Control of Lymphoproliferative and Autoimmune Disease in MRL-lpr/lpr Mice by Brequinar Sodium: Mechanisms of Action

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ABSTRACT
Brequinar sodium (BQR) was originally developed as an antitumor drug and subsequently as an immunosuppressant for controlling transplant rejection. It has been widely accepted that the antitumor and immunosuppressive activities of BQR are dependent on its ability to inhibit the enzymatic activity of dihydroorotate dehydrogenase, the fourth enzyme in the de novo pyrimidine synthesis pathway. Recently, we discovered that BQR has the ability to inhibit protein tyrosine phosphorylation in anti-CD3-stimulated murine T lymphocytes and to inhibit the activity of src-related protein tyrosine kinases, p56lck and p59fyn. We examined the in vivo effects of BQR in MRL-lpr/lpr mice. We report that the dose of BQR (10 mg/kg/day) that induced anemia, controlled lymphadenopathy and inhibited autoantibody production, also selectively reduced the pyrimidine nucleotide levels in the bone marrow and in the lymph nodes. Coadministration of uridine (1000 mg/kg/day) with BQR completely normalized pyrimidine nucleotide levels in the bone marrow and lymph nodes, and prevented BQR-induced anemia. However, coadministration of uridine with BQR only partially reversed the anti-proliferative effects of BQR, and did not antagonize the inhibitory effect of BQR on autoantibody production. Finally, we report that BQR markedly reduced protein tyrosine phosphorylation in lymph nodes of MRL-lpr/lpr mice. These results collectively suggest that the control of lymphadenopathy and autoantibody production in MRL-lpr/lpr mice by BQR is only partially dependent on inhibition of pyrimidine nucleotide synthesis, and suggest a critical role for in vivo inhibition of protein tyrosine phosphorylation.

MRL-lpr/lpr mice develop a spontaneous autoimmune disease that is similar to SLE in humans (Theofilopoulos and Dixon 1985). These mice also develop lymphadenopathy due to the expansion of a CD3+CD4+CD8−B220+ T cell subset (Morse et al., 1982; Davidson et al., 1986). Molecular cloning and mapping of the lpr recessive locus revealed a mutation in fas, a gene encoding a transmembrane protein that can trigger apoptosis in lymphocytes (Watanabe-Fukunaga et al., 1992; Adachi et al., 1993; Drappa et al., 1993; Nagata and Suda 1995). In addition, CD3−CD4+CD8+B220+ T cells from MRL-lpr/lpr mice exhibit characteristics of impaired intracellular signaling reminiscent of hyperresponsive T cells. These include increased expression of p59fyn (Katagiri et al., 1989), constitutively tyrosyl-phosphorylated ζ chain of TCR/CD3 complex (Samelson et al., 1986) and elevated intracellular IP3 production (Tomita-Yamaguchi and Santoro 1990).

BQR (NSC368390; DuP785) [6-fluoro-2-(2′-fluoro-1′-bi-phenyl-4-yl)-3-methyl-4-quinoline carboxylic acid sodium salt] was originally developed as an anticancer drug and later developed as an immunosuppressant for the control of transplant rejection (Dexter et al., 1985; Alison and Eugui 1993; Thomson and Starzl 1993). The mechanism of BQR-mediated antiproliferative and immunosuppressive activities is reported to be the inhibition of the enzymatic activity of dihydroorotate dehydrogenase, the fourth enzyme of the de novo pyrimidine biosynthetic pathway (Chen et al., 1986; Peters et al., 1987, 1990a, 1990b). However, several lines of evidence argue against this hypothesis. First, the serum uridine in human and rodents [5–15 μM (Karle et al., 1980; Pizzorno et al., 1992)] could be converted to pyrimidine nucleotides by the salvage pathway, resulting in a normalization of intracellular pyrimidine nucleotide levels. Second, patients with a human genetic disease, hereditary orotic aciduria, are defective in the de novo pyrimidine synthesis, but do not appear to be significantly immunocompromised (Webster et al., 1995). The symptoms of hereditary orotic aciduria include megaloblastic anemia and accumulation of orotic acid (Webster et al., 1995). These observations suggest that the lymphoid function may not be selectively inhibited by defective de novo pyrimidine synthesis. Third, when BQR was used at the concentrations higher than 30 μM, the growth inhibition of a murine colon tumor cell line was no

