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THE ANTILYMPHOCYTIC ACTIVITY OF BREQUINAR SODIUM AND ITS POTENTIATION BY CYTIDINE

EFFECTS ON LYMPHOCYTE PROLIFERATION AND CYTOKINE PRODUCTION[1]

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Based on its capacity to inhibit de novo pyrimidine biosynthesis by blocking dihydroorotate dehydrogenase activity, the antitumor agent brequinar sodium (BQR) has emerged as a new immunosuppressive agent. Since BQR is known to prevent the synthesis of nucleotides during cell proliferation, we hypothesized that it would be highly effective in controlling strong lymphocyte proliferative responses but might be less effective in controlling comparatively weak responses that do not necessarily involve new nucleotide synthesis. We addressed this question by culturing murine spleen cells with different types of stimuli, including Con A, phorbol myristate acetate ± ionomycin, anti-CD3, and anti-Igs.

Addition of BQR (0.001 μg/ml to 10 μg/ml) at the start of a 72-hr culture period caused dose-dependent inhibition of strong proliferative responses, induced either by Con A (5 μg/ml) or PMA + ionomycin. A residual degree of proliferation persisted, however, even at the highest BQR concentrations. In contrast, no impairment of low-concentration Con A (0.5 or 0.1 μg/ml), anti-CD3, or anti-Igs responses was observed. In order to ascertain its role in arresting nucleotide synthesis, we attempted to reverse the inhibitory effect of BQR by adding exogenous uridine or cytidine to lymphocyte cultures. BQR's inhibitory activity was reversed completely by adding uridine at 0.1 mM. In contrast, combination of BQR and cytidine (0.1 mM) potentiated BQR's activity and abrogated anti-CD3 or anti-Igs-induced lymphocyte proliferation in a dose-dependent manner. A synergistic inhibitory action between BQR and cytidine was observed when the BQR concentration was higher than 0.1 μg/ml and with cytidine at 0.1 mM. Production of interleukin-2 and IL-4 was only slightly affected by BQR, but was significantly suppressed by coadministration of BQR and cytidine. Neither BQR (5 μg/ml) on its own, however, nor combination of BQR with cytidine affected production of mRNA for IL-2, IL-4, or interferon-γ, as determined by reverse-transcription polymerase chain reaction. Our observations suggest that BQR may not only affect dihydroorotate dehydrogenase activity, but may also inhibit the enzyme cytidine deaminase, which converts cytidine to uridine. These antimetabolic effects of BQR complement the well-known cytokine synthesis inhibitory actions of FK506 or CsA. The combination of BQR and cytidine, however, offers a further possibility for inhibition of both cytokine production and T and B cell proliferation, and may have potential for the control of graft rejection.

Brequinar sodium (BQR)* is a novel substituted, 4-quinoline carboxylic acid derivative, known originally as an anticancer drug (*1–2*). It mediates antitumor activity by inhibiting the mitochondrial enzyme dihydroorotate dehydrogenase (DHO-DH) (*3–4*) leading to the arrest of cell growth. By inhibiting DHO-DH activity, BQR abrogates de novo pyrimidine nucleotide biosynthesis (*3–6*) (Fig. 1) and consequently synthesis of both RNA and DNA during replicative events in tumor cells. Like the purine nucleotide biosynthesis inhibitors azathioprine (*7*), mycophenolic acid (the active moiety of RS61443) (*8*), and mizoribine (*9*), BQR is likely to possess immunosuppressive properties (*10*). Although little has been reported concerning the influence of BQR on immune reactivity, there is recent evidence that the drug is effective in prolonging both experi-

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* BQR, brequinar sodium; CYT, cytidine; PCR, polymerase chain reaction; PMA, phorbol 12-myristate-13-acetate.

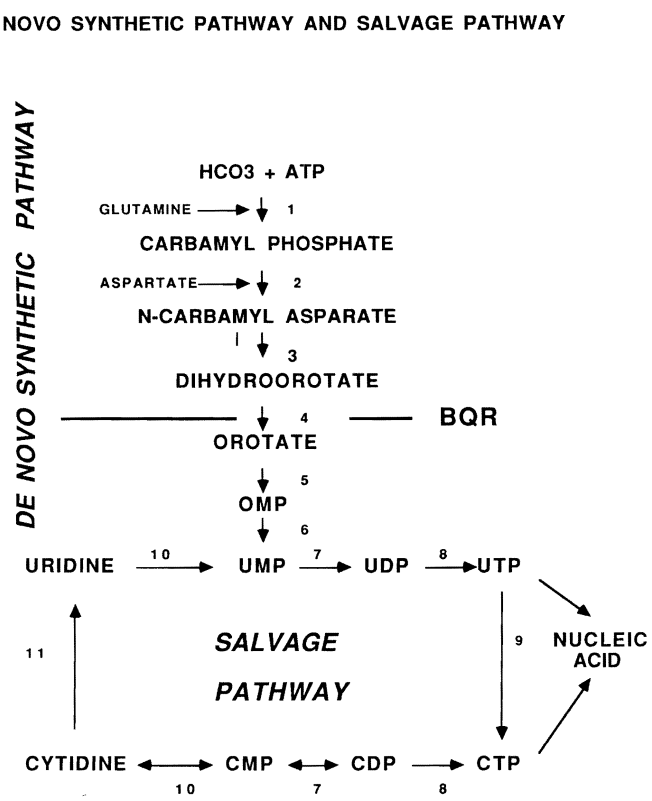


FIGURE 1. Flow diagram of the de novo pyrimidine synthetic pathway and the salvage pathway showing the site of inhibition by BQR. The eleven enzymes involved in these pathways are: (1) carbamylphosphate synthetase II; (2) aspartate transcarbamylase; (3) dihydroorotase; (4) dihydroorotate dehydrogenase; (5) orotate phosphoribosyltransferase; (6) orotidine 5′-monophosphate decarboxylase; (7) nucleoside monophosphate kinase; (8) nucleoside diphosphate kinase; (9) CTP synthetase; (10) uridine-cytidine kinase; and (11) cytidine deaminase.

mental allograft (*11*, *12*) and xenograft survival (*13*). In addition, BQR also has beneficial effects in alleviating experimental allergic encephalomyelitis (*14*).

