribonucleotide pools, as would be expected if its mechanism of action were mediated through conversion to 6MP. However, two experiments present very strong evidence that azathioprine is inhibiting the proliferation of purified T cells by a completely independent mechanism. First, the repletion of both adenine and guanine ribonucleotides is not effective in reversing the inhibition of cell proliferation and, second, the drug must be added within the first 24 hr of culture to have its effect. The latter observation differentiates azathioprine from each of the other immunosuppressive drugs we have studied. Further evidence supporting this difference was obtained by Dalke *et al.* (21), using HGPRT-deficient B lymphoblast cell lines. Azathioprine was approximately 8-fold more toxic to wild-type than to HGPRT-deficient B cells, whereas 6MP was as much as 100-fold more toxic to the same cells. Similar data were obtained in experiments on the mixed lymphocyte reaction of patients with congenital deficiency of HGPRT (22). It is clear, therefore, that azathioprine has a generalized antiproliferative effect separable from any effect on purine metabolism.

Our data demonstrate that the metabolic events resulting from mizoribine, MPA, and 6MP do not affect the T cell receptor-mediated signal transduction pathway. The results with azathioprine demonstrate that physiologically relevant concentrations of 10 and 50 μM must be present from time points at or near the start of the culture to inhibit proliferation. Although these data are compatible with the hypothesis that azathioprine could interfere with initial events in the physiologic T cell activation pathway, the fact that similar results are obtained with cells stimulated with PMA and ionomycin and with cells stimulated with anti-CD3 argues against this effect occuring via inhibition of GTP-binding protein activity.

Although both azathioprine and 6MP have been associated with bone marrow suppression and hepatotoxicity at effective doses, mizoribine appears to be more selective in its effect on the immune system (23–29). These data, in conjunction with the clear demonstration that azathioprine and 6MP have de-

monstrably different (and, by inference, perhaps less specific) mechanisms of inhibiting T cell proliferation, support further investigation into the use of mizoribine and other inhibitors of IMP dehydrogenase as immunosuppressive drugs.

