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Comparative In Vitro Studies on the Immunosuppressive Effects of Purine and Pyrimidine Synthesis Inhibitors

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OVER the past few decades the implementation of new immunosuppressive regimens has remarkably improved the success of clinical transplantation. Immunosuppressive drugs that can inhibit both cell-mediated and humoral immune responses have an important role in controlling acute cellular rejection and chronic rejection. In this report we compare the inhibitory effects of four antiproliferative drugs: two purine synthesis inhibitors, mycophenolic acid (MPA)[1] and mizorbine (MZR),[2] and two pyrimidine synthesis inhibitors, brequinar sodium (BQR)[3] and N-phosphonacetyl-L-aspartic acid (PALA).[4] A variety of in vitro culture systems have been used to determine the potency of these drugs including T-cell activation via calcium (Ca)-dependent (A23187) and Ca-independent (Interleukin-2 [IL-2] and Phorbol myristic acetate [PMA]) pathways. Transformed T and non-T cells were also used to test the effect of these antiproliferative drugs.

MATERIALS AND METHODS
Cells

Peripheral blood lymphocytes were isolated by Ficoll-Hypaque gradient centrifugation of heparinized blood from healthy donors. Cells were resuspended in RPMI 1640 tissue culture medium (TCM) supplemented with 25 nmol/L Hepes buffer and 100 U/mL gentamicin and 5% normal human serum.

Reagents

FK 506 and cyclosporin A (CyA) (Sandoz, Basel, Switzerland) were dissolved in methanol (1 mg/mL). The MPA (Sigma Chemical Co, St Louis, Mo), and MZR were dissolved in ethanol and water, respectively, before use. The BQR and PALA were suspended in distilled water.

Proliferative Responses of Lymphocytes

Mononuclear cells were cultured in TCM at 10^5 cells/well in the presence of A23187 (1 μg/mL) or PMA (0.1 μg/mL) in a volume of 200 μL for 3 days. Interleukin-2-induced proliferation was measured by culturing alloreactive T cells (2×10^4 cells/well) in the presence of 30 IU of IL-2 for 3 days.

Mixed lymphocyte reaction (MLR assays) were established with equal numbers of responder cells (5×10^4 cells/well) and irradiated stimulator cells in TCM and incubated for 6 days. For the secondary proliferative response (PLT), alloreactive T cells (2×10^4 cells/well) were incubated with irradiated stimulator cells for 3 days.

Proliferative Responses of Various Cell Lines

T-lymphoma cell lines (DND41, Peer), Epstein-Barr (EB) virus-transformed lymphoblastoid cell lines, a B-lymphoma cell line (RPMI-1788), an erythroleukemia cell line (K562), and a promyelocytic cell line (HL-60) were maintained in 10% TCM with fetal calf serum. Proliferation was measured after 72 hours of incubation by ^3H-thymidine incorporation.

Drug Inhibition Assays

The inhibitory effects of various drugs alone or in combination were measured at different concentrations. The percent inhibition was calculated as follows: % inhibition = (1 − {cpm with drug/cpm without}) × 100.

RESULTS

Table 1 summarizes the drug dose required to obtain 50% inhibition (IC50) for five immunosuppressive drugs. Both BQR and MPA showed similar IC50 (80 to 100 nmol/L) in both Ca-dependent and Ca-independent T-cell proliferation. The IC50 for MZR was about 10^2-fold higher than that observed for MPA or BQR. FK 506 and CyA exhibited significant inhibition of Ca-ionophore stimulation (IC50 0.18 nmol/L and 23 nmol/L, respectively) but had no effect on IL-2 and PMA stimulation. These data suggest that BQR and MPA have similar in vitro potency for suppressing both Ca-dependent and Ca-independent T-cell activation pathways. The PALA, which was also tested in

Table 1. Inhibitory Effects of Immunosuppressive Drugs on T-Cell Proliferation

	IC 50 nmol/L*				
	FK 506	CyA	BQR	MPA	MZR
Mitogen					
A23187	0.18	23	88	81	NT
IL-2	No Inhibition	75	119	7,637	
PMA	No Inhibition	100	81	14,861	
Allostimulation					
Primary (MLR)	0.1	10	50	103	14,706
Secondary (PLT)	0.17	29	18	163	28,761

*Concentration of drugs required to obtain 50% Inhibition IC50.

various T-cell activation assays, had minimal inhibitory effect even at a high dose of 10 μg/mL (data not shown).