Prolonged exposure of tumor cells to BQR results in depletion of the nucleotides CTP and UTP (*5*, *15*); its effect on the residual pyrimidine pool in normal or activated lymphocytes, however, has not been evaluated. It is known that the magnitude of lymphocyte activation in vitro, and perhaps also in vivo, depends on the types of signal transduction pathway involved (*16–20*). As BQR may be effective in controlling nucleotide biosynthesis induced by strong rather than weak stimuli (which do not require increases in nucleotide pools) a differential inhibitory activity of the drug rather than a generalized antiproliferative action on responses to different types of stimulation might be expected.

According to the role of BQR in inhibiting pyrimidine synthesis, the addition of exogenous uridine or cytidine might be expected to bypass the pyrimidine biosynthesis pathway and reverse the antiproliferative activity of BQR (salvage pathway [Fig. 1]). Thus uridine is converted to UMP and ultimately to UTP, from which it is transformed to CTP by CTP synthetase. Exogenous cytidine on the other hand, can be deaminated to uridine by cytidine deaminase and converted eventually to UTP (*21*). Alternatively, cytidine may be converted through CMP to CTP. Here, however, we present evidence for differential effects of exogenous uridine and cytidine on lymphocyte responses that is consistent with inhibition of cytidine deaminase activity by BQR. The disruption in intracellular nucleotide balance induced by cytidine in the presence of BQR potentiates the antilymphocytic activity of BQR. In this study, we address the possibilities and present evidence that (1) the inhibitory activity of BQR is dependent on the strength of stimulation and not on the type of signal transduction pathway and that (2) cytidine can potentiate BQR's antilymphocytic activity, both in terms of cell proliferation and cytokine production.

MATERIALS AND METHODS

Animals. Adult, 8–12-week-old, male C57BL/10SnJ mice were purchased from Jackson Laboratory, Bar Harbor, ME. They were maintained in the Central Animal Facility of the University of Pittsburgh Health Science Center.

Reagents. Brequinar sodium (DuPont Merck Pharmaceutical Company, Wilmington, DE) was kindly provided by Dr. L. Makowka, Cedars-Sinai Medical Center, Los Angeles, CA. It was dissolved fresh daily in filter-sterilized saline at 1 μg/ml and diluted to working concentrations with RPMI-1640 (Gibco, Grand Island, NY), supplemented with 2 mM glutamine, 50 U/ml penicillin G sodium, 50 mg/ml streptomycin sulfate, 25 mM Hepes, and 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY). FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan) was dissolved initially in absolute ethanol and diluted subsequently in RPMI-1640 with 10% FBS. Concanavalin A was purchased from Sigma Chemical Co., St. Louis, MO, and was dissolved in sterile saline, followed by further dilution in RPMI-1640 with 10% FBS. Anti-CD3 monoclonal antibody, derived from clone KT3, was purchased from Serotec, Oxford, England, UK. Goat anti-mouse polyvalent immunoglobulin was purchased from Sigma and was used as a B cell mitogen. Phorbol 12-myristate-13-acetate (PMA), ionomycin, uridine, and cytidine were purchased from Sigma, and were dissolved in absolute ethanol (PMA, ionomycin) or saline (uridine, cytidine), and finally diluted in RPMI-1640 with 10% FBS.

Lymphocyte culture. Spleen cells were used for setting up lymphocyte cultures. Briefly, spleens were removed aseptically, then disrupted mechanically using a pair of sterile forceps. The cells were washed, followed by lysis of erythrocytes in Tris-NH_4Cl solution, pH 7.2. After further washes, the cells were resuspended finally in RPMI-1640 with 10% FBS. The number of spleen cells was adjusted and 5×10^5 cells (final volume 0.2 ml) were added to triplicate wells of 96-well round-bottomed microtiter plates (Corning Glass Works, Corning; NY). Con A, PMA, and ionomycin were used at 5 μg/ml, 1 ng/ml, and 0.5 μM, respectively, and anti-CD3 and anti-Igs antibodies were used at final dilutions of 1/100 and 1/50, respectively. For addition of exogenous uridine or cytidine, each was used at 0.1 mM (final concentration) unless specified. Cells were cultured for 24–120 hr, and 1 μCi [^{3}H]-thymidine was added to wells 24 hr before cell harvesting. The degree of radioactive thymidine incorporation was measured by a liquid scintillation counter 1205 Betaplate (Pharmacia, Gaithersburg, MD), and results expressed as cpm ± 1 SD.

In cultures set up to determine cell viability, 50 μl cell suspension was collected at 24, 48, and 72 hr, and the number of viable cells quantitated by the trypan blue exclusion test.

Generation of supernatants for cytokine measurement. Fifty million spleen cells were cultured with Con A (5 μg/ml) for 20 hr with/without BQR in Falcon tissue-culture tubes (Becton Dickinson, NJ). In some cultures, 0.1 mM cytidine was added. After incubation, cultures were centrifuged, and supernatants were collected and stored at −80°C until analysis.