Acknowledgments

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References

1. Amemiya, H., S. Suzuki, S. Niiya, H. Watanabe, and T. Kotake. Synergistic effect of cyclosporine and mizoribine on survival of dog renal allografts. *Transplantation (Baltimore)* **46**:768–771 (1988).
2. Inou, T., R. Kusaba, I. Takahashi, H. Sugimoto, K. Kuzuhara, Y. Yamada, J. Yamauchi, and O. Otsubo. Clinical trial of bredinin in renal transplantation. *Transplant. Proc.* **12**:526–528 (1980).
3. Mizuno, K., M. Tsujino, M. Takada, M. Hayashi, K. Atsumi, K. Asano, and T. Matsuda. Studies of bredinin: isolation, characterization and biological properties. *J. Antibiot. (Tokyo)* **27**:775–782 (1974).
4. Koyama, H., and M. Tsuji. Genetic and biochemical studies on the activation and cytotoxic mechanism of bredinin, a potent inhibitor of purine biosynthesis in mammalian cells. *Biochem. Pharmacol.* **32**:3547–3553 (1983).
5. Sakaguchi, K., M. Tsujino, M. Hayashi, K. Kawai, J. Mizuno, and K. Hayand. Mode of action of bredinin with guanylic acid on L5178Y mouse leukemia cells. *J. Antibiot. (Tokyo).* **29**:1320–1327 (1976).
6. Turka, L. A., J. Dayton, G. Sinclair, C. B. Thompson, and B. S. Mitchell. Guanine ribonucleotide depletion inhibits T cell activation: mechanism of action of the immunosuppressive drug mizoribine. *J. Clin. Invest.* **87**:940–948 (1991).
7. Graves, J. D., and D. A. Cantrell. An analysis of the role of guanine nucleotide binding proteins in antigen receptor/CD3 antigen coupling to phospholipase C. *J. Immunol.* **146**:2102–2107 (1991).
8. Kvanta, A., C. Nordstedt, I. van der Ploeg, M. Jondal, and B. B. Fredholm. CD3/T cell receptor coupling to a pertussis and cholera insensitive G-protein. *FEBS Lett.* **250**:536–540 (1989).
9. Turka, L. A., J. A. Ledbetter, K. Lee, C. H. June, and C. B. Thompson. CD28 is an inducible T cell surface antigen that transduces a proliferative signal in $CD3^+$ mature thymocytes. *J. Immunol.* **144**:1646–1653 (1990).
10. Sidi, Y., and B. S. Mitchell. 2′-Deoxyguanosine toxicity of B and mature T lymphoid cell lines is mediated by guanine ribonucleotide accumulation. *J. Clin. Invest.* **74**:1640–1648 (1984).
11. Ledbetter, J. A., C. H. June, L. S. Grosmaire, and P. S. Rabinovich. Cross-linking of surface antigens causes mobilization of intracellular ionized calcium in T lymphocytes. *Proc. Natl. Acad. Sci. USA* **84**:1384–1388 (1987).
12. Morris, R. E., and J. Wang. Comparison of the immunosuppressive effects of mycophenolic acid and the morpholinoethyl ester of mycophenolic acid (RS-61443) in recipients of heart allografts. *Transplant. Proc.* **23**:493–496 (1991).
13. Eugui, E. M., S. J. Almquist, C. D. Muller, and A. C. Allison. Lymphocyte-selective cytostatic and immunosuppressive effects of mycophenolic acid *in vitro:* role of deoxyguanosine nucleotide depletion. *Scand. J. Immunol.* **33**:161–173 (1991).
14. Eugui, E. M., A. Mirkovich, and A. C. Allison. Lymphocyte-selective antiproliferative and immunosuppressive effects of mycophenolic acid in mice. *Scand. J. Immunol.* **33**:175–183 (1991).
15. Lowe, J. K., L. Brox, and J. F. Henderson. Consequences of inhibition of guanine nucleotide synthesis by mycophenolic acid and virazole. *Cancer Res.* **37**:736–743 (1977).
16. Wolberg, G. Antipurines and purine metabolism. *Handb. Exp. Pharmacol.* **85**:517–533 (1988).
17. Elion, G. B., and G. H. Hitchings. Azathioprine. *Handb. Exp. Pharmacol.* **38**:404–425 (1975).
18. Sauer, H., U. Hantke, and W. Wilmanns. Azathioprine lymphocytotoxicity: potentially lethal damage by its imidazole derivatives. *Arzneim. Forsch.* **38**:820–824 (1988).
19. Al-Safi, S. A., and J. L. Maddocks. Azathioprine and 6-mercaptopurine (6-MP) suppress the human mixed lymphocyte reaction (MLR) by different mechanisms. *Br. J. Clin. Pharmacol.* **17**:417–422 (1984).
20. Al-Safi, S. A., and J. L. Maddocks. Effects of azathioprine on the human mixed lymphocyte reaction (MLR). *Br. J. Clin. Pharmacol.* **15**:203–209 (1983).
21. Dalke, A. P., I. S. Kazmers, and W. N. Kelley. Hypoxanthine-guanine phosphoribosyltransferase-independent toxicity of azathioprine in human lymphoblasts. *Biochem. Pharmacol.* **33**:2692–2695 (1984).
22. Szawlowski, P. W. S., S. A. Al-Safi, T. Dooley, and J. L. Maddocks. Azathioprine suppresses the mixed lymphocyte reaction of patients with Lesch-Nyhan syndrome. *Br. J. Clin. Pharmacol.* **20**:489–491 (1985).
23. Tajima, A., M. Hata, N. Ohta, Y. Ohtawara, K. Suzuki, and Y. Aso. Bredinin treatment in clinical kidney allografting. *Transplantation (Baltimore)* **38**:116–118 (1984).
24. Kusaba, R., O. Otubo, H. Sugimoto, I. Takahashi, Y. Yamada, J. Yamauchi, N. Akiyama, and T. Inou. Immunosuppressive effect of bredinin in the management of patients with renal transplantation. *Proc. Eur. Dial. Transplant. Assoc.* **18**:420–425 (1981).
25. Aso, K., H. Uchida, K. Sato, K. Yokota, T. Osakabe, Y. Nakayama, M. Ohkubo, K. Kumano, T. Endo, K. Koshiba, K. Watanabe, and N. Kashiwagi. Immunosuppression with low-dose cyclosporine combined with bredinin and prednisolone. *Transplant. Proc.* **19**:1955–1958 (1987).
26. Marumo, F., M. Okubo, K. Yokota, H. Uchida, K. Kumano, T. Endo, K. Watanabe, and N. Kashiwagi. A clinical study of renal transplant recipients receiving triple-drug therapy, cyclosporine A, mizoribine, and prednisolone. *Transplant. Proc.* **20**:406–409 (1988).
27. Takahara, S., T. Fukunishi, Y. Kokado, Y. Ichikawa, M. Ishibashi, S. Nagano, and T. Sonoda. Combined immunosuppression with low-dose cyclosporine, mizoribine, and prednisolone. *Transplant. Proc.* **20**:147–151 (1988).
28. Amemiya, H., S. Suzuki, H. Watanabe, R. Hayashi, and S. Niiya. Synergistically enhanced immunosuppressive effect by combined use of cyclosporine and mizoribine. *Transplant. Proc.* **21**:956–958 (1989).
29. Osakabe, T., H. Uchida, Y. Masaki, K. Sato, Y. Nakayama, M. Ohkubo, K. Kumano, T. Endo, K. Watanabe, and K. Aso. Studies on immunosuppression with low-dose cyclosporine combined with mizoribine in experimental and clinical cadaveric renal allotransplantation. *Transplant. Proc.* **21**:1598–1600 (1989).

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