ABBREVIATIONS: ATP, adenosine-5′-triphosphate; BQR, brequinar sodium; CTP, cytidine-5′-triphosphate; DHO-DHase, dihydroorotate dehydrogenase; GTP, guanosine-5′-triphosphate; SLE, systemic lupus erythematosus; UTP, uridine-5′-triphosphate.
longer be reversed by the addition of exogenous uridine (Peters et al., 1992). These data collectively suggest that BQR may exert its antiproliferative and immunosuppressive activities via mechanisms independent of inhibition of de novo pyrimidine synthesis. We have recently identified a novel activity of BQR-inhibition of protein tyrosine phosphorylation (Xu et al., 1997b). In this study, we investigate the relative contribution of de novo pyrimidine synthesis and of inhibition of protein tyrosine phosphorylation to the immunosuppressive and anti-proliferative activities of BQR in vivo.

Materials and Methods

Reagents. BQR was a kind gift from the DuPont Merck Pharmaceutical Company (Wilmington, DE). BQR was dissolved in ethanol (200 proof) at the concentration of 80 mg/ml and stored at -20°C. Uridine was purchased from Sigma Chemical Co. (St. Louis, MO), it was dissolved in 0.9% NaCl for in vivo studies and in PBS (pH 7.4) for in vitro studies. Before i.p. injection, BQR was diluted in 0.9% NaCl. Anti-phosphotyrosine monoclonal antibody, 4G10, was purchased from UBI (Placid Lake, NY). Double-stranded calf thymus DNA, poly-L-lysine, and poly-L-glutamic acid were purchased from Sigma. Horseradish peroxidase-conjugated anti-mouse IgG or IgM was purchased from Southern Biotechnology, Inc. (Birmingham, AL).

In vitro stimulation of splenic T cells. Spleen cells from BALB/c mice were depleted of B cells by adherence to goat-anti-mouse IgG (10 μg/ml) coated plates. The nonadherent cells were harvested and cultured at 5 × 10⁶ cells/ml in RPMI 1640 medium supplemented with 10% fetal bovine serum. Cells were then stimulated with 2 μg/ml Con A (Sigma) in the presence of the indicated concentrations of BQR or uridine. After 40 hr incubation, the cells were harvested, nucleotides were extracted and quantitated as described below.

Treatment of MRL-lpr/lpr mice. Female MRL/lpr/lpr and MRL/MpJ-lpr/lpr mice were purchased from The Jackson Laboratories (Bar Harbor, ME). MRL/MpJ-lpr/lpr mice at the age of 10 wk were divided into four groups, with four to six mice per group. Mice were left untreated or were treated with BQR (10 mg/kg/day) and/or uridine (500 mg/kg, twice per day). Four hours after last treatment with BQR, mice were killed. Blood samples were collected, serum samples prepared and stored at -80°C. Lymph nodes and thymus were collected for Western blotting and nucleotide quantitation. Bone marrow and spleen were collected for quantitation of nucleotide triphosphate levels.

Western blotting and protein tyrosine phosphorylation. About 10 mg of lymph node was directly lysed in NP-40 buffer (50 mM Heps-HCl, pH 8.0; 150 mM NaCl; 1% Nondit P-40; 5 mM EDTA; 1 mM sodium vanadate; 5 mM NaF; 1 mM PMSF; 10 μg/ml of aprotinin and leupeptin each), and postnuclear lysates were prepared. Protein concentration in cell lysates was measured by using a Bio-Rad protein assay kit (Bio-Rad Lab., Hercules, CA). Thirty μg protein of each sample were separated on an SDS-polyacrylamide gel, and then transferred onto a nitrocellulose membrane. Protein tyrosine phosphorylation was monitored by using anti-phosphotyrosine mAb, 4G10, and enhanced chemiluminescence (Amer sham Corp., Arlington Heights, IL).