Quantitation of IL-2 and IL-4 by "sandwich" ELISA. A murine IL-2 ELISA kit (Collaborative Research, Bedford, MA) and a murine IL-4 ELISA kit (Endogen, Boston, MA) were employed for measurement of cytokine levels in culture supernatants. Briefly, IL-2 or IL-4 in diluted supernatants was captured by specific antibodies precoated on wells of

ELISA plates. After incubation and washing, bound IL-2 and IL-4 were labeled with peroxidase-conjugated antimurine IL-2 or IL-4 antibodies, and the presence of the cytokine-antibody complex was detected after addition of substrate o-phenylene-diamine. The reaction was stopped by addition of 2 M sulfuric acid, and the absorbance was measured at wavelength 490 nM using an ELISA plate reader (MR5000, Dynatech, Guernsey, Channel Islands). The amount of cytokine was derived from standard curves obtained with known IL-2 and IL-4 concentrations and performed on the same ELISA plates.

Detection of cytokine gene expression. Cells were harvested 20 hr after Con A stimulation and total RNA extracted by the guanidinium-isothiocyanate method. A 1 μg RNA sample was reverse-transcribed into cDNA using oligo-dT primer and MMLV reverse transcriptase (Gibco BRL). This template cDNA was used for 30 cycles of PCR amplification, using specific IL-2, IL-4, and IFN-γ oligonucleotide primers synthesized at the University of Pittsburgh DNA synthesis facility. The PCR amplification was carried out using Taq polymerase (Perkin-Elmer) and a Model 480 DNA Thermal Cycler (Perkin-Elmer). The products of amplification, along with molecular weight markers for sizing, were separated on 2% agarose gels stained with ethidium bromide and photographed in UV light. Semiquantitative analysis was performed by comparing visibility of bands for serial ten-fold dilutions of input template DNA. β-actin primer was used in a separate PCR reaction to control for amount of template cDNA.

RESULTS

Differential effects of BQR on lymphocyte activation induced by strong and weak stimuli. In order to address the question of the relationship between upregulation of nucleotide biosynthesis during cell proliferation and the inhibitory action of BQR, lymphocytes were cultured with either strong (Con A, PMA + ionomycin) or weak (anti-CD3, anti-Igs) stimuli. This induced different degrees of lymphocyte activation that required different extents of de novo nucleotide biosynthesis for cell proliferation. Maximal stimulation of ^{3}H-thymidine incorporation over 72 hr in response to Con A was achieved using a mitogen concentration of 5 μg/ml. High ^{3}H-thymidine incorporation was also achieved with PMA (1 ng/ml) plus ionomycin (0.5 μM). Addition of BQR at the start of cultures resulted in concentration-dependent inhibition of DNA synthesis in response to either Con A or PMA + ionomycin (Fig. 2a). In response to either of these stimulants, the minimally effective concentration of BQR was 0.1 μg/ml, whereas the IC_{50}s for Con A- or PMA + ionomycin-stimulated cells were 0.2 and 0.5 μg/ml BQR, respectively. Although a sigmoidal dose-response effect, with approximately 80–90% inhibition at high concentrations of BQR (>5 μg/ml) was obtained, persistent residual augmentation of DNA synthesis corresponding to 30,000–50,000 cpm (stimulation index 10–20) was observed consistently in these mitogen-stimulated cultures.

In contrast to the strong inhibitory effect demonstrated by BQR on either Con A- or PMA+ionomycin-induced DNA synthesis, mitogenic responses of mouse lymphocytes to weak stimuli, including anti-CD3 and anti-Igs, showed resistance to BQR (Fig. 2b). The extent of thymidine incorporation induced by these stimulants was substantially less (by approximately one order of magnitude) than that achieved with Con A or PMA + ionomycin. BQR (at concentrations that profoundly inhibited the response to 5 μg/ml Con A) failed to inhibit anti-CD3-induced thymidine incorporation, while no suppression but some augmentation of anti-Ig-induced DNA synthesis was observed. In contrast, anti-CD3-induced thymidine incorporation was very sensitive to the anti-T cell agent FK506 (Fig. 2c). In order to ascertain whether this differential effect on Con A, PMA + ionomycin, anti-CD3, and anti-Igs responses was due to differences in activation pathways or to the magnitude of activation and upregulation of nucleotide synthesis required for cell proliferation, different concentrations of Con A were added to cultures to provide different degrees of stimulation. As shown in Fig. 2d, the inhibitory effect of BQR on Con A responses was dependent both on the concentration of the mitogen and the strength of response. The inhibitory effect of BQR was attenuated and eventually lost as the Con A concentration was reduced from 5.0 to 0.5 μg/ml.

Cytotoxicity of BQR. To confirm that the antiproliferative effect of BQR was not due to a cytotoxic effect at high drug concentration, the number of viable cells was measured following 72 hr culture of either unstimulated or Con A (5 μg/ml)-stimulated cells with BQR. As shown in Figure 3, BQR at inhibitory concentration (5 μg/ml) did not affect the viable cell number in unstimulated cultures (Fig. 3a). It did, however, reduce the viable cell number (to approximately that of unstimulated cells) in Con A-stimulated cultures, indicating that BQR was not toxic to resting cells but exerted antiproliferative activity on Con A-activated lymphocytes.

Kinetics of the BQR inhibitory effect. To ascertain whether the difference in responsiveness to Con A or anti-CD3/Igs in the presence of BQR was due to differences in kinetics of the respective DNA synthesis, cells were harvested at various times after stimulation in the presence of the drug. Inhibitory concentrations of 0.5–5.0 μg/ml BQR at 72 hr were highly effective in suppressing Con A-induced thymidine incorporation throughout the culture period, while 0.1 μg/ml BQR had only a partial effect (data not shown). In contrast, harvesting of cells 24–120 hr after stimulation with anti-CD3 or anti-Igs showed no significant inhibitory effect of BQR, even when BQR was at 10 μg/ml (data not shown).