All five immunosuppressive drugs inhibited the primary MLR and the secondary PLT proliferation of alloreactive T cells. In these assays BQR was two- to fourfold more potent than MPA whereas MZR and PALA were about 10^3- and 10^4-fold, respectively, less active than BQR (Table 1). Combinations of low doses of BQR, MPA, or MZR with FK 506 showed, at best, an additive effect in the suppression of PLT responses (data not shown).

The MPA uniformly inhibited all cell lines tested at drug concentrations of 90 to 365 nmol/L, whereas BQR appeared to have a more restricted pattern of inhibition. Proliferation of EB virus B-cell lines and HL-60 cells were inhibited by BQR at a concentration of 250 nmol/L, whereas T-lymphoma cell lines were resistant even at high doses of BQR (100 μg/mL). All cell lines tested were resistant to the inhibitory effects of PALA.

DISCUSSION

This study demonstrates that BQR, MPA, and MZR inhibit the proliferative responses of normal T cells to mitogenic and allogeneic stimulation, whereas PALA has a minimal effect. The proliferative responses of T cells induced by T-cell receptor, IL-2, and protein kinase C are equally affected by BQR, MPA, and MZR, since these drugs block DNA synthesis.[1-4] In contrast, FK 506 and CyA are more efficient in blocking signal transduction via T-cell receptors by inhibiting the transcription of early activation genes.[5,7]

T and B cells lack the salvage pathway found in other types of cells and depend solely on the de novo pathway of purine and pyrimidine synthesis.[8,9] Although both BQR and PALA efficiently inhibit the de novo pyrimidine synthesis, T cells are 10^3 to 10^4 times more sensitive to the immunosuppressive effects of BQR than PALA. The BQR, an anticancer drug, exerts its antiproliferative activity by noncompetitively inhibiting the activity of dehydroorotate dehydrogenase, the fourth enzyme in the de novo pyrimidine biosynthetic pathway.[3,9] The PALA inhibits DNA synthesis by blocking the second enzyme aspartate transcarbamylase in the de novo pyrimidine pathway.[4] Growth inhibition of PALA can be easily reversed by carbamyl-L-asparate, whereas BQR inhibitory activity is not affected by the addition of dihydroorotic acid.[10,11] The BQR inhibits the growth of a broad spectrum of murine and human solid tumors, whereas PALA is effective against a limited number of solid tumors.[12] Consistent with these findings are our observations that BQR blocks the proliferative responses of many lymphoid and nonlymphoid cell lines, while PALA, even at 100 μg/mL, had only a minimal inhibitory effect on nonlymphoid cell lines.

Other antiproliferative drugs such as MPA and MZR have also been shown to have both antitumor and immunosuppressive activities.[13,14] The MPA, an antibiotic, inhibits reversibly and noncompetitively only inosine mono-

phosphate dehydrogenase (IMPDH), an enzyme in the de novo purine biosynthetic pathway. Mizorbine, which is an imidazole nucleoside, also inhibits IMPDH but is 10^2- to 10^3-fold less active in blocking T-cell proliferation than MPA or BQR, respectively.

All anti-DNA synthesis drugs tested act at a late stage of the cell activation pathway by blocking the movement of cells from G1 to S, whereas FK 506 and CyA act at an early stage of T-cell activation by inhibiting cell division cycle at G0/G1 interface. Since T cells and B cells depend on de novo nucleotide synthesis, both humoral responses mediated primarily by B cells and cellular immune responses governed by T cells could be significantly impaired in the presence of purine and pyrimidine inhibitors.[15,16] The MPA and its acid derivative RS 61443 have shown promising results in preventing acute cellular rejection in an experimental canine renal allograft model and in suppressing chronic rejection associated with vasculitis in a rat heart allograft model.[17] Also BQR is effective in suppressing the development of contact sensitivity and adjuvant arthritis in rodent models and preventing kidney, heart, and liver allograft rejection in rats.[16]

Although no synergism between BQR, MPA, or MZR with FK 506 was observed in inhibition of allogeneic T cell proliferation, an additive effect was seen.[18] Thus, the antiproliferative drugs may offer an alternative regimen when used in combination with other immunosuppressive drugs in treatment of acute and chronic cellular rejection. However, the most important benefit from the introduction of the experimental antimetabolite drugs or the established anti-DNA synthesis drug cyclophosphamide, is the ability to break down the antibody barrier to xenotransplantation.[19] Murase et al[19] have recently shown that the combination of FK 506 with either one of the anti-DNA synthesis inhibitors significantly prolongs the survival of hamster to rat heart or liver xenotransplantation by suppressing the anti-hamster antibody response.

ACKNOWLEDGMENT

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