High-performance liquid chromatography analyses of intracellular nucleotide pool. Lymph node or spleen (60 mg per sample) were briefly homogenized in 540 μl of 0.4 M trichloric acid, nucleotides were extracted by centrifugation and then neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon 113 as previously described (Olempaska-Beer and Freese 1984). Bone marrow cells were collected by flushing both femurs with Hank’s-buffered salt solution, then washed twice with 1 ml of Hanks’-buffered salt solution. Bone marrow cells or splenic T lymphocytes harvested from in vitro cell cultures were spun down, lysed in 0.4 ml of 0.4 M trichloric acid and incubated on ice for 20 min. The lysates were centrifuged, and the pellet saved for protein quantitation, the supernatants were transferred and neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon 113. After centrifugation, the upper aqueous phase was collected and used for the nucleotide quantitation. Nucleotides were analyzed on a Waters HPLC system with a 616 pump, a 600S gradient controller, a 717 plus autosampler and 996 PDA detector (Milford, MA). The separation was achieved by a linear gradient elution of potassium phosphate buffer, pH 4.3 (10–500 mM) on a Whatman anion exchange column, partisol 10 SAX (Alltech, Deerfield, IL). The corresponding peaks of the four nucleotides were integrated and the concentrations were calculated based on a standard curve. Nucleotide levels in bone marrow cells and splenic T cells were normalized by protein concentrations.

Quantitation of anti-DNA antibodies. The anti-double-strand (ds) DNA antibodies in serum samples were quantitated by using an ELISA assay as previously described (Zhou et al., 1993). Briefly, the 96-well microplates were precoated with poly-L-lysine (10 μg/ml), or with poly-L-glutamic acid (10 μg/ml) as a negative control and then coated with calf thymus dsDNA (10 μg/ml) for 20 hr at 4°C. The serum was diluted in a 2-fold series in PBS containing 5% bovine serum albumin, starting at 1:2000 dilution. The bound antibodies were detected with horseradish peroxidase-conjugated anti-mouse IgG or anti-mouse IgM, followed by colorimetric development of the substrate, ABTS (2,2-azide-bis-(3-ethylbenzthiazoline-6-sulfonic acid)). The optical density was read on an ELISA reader (Bio-Rad, Richmond, CA).

Hematocrit. Mice were bled through the orbital vein using a microhematocrit capillary tube (Baxter, Deerfield, IL), and percent packed cell volumes were determined with a micro-hematocrit capillary tube reader (Critocaps, Oxford Lab., Baxter, Deerfield, IL).

Quantitation of tyrosine phosphorylation. The exposed X-Omat film containing phosphotyrosine proteins detected by Western blotting were scanned in a Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA). The peaks corresponding to the bands of interest were integrated to determine the relative amounts of phosphorylation.

Statistical analysis. All analysis to determine significant differences between treatment and control groups were performed using the SuperANOVA program for Macintosh (Abacus Concepts Inc., Berkeley, CA). Significant differences were concluded when α ≤ .05 by the analysis of variance and a post hoc Tukeys compromise test.

Results

Effect of BQR and uridine on intracellular nucleotide levels in vitro. The mechanism of action of BQR is generally accepted as inhibition of de novo pyrimidine nucleotide synthesis (Chen et al., 1986; Peters et al., 1990a, 1990b, 1992). Thus, addition of exogenous uridine should normalize pyrimidine nucleotide levels in BQR-treated cells. We stimulated murine splenic T lymphocytes with Con A in the presence of BQR and various concentrations of uridine. After incubation for 40 hr, cells were harvested, nucleotides were extracted and nucleotide triphosphates were quantitated by high-performance liquid chromatography. As previously reported (Fairbanks et al., 1995), activation of T lymphocytes resulted in a 2- to 4-fold increase in nucleotides levels (data not shown). BQR treatment inhibited this increase in pyrimidine nucleotide levels by approximately 70%, the addition of exogenous uridine (1–40 μM, final concentration in supernatant) restored the UTP and CTP levels in a dose-dependent manner (fig. 1).

Control of lymphadenopathy by BQR. We examined the in vivo antiproliferative effects of BQR on constitutively proliferating Fas-mutated lymphocytes in MRL-lpr/lpr mice.
A dose of uridine was chosen for the treatment of MRL-lpr/lpr anemia caused by BQR treatment (20 mg/kg/day) (Xu et al., 1997). Our earlier toxicity studies indicated that uridine at the dose of 500 mg/kg, twice daily, was able to completely overcome the toxicity of BQR. Our earlier studies showed that uridine at 500 mg/kg/day was able to completely reverse the antiproliferative effects of BQR. Our earlier studies also showed that uridine nucleotide synthesis, coadministration of uridine with BQR in vivo, is consistent with the observation that uridine (1000 mg/kg/day) with BQR is able to completely normalize pyrimidine nucleotide levels in all lymphoid tissues examined.