Influence of exogenous nucleosides on the antiproliferative effect of BQR. Since BQR is known to arrest pyrimidine synthesis, its inhibitory effect on cell thymidine incorporation might be expected to be reversed by the addition of exogenous pyrimidines. When added together with BQR at the initiation of Con A-stimulated cultures, however, the nucleosides uridine and cytidine had differential effects on lymphocyte thymidine incorporation (Fig. 4). While uridine (0.1 mM) prevented completely the growth inhibitory effect of BQR, the addition of cytidine (0.1 mM) resulted in augmentation of the anti-DNA synthesis activity of BQR. Indeed, in comparison with BQR alone, BQR and cytidine together totally inhibited ^{3}H-thymidine incorporation in the BQR concentration range 0.5–10 μg/ml. When added alone to cultures, little (cytidine) or no (uridine) inhibitory effect of nucleosides was observed. Neither BQR nor cytidine had any direct cytotoxic effect on Con A-stimulated spleen cell cultures (Fig. 3, b and c). Examination of the interaction between BQR and cytidine at various concentrations of each agent showed synergistic effects between the drugs when the concentration of cytidine was 0.1 mM and that of BQR was >0.1 μg/ml (Fig. 4b). The cpm for untreated Con A-stimulated cultures was 154,396±13,147. Addition of cytidine at 0.1 mM had very little effect on thymidine incorporation (cpm = 141,513±4403). However, the presence of cytidine substantially reduced the thymidine incorporation observed with BQR alone (0.5 mg/ml) from cpm 52,630±1490 to 3151±371, approaching the baseline level of unstimulated cultures. Although a positive interaction between BQR and cytidine was observed consistently, the addition of cytidine did not

