IMMUNOSUPPRESSIVE EFFECT OF COMBINATION SCHEDULES OF BREQUINAR WITH LEFLUNOMIDE OR TACROLIMUS ON RAT CARDIAC ALLOTRANSPLANTATION

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Drug toxicity is one of the major problems in clinical immunosuppression. Combining two immunosuppressants in low or ineffective doses is an attractive strategy if it helps to reduce drug-related toxicity. We examined the immunosuppressive efficacy of brequinar (BQR) in combination with leflunomide (Lef) or tacrolimus (FK) in a heterotopic rat cardiac allotransplantation model. Abdominal heterotopic heart grafts (DA × LEW) were immunosuppressed from the time of transplantation and continued until the ninth posttransplant day (POD) in experiments examining prophylaxis of rejection treatment (PRT). In a separate series of experiments designed to test rescue treatment (RT), immunosuppression was begun on POD 4 and continued for 10 days; transplanted rats were sacrificed the following day intentionally. Cardiac rejection was monitored by palpation and documented by light microscopy. Immunosuppressive drugs (BQR 3 mg/kg and 12 mg/kg; BQR 3 mg/kg + Lef 5 mg/kg; BQR 3 mg/kg + FK 0.5 mg/kg) were given orally by gavage; thrice weekly according to the monotherapy or dual-therapy dosing protocol. Median survival time of the cardiac graft for controls (no treatment) was 5 days. BQR monotherapy 3 mg/kg (low dose) improved graft survival ($P = 0.003$); graft histology showed moderate acute rejection. BQR monotherapy 12 mg/kg (therapeutic dose) application in the PRT or RT treatment arms of the study design resulted in aortic-graft ruptures and clinical toxicity in each treatment arm due to overimmunosuppression; normal graft morphology was maintained. Successful rescue of rejecting grafts was histologically documented. Combining BQR with Lef or FK in the PRT protocol showed prolonged graft survival in both drug combination groups (median survival time, 14 days; $P = 0.009$ and 0.014, respectively). Using an identical combination protocol for RT, all grafts achieved a 14-day graft survival; cardiac histology showed reversible moderate acute rejection. BQR given in the presence of Lef or FK not only prevented acute rejection but intercepted it so long as it was administered; grafts were rejected within 4 days of stopping immunosuppression in the PRT study. These combinations using low or subtherapeutic doses may be important for controlling transplant rejection and rescuing ongoing graft rejection. The need for continuing treatment in this strongly allogeneic model is highlighted.

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Successful solid organs transplantation depends primarily on graft viability at the time of transplantation and the ability of immunosuppressive agents to prevent the postoperative cellular and humoral immune response. Cyclosporin (CsA) and, more recently, tacrolimus (FK) have been successfully established as antirejection drugs for vascularized organ transplantation, resulting in dramatic improvement in graft survival. However, prolonged administration of these drugs is associated with the appearance of significant toxic side effects. The need of less toxic and more powerful and specific drugs has stimulated an active search for new immunosuppressive agents that could either replace or be used in combination with CsA or FK.[1]

Brequinar sodium (BQR) is a novel immunosuppressive agent that blocks de novo pyrimidine synthesis via a noncompetitive inhibition of the enzyme dihydroorotate dehydrogenase.[2,3] This drug was originally developed as an antimetabolite for the clinical treatment of patients with cancer, psoriasis, and rheumatoid arthritis.[4] BQR was later found to inhibit heart, liver, and kidney allograft rejection in the rat either when used alone[5] or in combination with CsA.[6]

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Leflunomide (Lef) (HWA486), an isoxazole derivative, is a prodrug that is transformed into the immunologically active primary metabolite A77 1726 and was originally developed as an antirheumatic drug.[7] In vitro, it is similar to rapamycin, but in contrast to CsA and FK, Lef suppresses human T-cell proliferation primarily by inhibiting T-cell responsiveness to IL-2. In vivo studies have shown that Lef prevents acute allograft rejection of skin, kidney, and heart transplants in rats.[8–10]

Because the immunosuppressive activity of BQR is distinctively different from those of FK and Lef, the combination of these drugs theoretically predicts good results on graft survival with correspondingly less toxicity when they are used at lower doses. Previous experience of the combination of these three drugs from this laboratory was used to design the present study.[11] The present study was designed to examine the immunosuppressive efficacy of BQR in combination therapy with Lef or FK, in subtherapeutic doses, when applied in the prophylaxis of acute rejection (PRT) or in the interception and reversal of established acute rejection—rescue therapy (RT).