The therapeutic effects of BQR on the lymphadenopathy of MRL-lpr/lpr mice was dependent on inhibition of pyrimidine nucleotide synthesis, coadministration of uridine with BQR for 3 to 5 wk had hematocrits comparable to those of untreated mice. Uridine by itself had no effect on the hematocrit levels of MRL-lpr/lpr mice; uridine coadministered with BQR completely prevented BQR-induced anemia. This result is consistent with the observation that uridine (1000 mg/kg/day) coadministered with BQR was able to normalize pyrimidine nucleotide levels in bone marrow cells and increased the levels of pyrimidine nucleotides in the lymph nodes and spleen by approximately 200% (P < .05). The purine nucleotide levels in the lymph nodes and bone marrow of mice treated with various agents essentially remained unchanged, whereas the purine levels in the spleens of mice treated with uridine plus BQR were significantly increased (P < .05). These results indicate that BQR interferes with pyrimidine nucleotide synthesis in vivo. In addition, coadministration of uridine (1000 mg/kg/day) with BQR is able to completely normalize pyrimidine nucleotide levels in all lymphoid tissues examined.

We reasoned that if control of lymphadenopathy in MRL-lpr/lpr mice by BQR is dependent on inhibition of pyrimidine nucleotide synthesis, coadministration of uridine with BQR should reverse the antiproliferative effects of BQR. Our earlier toxicity studies indicated that uridine at the dose of 500 mg/kg, twice daily, was able to completely overcome the anemia caused by BQR treatment (20 mg/kg/d) (Xu et al., submitted for publication). In the present studies, the same dose of uridine was chosen for the treatment of MRL-lpr/lpr mice. As shown in table 1, uridine alone did not affect the weights of lymph nodes. Coadministration of uridine with BQR reduced the weights of lymph nodes and thymy by 57.6 and 40.6%, respectively (P < .05), in comparison to the untreated control. Comparing the weights of lymph nodes and thymy of BQR plus uridine-treated mice with that of BQR-treated mice, it was found that uridine significantly antagonized the therapeutic effects of BQR on the lymphadenopathy of MRL-lpr/lpr mice in lymph nodes (P < .05) but not of the thymy (P > .05). These observations indicate that inhibition of pyrimidine nucleotide synthesis partially contributes to the ability of BQR to control lymphoproliferative disease in MRL-lpr/lpr mice.

### Nucleotide levels in bone marrow, lymph nodes and spleen

To monitor the in vivo inhibitory effect of BQR on pyrimidine nucleotide synthesis and to ascertain that the dose of uridine given to mice was sufficient to normalize pyrimidine nucleotide synthesis in lymphoid tissues, nucleotides triphosphate concentrations in bone marrow cells, lymph nodes and spleens, were quantitated. As shown in table 2, the UTP and CTP levels in uridine-treated mice were increased by 35% in bone marrow cells, and were increased by approximately 300% in the spleen and lymph nodes. These increases in pyrimidine nucleotide triphosphate levels were statistically significant (P < .05). In BQR-treated mice, the UTP and CTP levels were decreased by 33 to 43% in bone marrow cells and in the lymph nodes (P < .05), but were essentially unchanged in spleen (P > .05). Coadministration of uridine and BQR normalized the pyrimidine nucleotide levels in bone marrow cells and increased the levels of pyrimidine nucleotides in the lymph nodes and spleen by approximately 200% (P < .05). The purine nucleotide levels in the lymph nodes and bone marrow of mice treated with various agents essentially remained unchanged, whereas the purine levels in the spleens of mice treated with uridine plus BQR were significantly increased (P < .05). These results indicate that BQR interferes with pyrimidine nucleotide synthesis in vivo. In addition, coadministration of uridine (1000 mg/kg/day) with BQR is able to completely normalize pyrimidine nucleotide levels in all lymphoid tissues examined.