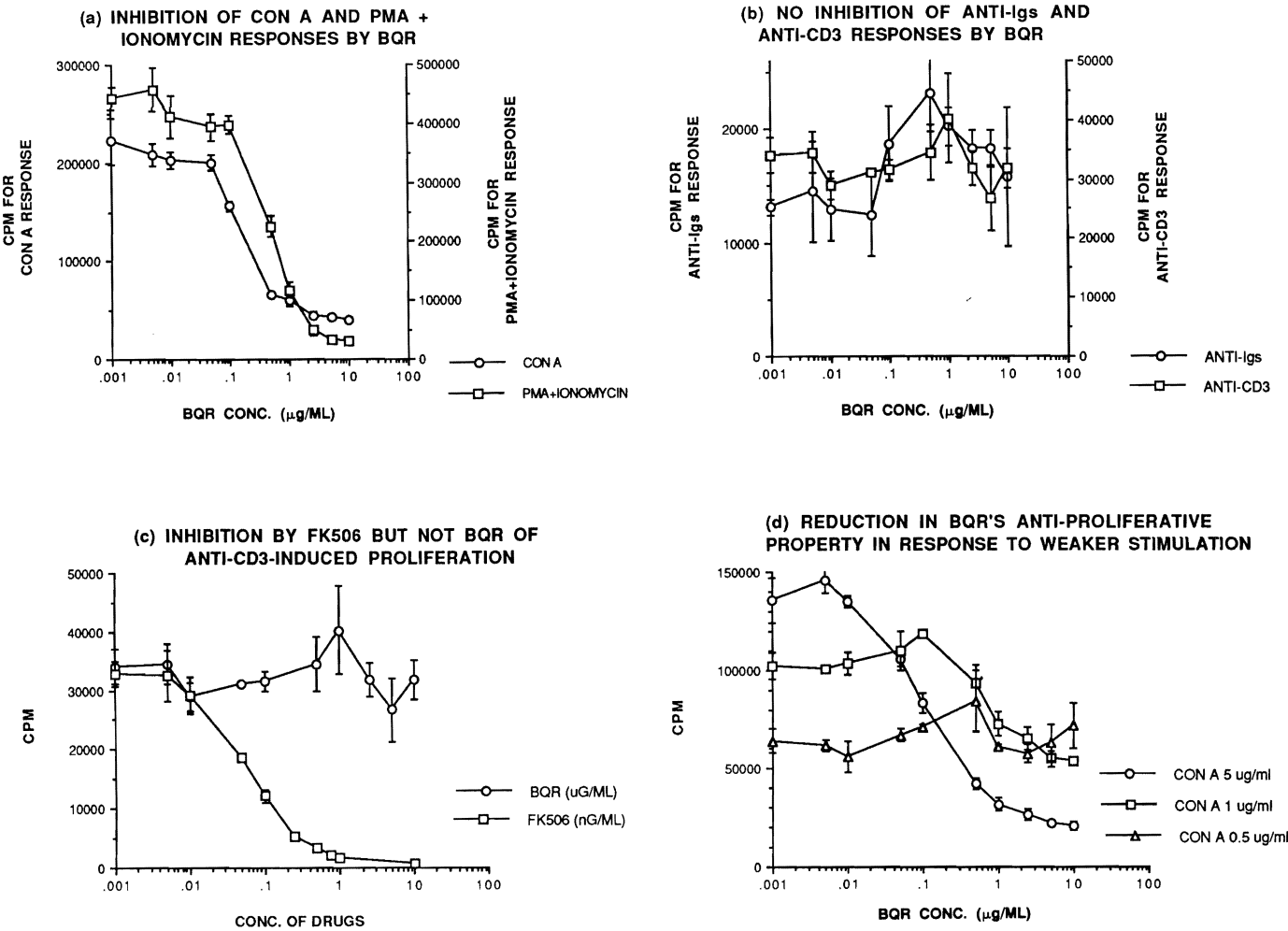


FIGURE 2. Differential effects of BQR on DNA synthesis induced by strong stimuli—optimal concentrations of Con A or PMA + ionomycin (a), and weak stimuli—optimal concentrations of anti-CD3 and anti-Igs antibodies (b). Anti-CD3-induced DNA synthesis, though resistant to BQR, is highly susceptible to the anti-T cell agent FK506 (c). The magnitude of BQR's inhibitory effect is dependent on the strength of stimulus, as BQR is very effective in inhibiting thymidine incorporation induced by 5 μg/ml Con A but is less effective at 1 μg/ml Con A and ineffective at 0.5 μg/ml Con A (d). The cpm of unstimulated cultures in (a) was 1985±372. The cpm for cells cultured with Con A or PMA + ionomycin alone in (a) were 220,498±7209 and 414,394±43,946, respectively. The cpm for unstimulated cultures in (b) was 2607±814. The cpm for cells cultured with anti-CD3 or anti-Igs alone in (b) were 34,023±2990 and 14,475±1777, respectively. The cpm for unstimulated cultures in (c) was 2753±259. The cpm for cultures of cells with Con A at 5, 1, and 0.5 μg/ml in (d) were 138,097±4352, 92,903±15,159, and 55,410±11,048, respectively. The cpm for unstimulated cultures in (d) was 3667±1022. Figures shown are representative results from 3 separate experiments.

lower the minimum inhibitory concentration of BQR. Indeed, the occurrence of this positive interaction was dependent on the concentration of BQR.

Due to its augmented anti-DNA synthesis activity against Con A–stimulated lymphocytes in the presence of cytidine, a combination of BQR with cytidine (0.1 mM) was used to test otherwise BQR-resistant anti-CD3 and anti-Igs responses. As shown in Figure 5, a and b, the combination of BQR with cytidine (0.1 mM) suppressed these responses (Fig. 5, a and b). As with suppression of Con A responses, the inhibition of anti-CD3 and anti-Igs responses was BQR-concentration-dependent.

The effects of BQR and cytidine on cytokine production. To ascertain whether the combination of BQR and cytidine could inhibit lymphocyte responses other than DNA synthesis, levels of two T cell–derived cytokines (IL-2 and IL-4) were determined 20 hr after Con A stimulation. Although slight reductions in IL-2 and IL-4 levels were observed in cultures with BQR or cytidine alone, the combination of BQR and cytidine resulted in strong and significant inhibition ($P < 0.05$) of IL-2 and IL-4 production (Fig. 6, a and b) when compared with BQR alone or cytidine alone. The degree of IL-2 inhibition achieved with the drug combination (mean: 63.85%) was significantly higher than the added effects of either BQR (mean: 21.95%) or cytidine (mean: 16.7%) alone. Similarly, the degree of inhibition of IL-4 production achieved with drug combination (mean: 86.4%) was higher than the added effects of either BQR (mean: 13.2%) or cytidine (mean: 7.3%) alone, indicating a synergistic inhibitory effect on cytokine production.

The effects of BQR and cytidine on cytokine gene expression. The expression of mRNA for IL-2, IL-4, and IFN-γ in Con A–stimulated lymphocytes treated with either BQR or BQR and

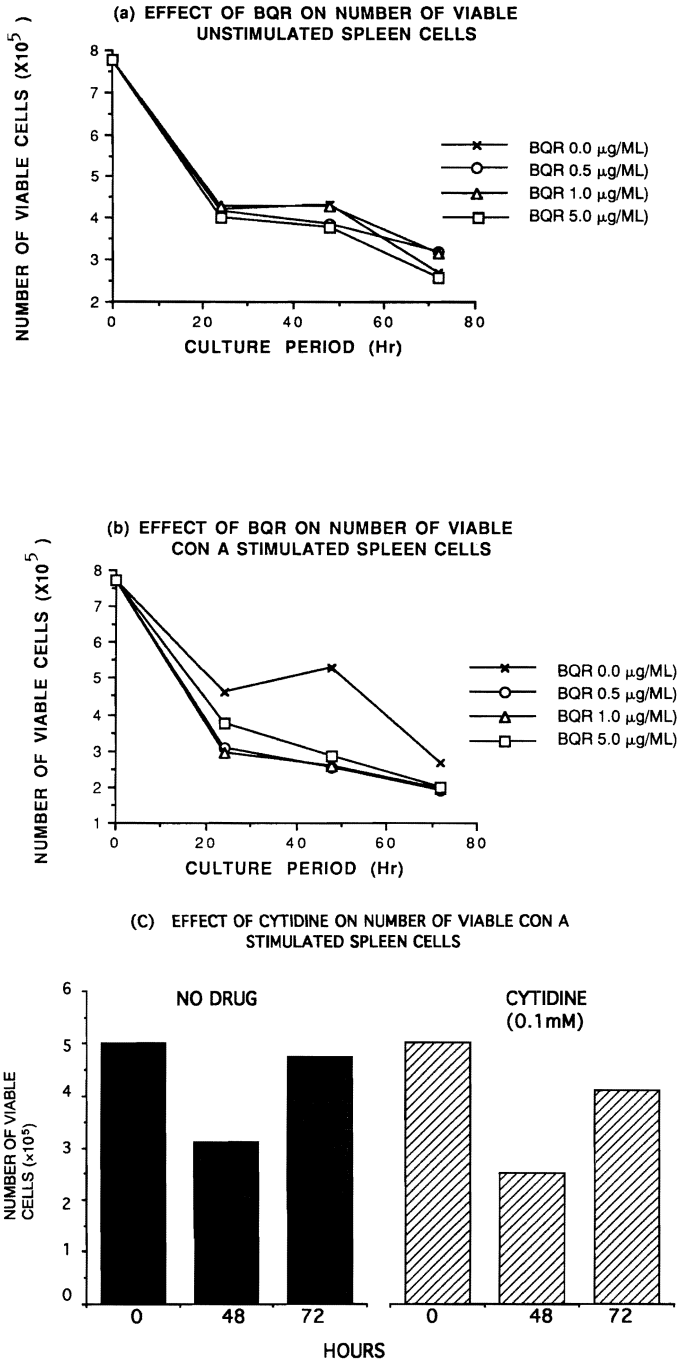


FIGURE 3. Total number of viable cells following culture of unstimulated cells with BQR-(a) or Con A (5 μg/ml)-stimulated cells with BQR (b), or Con A-stimulated cells with cytidine (0.1 mM) (c) for a period of 72 hr. Viability of cells was determined by trypan blue exclusion. At least 200 cells were counted for each sample.

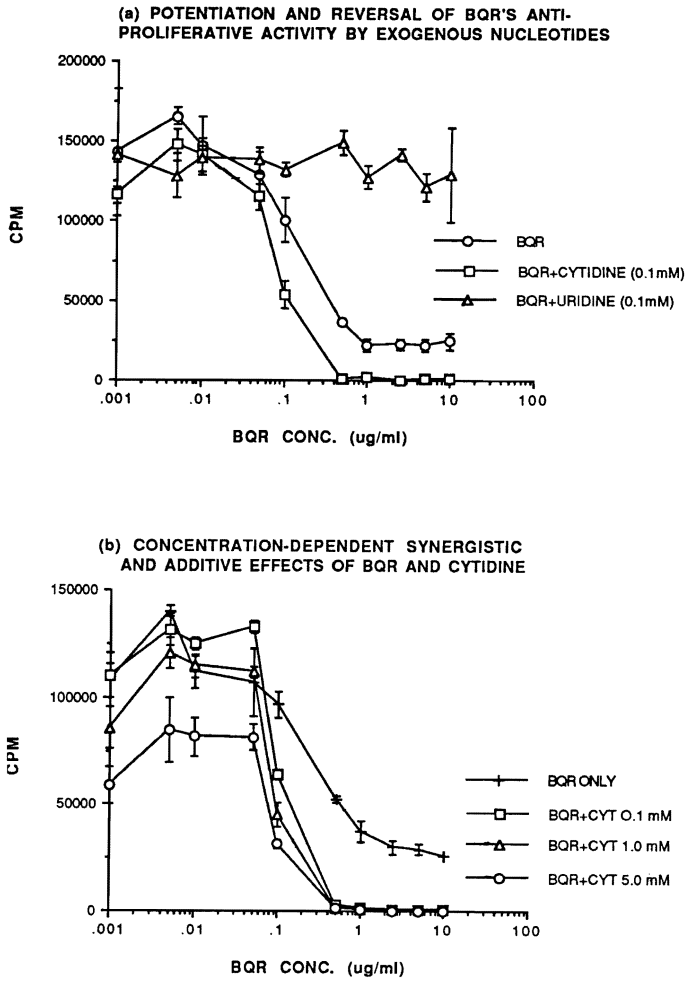
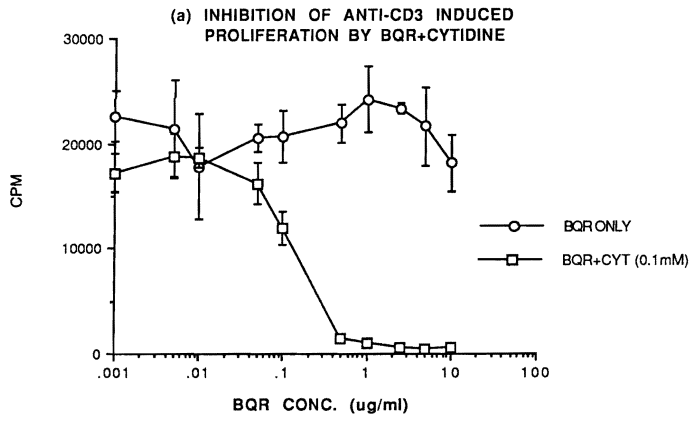


FIGURE 4. Differential effects of exogenous uridine and cytidine (CYT) in Con A-stimulated cultures. The cpm for unstimulated spleen cell cultures and Con A-stimulated cultures were 5044±1353 and 134,124±13,683, respectively. Uridine at 0.1 mM totally reversed BQR's inhibitory effect, but cytidine at 0.1 mM further potentiated BQR's inhibitory action (a). In (b), a synergistic effect between cytidine (0.1 mM) and BQR (>0.1 μg/ml) was observed, while at higher concentrations of cytidine, an additive effect was obtained. The cpm of cultures of Con A-stimulated cells, either alone or in the presence of uridine or cytidine, were 165,859±5371, 134,123±13,683, and 135,057±16,069, respectively. Figures shown are representative results from 3 separate experiments.

cytidine was investigated using the reverse transcriptase PCR. As shown in Figure 7 (lanes 5,6), BQR alone did not suppress message for any of the cytokines investigated. Although BQR plus cytidine demonstrated strong inhibition of cytokine production (Fig. 6), a strong signal of cytokine mRNA expression was still detectable using this drug combination (lanes 7,8) as compared with either BQR or cytidine alone. This suggests that very early events in lymphocyte activation are not susceptible to BQR or BQR plus cytidine, and that the inhibition of cytokine production we observed depends on the antiproliferative activity of the drug combination.

DISCUSSION

In this study we have confirmed the antiproliferative activity of BQR based on its inhibitory action on lymphocyte responses stimulated by optimal concentrations of the T cell mitogen, Con A. These observations are consistent with the documented capacity of BQR to inhibit in vitro tumour cell growth (*1*, *2*) and with a recent report of its suppressive effect on allogeneic mixed lymphocyte reactions (*10*). We found that, as with Con A responses, BQR inhibited strong mitogenic responses elicited by PMA and ionomycin, which together stimulate lymphocytes via various intracellular transduction pathways involving protein kinase C (PKC) activation and the upregulation of intracellular Ca^{++} levels.