### Reversal of BQR-induced anemia by co-administration with uridine

One of the major side-effects caused by BQR is myelosuppression, resulting in anemia (Thomson and Starzl 1993). We next tested whether coadministration of uridine with BQR could prevent BQR-mediated anemia. As shown in figure 2, mice treated with BQR (10 mg/kg/day) for 2 wk became anemic; the hematocrits (42%) were significantly reduced in comparison to that in untreated mice (62%; P < .05). Uridine by itself had no effect on the hematocrit levels of MRL-lpr/lpr mice; uridine coadministered with BQR completely prevented BQR-induced anemia. This result is consistent with the observation that uridine (1000 mg/kg/day) coadministered with BQR was able to normalize pyrimidine nucleotide levels in bone marrow cells. Mice treated with BQR for 3 to 5 wk had hematocrits comparable to that in untreated control (only the hematocrit levels measured on day 31 are shown), probably due to extramedullary hematopoiesis in spleen as described in our earlier studies (Xu et al., 1997b).

### Inability of uridine to reverse the inhibition of autoantibody production mediated by BQR

Autoantibodies play an important role in the initiation of autoimmune disease in the MRL-lpr/lpr mice (Andrew et al., 1978). We tested whether BQR could inhibit autoantibody production,
were obtained. The results represent mean ± S.E. from four to six mice in each group. This experiment was repeated twice, and similar results were obtained.

The concentration of nucleotides in bone marrow cells was calculated as ng per mg of precipitated protein.

The concentration of nucleotides in lymph node and spleen was calculated as ng per 10 mg of wet tissue.

Urinary nucleotide levels in MRL-1pr/1pr mice treated with various agents are shown in Table 2.

![Graph showing hematocrit over time](image)

**Fig. 2.** Reversal of BQR-induced anemia by co-administration of uridine. MRL-lpr/lpr mice (four to six mice/group) were treated with uridine only (500 mg/kg, twice daily), BQR only (10 mg/kg/day) or BQR (10 mg/kg/day) plus uridine (500 mg/kg, twice per day) for 7 wk. Blood samples were collected from the orbital vein once per week, then simultaneously spin down at 1600 × g 15 min. Percent packed cell volumes (hematocrits) were determined with a micro-hematocrit capillary tube reader. The results represent the mean of hematocrits of four to six animals in each group on day 13 and 31 after starting treatment.

The relative density of each band was determined and compared to the level of tyrosine phosphorylation of untreated individuals. The tyrosine phosphorylation levels of these proteins were further quantitated by densitometric scanning. The results suggest that in vivo inhibition of protein tyrosine phosphorylation by BQR is unrelated to its inhibitory effect on pyrimidine nucleotide synthesis.

In vivo inhibition of protein tyrosine phosphorylation by BQR. Observations of the inability of uridine to completely reverse BQR-mediated control of lymphadenopathy and autoantibody production in MRL-lpr/lpr mice suggest that BQR may exert its in vivo antiproliferative activity via a mechanism independent of depletion of intracellular pyrimidine nucleotides. We have recently made the novel observations that BQR is able to inhibit protein tyrosine phosphorylation (Xu et al., 1997b). In the present study, we examined the protein tyrosine phosphorylation in the cell lysates prepared from the lymph nodes by Western blotting analysis. Consistent with a previous report (Katagiri et al., 1989), our results in figure 4 show that several proteins from lymphocytes of untreated MRL-lpr/lpr mice with molecular masses of 120-, 90-, 70- and 60-kDa were heavily phosphorylated on tyrosine residues. Uridine treatment (1000 mg/kg/day) did not reduce tyrosine phosphorylation of these proteins, whereas BQR treatment (10 mg/kg/day) or BQR plus uridine treatment significantly reduced tyrosine phosphorylation of all these proteins in all four mice tested. There was some variation in protein tyrosine phosphorylation among individuals, the tyrosine phosphorylation levels of these proteins were further quantitated by densitometric scanning. The relative density of each band was determined and compared to the level of tyrosine phosphorylation of untreated MRL-lpr/lpr lymphocytes. Treatment of MRL-lpr/lpr mice with BQR significantly reduced the level of tyrosine phosphorylation of the 120-, 90-, 70- and 60-kDa proteins by 89, 80, 92 and 88%, respectively (P < .05; fig. 4). Uridine treatment alone did not affect the levels of tyrosine phosphorylation in the lymph node cells. Treatment of mice with uridine and whether uridine could reverse the effects of BQR. The results in figure 3 show that BQR treatment significantly prevented the production of anti-DNA IgM and IgG antibodies. The titers of anti-IgG and anti-IgM in BQR-treated animals were about 3 and 12% of untreated animals. Uridine treatment alone slightly reduced autoantibody levels. Co-administration of uridine with BQR did not reverse BQR-induced reduction in the titers of either anti-DNA IgM or anti-DNA IgG. These results suggest that control of autoantibody production by BQR is unrelated to its inhibitory effect on pyrimidine nucleotide synthesis.
and BQR reduced tyrosine phosphorylation of the 120-, 90-, 70- and 60-kDa proteins by 71, 81, 39 and 63%, respectively. Statistical analyses indicate that the tyrosine phosphorylation levels of these intracellular proteins, except the 70-kDa protein, were not significantly different from that in BQR-treated mice (P > .05). These observations also suggest that inhibition of protein tyrosine phosphorylation by BQR is largely independent of its inhibitory effect on pyrimidine nucleotide synthesis.