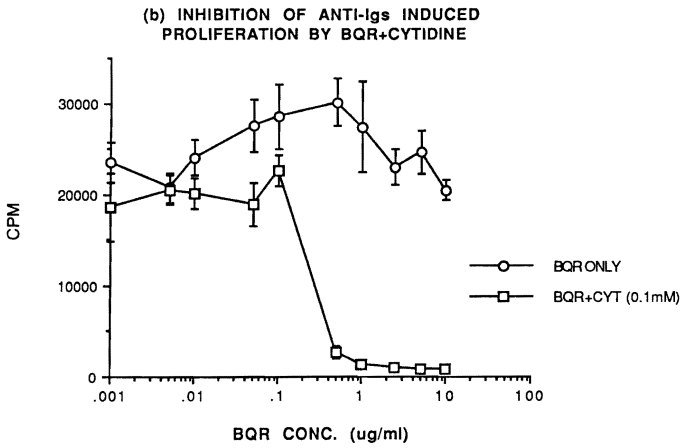
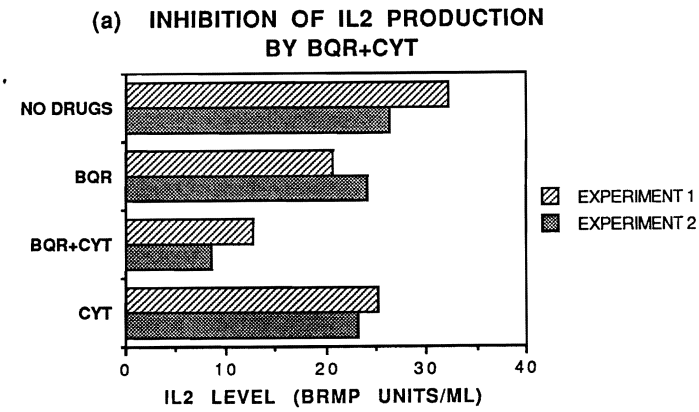


FIGURE 5. Inhibition of anti-CD3–induced (a) or anti-Igs–induced (b) lymphocyte DNA synthesis by BQR + cytidine, but not by BQR alone. The mean cpm of cultures with anti-CD3 alone or anti-Igs alone were 19,997±8141 and 24,911±2135, respectively, and with anti-CD3 + cytidine or anti-Igs + cytidine they were 16,603±807 and 17,323± 1413, respectively. Figures shown are representative results from 3 separate experiments.



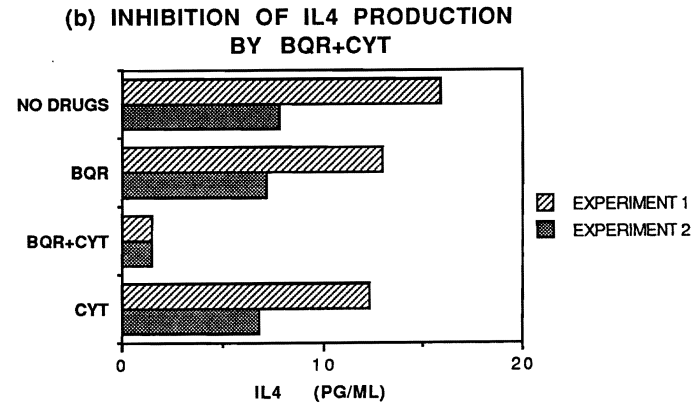


FIGURE 6. Results from 2 separate experiments on IL-2 (a) and IL-4 (b) production following 20 hr Con A stimulation in the presence of BQR, cytidine, or both drugs. In both experiments, slight inhibition of IL-2 and IL-4 production was obtained with either BQR (5 μg/ml) alone or cytidine (0.1 mM) alone, while strong inhibition was achieved by the combination of BQR and cytidine.

Compared with Con A responses, weaker T cell (anti-CD3)–specific and B cell (anti-Igs)–specific responses were insensitive to BQR, as judged by quantitation of DNA synthesis. This differential sensitivity to the drug may simply reflect (in the case of weaker mitogenic responses) the availability of sufficient pyrimidine nucleotide reserve during the culture period to support nucleic acid synthesis. Stronger stimuli, however, evoke proliferative responses that require de novo pyrimidine biosynthesis, which is blockaded (via inhibition of dihydroorotate dehydrogenase activity) by BQR. Failure of BQR (10 μg/ml) to inhibit Con A– or PMA + ionomycin–induced responses completely (Fig. 2a) may be attributed to use of the pyrimidine reserve. We have been unable to locate any previously published report demonstrating a similar phenomenon with regard to other pyrimidine inhibitors.