**Discussion**

The MRL-lpr/lpr mice develop a spontaneous lymphadenopathy that is caused by an overexpansion of a subset of nonapoptotic T lymphocytes (Morse et al., 1982; Davidson et al., 1986). These mice also develop to an autoimmune disease that is caused by the spontaneous production of autoantibodies cause tissue damage and dysfunction, such as glomerulonephritis (Andrew et al., 1978). Thus, these MRL-lpr/lpr mice have served as an excellent model for identifying immunosuppressive or antiproliferative drugs and for defining the in vivo activity of these drugs (Popovic and Bartlett 1986; Weinberg et al., 1994; Edward et al., 1996). Using this unique model, we have examined the in vivo mechanisms of action of BQR.

Previous studies have demonstrated that BQR is a potent inhibitor of DHO-DHase activity and that it selectively depletes pyrimidine nucleotide pools in lymphocytes and tumor cells (Chen et al., 1986; Peters et al., 1990a, 1990b, 1992; Xu et al., 1996). We report that BQR reduced the pyrimidine nucleotide levels in Con A-stimulated murine T lymphocytes in vitro. In addition, we demonstrate the ability of BQR to reduce pyrimidine nucleotide levels in the lymph nodes and bone marrow of MRL-lpr/lpr mice. The observations of reduced pyrimidine nucleotide levels are consistent with the concept that BQR is an inhibitor of de novo pyrimidine nucleotide synthesis.

Pyrimidine nucleotides that are essential for DNA and RNA synthesis can be synthesized either by the de novo pathway or salvaged from exogenous nucleosides. It has previously been reported that the antiproliferative effect of BQR in vitro can be countered by exogenous uridine (Peters et al., 1992; Forrest et al., 1994). We demonstrate that exogenous uridine normalized pyrimidine nucleotide levels in BQR-treated Con A-stimulated murine T lymphocytes. Similarly, our in vivo studies with MRL-lpr/lpr mice show that coadministration of BQR with uridine (1000 mg/kg/day) also normalized pyrimidine nucleotide levels in the lymph nodes and bone marrow.

We reasoned that if the in vivo effects of BQR in MRL-lpr/lpr mice are solely dependent on inhibition of pyrimidine nucleotide synthesis, coadministration of uridine should reverse the antiproliferative and immunomodulatory effects of BQR. We observed that uridine coadministration completely...

**Fig. 3.** Autoantibody production in MRL-lpr/lpr mice treated with BQR and/or uridine. MRL-lpr/lpr mice (four to six mice/group) were treated with uridine only (500 mg/kg, twice daily), BQR only (10 mg/kg/day) or BQR (10 mg/kg/day) plus uridine (500 mg/kg, twice per day) for 7 wk. Controls were age-matched untreated MRL-lpr/lpr mice. Mice were killed and blood samples were collected. Anti-IgG and anti-IgM antibodies specific for dsDNA in serum samples were quantitated by using ELISA as described in “Materials and Methods.” The results were mean optical density ± S.E. of four to six mice in each group. This experiment was repeated twice and similar results were obtained.

**Fig. 4.** Inhibition of protein tyrosine phosphorylation in vivo. MRL-lpr/lpr mice (four to six mice/group) were treated with uridine only (500 mg/kg, twice daily), BQR only (10 mg/kg/day) or BQR (10 mg/kg/day) plus uridine (500 mg/kg, twice per day) for 7 wk. Controls were age-matched untreated MRL-lpr/lpr mice. Lymph nodes were collected, single cell suspension prepared. Cells (1 × 107/sample) were lysed in NP-40 lysis buffer, protein concentrations measured, and equal amounts of protein were separated on a 10% SDS-polyacrylamide gel. The proteins were transferred onto a nitrocellulose membrane, and tyrosine phosphorylated proteins were detected by the anti-phosphotyrosine mAb, 4G10, and ECL, followed by exposure to the X-Omat film. The results shown are from randomly picked two to four mice from each group. To quantitate the percent inhibition of protein tyrosine phosphorylation, the exposed X-Omat films containing phosphotyrosine protein bands were scanned in a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). The peaks corresponding to the bands of several intracellular proteins were integrated to determine the relative amounts of phosphorylation. The mean relative densities of each protein band, from four to six animals in each group, were compared with that of the untreated control. The P value was calculated by using a **post hoc** Tukeys compromise test in the SuperANOVA program. The experiments were repeated twice, and similar results were obtained.
prevented BQR-induced anemia but only partially antagonized the antiproliferative effects of BQR. In addition, uridine was unable to mitigate the inhibitory effects of BQR on autoantibody production, and the skin lesions that were inhibited by BQR were unaffected by uridine coadministration (data not shown). These observations suggest that inhibition of hematopoiesis is largely due to the depletion of pyrimidine nucleotides by BQR, although the control of lymphadenopathy is only partially dependent on this activity of BQR. In addition, we recently observed that control of allograft rejection by BQR in a murine BALB/c→C3H heart transplant model is unaffected by uridine coadministration (Xu et al., 1997b). Thus, it appears that the immunomodulatory activity of BQR is largely independent of inhibition of the de novo pyrimidine nucleotide synthesis. An alternative explanation may also be that cells undergoing hematopoiesis and T cells in the lymph node may be more sensitive to BQR or less effective in salvaging uridine.

Consistent with our previous findings that BQR is able to inhibit in vitro protein tyrosine phosphorylation in anti-CD3-stimulated murine T cells and to inhibit the activity of src-related tyrosine kinases, p56lck and p59fyn (Xu et al., 1997b), we here demonstrate that BQR significantly reduced the protein tyrosine phosphorylation levels of lymph node cells from MRL-lpr/lpr mice. Because protein tyrosine phosphorylation plays an important role in cell activation and proliferation, our results suggest that inhibition of protein tyrosine phosphorylation may be the major factor contributing to the control of the lymphoproliferative disease in MRL-lpr/lpr mice. Although not statistically significant, the protein tyrosine phosphorylation levels in the lymph nodes of mice treated with uridine and BQR was slightly increased in comparison to that of BQR-treated mice. We speculate that because the volume of lymph nodes in uridine plus BQR-treated mice was much larger than that in BQR-treated mice, the effective concentrations of BQR in lymph nodes of mice treated with uridine plus BQR-treated mice might be lower compared to that in BQR-treated mice.

Leflunomide is a novel immunosuppressant with many striking similarities to BQR (Cherwinski et al., 1995a, 1995b; Xu et al., 1995, 1996). It has two biochemical activities: inhibition of protein tyrosine phosphorylation and interference with pyrimidine nucleotide synthesis (Xu et al., 1995, 1996; Elder et al., 1997). The ability of leflunomide to inhibit src-related tyrosine kinase is comparable to that of BQR; however, its ability to inhibit DHO-DHase activity is about 10- to 50-fold less potent than BQR's (Xu et al., 1996). Our recent in vivo studies with the MRL-lpr/lpr model indicate that the control of lymphadenopathy by leflunomide is completely independent of its effect on pyrimidine nucleotide synthesis, and may be largely dependent on inhibition of protein tyrosine phosphorylation (Xu et al., 1997a). Thus our results with BQR and leflunomide collectively suggest that inhibition of tyrosine phosphorylation is sufficient to achieve immunosuppression, whereas inhibition of pyrimidine nucleotide synthesis, which has a more profound effect on bone marrow cells, is responsible for their side-effects such as myelosuppression. These observations are of clinical significance because they suggest that the toxicities associated with BQR administration may be countered with uridine, but without the attenuation of its immunomodulatory activities.

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References


nucleotide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthe-


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