We had anticipated that supplementation of the lymphocyte cultures with either uridine or cytidine would prevent the anti-DNA synthesis activity of BQR. While the inhibitory effect of BQR was reversed by uridine as expected, the unexpected capacity of cytidine to potentiate the action of BQR leads us to speculate that BQR may inhibit the activity of cytidine deaminase, the enzyme that mediates the salvage pathway allowing conversion of cytidine to uridine and biosynthesis of both CTP and UTP (Fig. 1). This argument is consistent with our observation that addition of exogenous uridine to BQR-treated cultures must replenish the necessary UTP and CTP—in the latter instance through the action of CTP synthetase. Although both uridine and cytidine are able to restore cell growth in BQR-treated WiDR human adenocarcinoma cells (*5*), cytidine alone is not able to prevent growth inhibition induced by BQR in cytidine deaminase-free L1210 mouse leukemia cells (*3*). If, as we suggest, cytidine deaminase is blockaded by BQR, the supplement of cytidine can only form CTP instead of both nucleotides. Such an imbalance in the UTP/CTP ratio would predispose to further adverse effects on cell function, including inhibition of aspartate transcarbamylase, an enzyme that regulates an earlier step in the pyrimidine biosynthesis pathway (*22, 23*). This is supported by the observation that in WiDR cells, the lowest UTP/CTP ratio following culture with BQR corresponds to the largest growth inhibition (*5*). Feedback suppression of uridine kinase activity by the formation of CTP from exogenous cytidine may offer an alternative explanation of cytidine's "enhancing effect." We observed, however, that the augmentory effect of cytidine occurred only with inhibitory BQR concentrations (0.1 μg/ml or

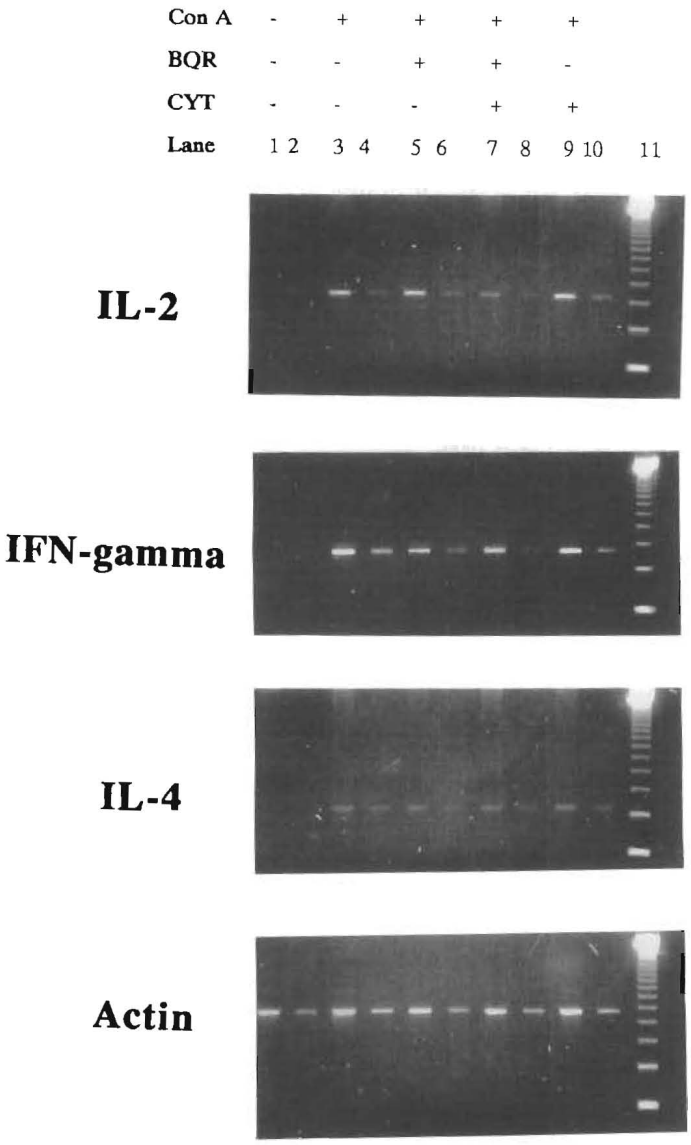


FIGURE 7. The expression of cytokine mRNAs in cultured cells was measured by semiquantitative PCR. Aliquots of amplification products from 5 ng RNA (lanes 1,3,5, and 7) and 0.5 ng DNA (Lanes 2,4,6, and 8) were separated on 2% agarose gel. (Lanes 1 and 2) unstimulated mononuclear cells, (lanes 3 and 4) 20-hr Con A–stimulated cells, (lanes 5 and 6) Con A–stimulated cells in the presence of 5 μg/ml BQR (lanes 7 and 8) Con A–stimulated cells in the presence of 5 μg/ml BQR + 0.1 mM cytidine, (lanes 9 and 10) Con A–stimulated cells with 0.1 mM cytidine, (lane 11) molecular weight ladder. Figures shown are representative results from 3 separate experiments.

above). This suggests that certain enzymatic activity must first be abrogated before the potentiating effect of cytidine can be manifest. It adds credence to the view that the interaction between BQR and cytidine may be due, at least in part, to inhibition of cytidine deaminase and consequent accumulation of a relative excess of CTP. Complete inhibition of anti-CD3– or anti-Igs–induced proliferation may also be ascribed to gross imbalance in the UTP:CTP ratio as a result of blockade of pyrimidine biosynthesis and supplementation with exogenous cytidine. The influence of BQR on cytidine deaminase activity is now under investigation in our laboratory.

Earlier events in the lymphocyte cell cycle—e.g., cytokine gene expression—were not inhibited by BQR plus cytidine, despite strong inhibition of DNA synthesis and cytokine production. This finding indicates that, unlike cyclosporine or FK506, BQR-plus-cytidine does not effect early lymphocyte activation events. Its inhibitory effect on cytokine production would, therefore, appear to be based on the anti-DNA synthesis property of the drug combination, resulting in availability of fewer activated cells for cytokine production (Fig. 3). The antimetabolic effect is probably not restricted to cytokine production. It is likely, however, that the antimetabolic effect of BQR plus cytidine is selective toward metabolically active cells, in particular lymphocytes in sites of inflammatory/immune reactions. In vivo therapeutic administration of cytidine has been performed in cases of congenital pyrimidine deficiency, such as orotic aciduria (*24*). It remains to be determined whether a combination of BQR and cytidine may be an effective and safe approach to immunosuppressive therapy.

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