MATERIALS AND METHODS

Animals

Adult male DA ($RT1^a$) and Lewis-$RT1^1$ (LEW) rats weighing 200–250 g were used as donors and recipients, respectively. The subjects were commercially obtained from Charles River (U.K.) and cared for humanely during the course of the study according to prevailing Home Office Guidelines in the U.K. Rats were allowed unrestricted access to food and water and were housed in filter-top barrier housing cages in light- and temperature-controlled quarters. The procedures performed in this study were licensed by the U.K. Home Office governing body. All surgical procedures were carried out aseptically and each animal's postoperative condition was monitored a minimum of twice daily.

Surgical Model

Syngeneic exchanges were performed using DA and LEW rats. DA rats were always used as donors for allogeneic transplants to LEW recipients. This is a strong MHC mismatch strain combination that allows a better anticipation of the immunosuppressive efficacy of the drugs used. Heterotopic cardiac transplantation was carried out by a modified technique of Ono and Lindsey[12] under enflurane anesthesia. Our operative technique involved keeping a donor-specific transfusion (DST) effect to a minimum and substantially reducing the quantity of bronchus-associated lymphoid tissue (BALT) with the heart graft, after a rapid exsanguination and subsequent perfusion of the donor heart. Using this system, we were able to exclude the possible synergistic role of BQR, Lef, or FK with the DST effect in order to better understand the efficacy of these agents.

Donor cardiectomy was performed under terminal anesthesia after thoroughly venting the heart by severing the thoracic aorta soon after thoracotomy and clamping the thoracic suprahepatic inferior vena cava (IVC) in an effort to prevent inflow to the right heart. The heart was flushed immediately through the IVC with 5–6 ml of heparinized lactated Ringer's solution, which achieved an effective washout of blood from the organ. Using this approach, we were able to standardize the procurement procedure so as to last only 7 min from the time of skin incision. After a minimal cold ischemic time (less than 10 min), during which period the recipient's abdomen was surgically prepared and its aorta and IVC clamped together with a Lee clamp, the heart was implanted using a microsurgical technique with continuous 8-0/9-0 prolene or nylon microsutures. The recipient procedure was standardized so that revascularization of the graft was achieved within 12–15 min of clamping. In total, the graft was revascularized within a mean period of 24 min (range, 22–30 min) commencing from the donor skin incision. Transplanted hearts that did not beat strongly immediately after reperfusion and for the 2 days following transplantation were excluded from the study. The technical success in this study was 98%.

Postoperative Assessment and Endpoints

Postoperatively, the animals were kept in thermally regulated recovery cages until they were able to regulate their body temperature, at which time they were transferred to holding cages and permitted food and water ad libitum. Body weight and clinical behavior were monitored each morning; transplanted grafts function was assessed by palpation for the ventricular impulse and graded daily for rejection using a previously validated heart beat scoring system ranging from 4 (normal) to 0 (rejection).[13] These assessments were made in reference to a cohort of syngeneic heart transplants observed over an extended period. Hearts possessing a barely palpable impulse or complete cessation of ventricular motion were considered rejected. Rejection was confirmed by laparotomy and cardiac biopsy. Blood samples were obtained terminally for hematological parameters and liver and kidney function tests. Native liver and renal tissues were also obtained for histological assessment of drug toxicity. Recorded graft survival was arbitrarily designated as the total number of days until day of rejection minus one. Recipients with rejected grafts or adverse clinical signs were euthanized and tissues analyzed histologically. Results are expressed as median survival days and analyzed using nonparametric tests (Mann-Whitney test). A two-tail P value < 0.05 was considered significant.

Pharmacological Agents and Study Design

Brequinar sodium (DUP-785) (Dupont Pharmaceuticals, Wilmington, DE) was dissolved in distilled water and Tacrolimus (FK506) (Fujisawa Pharmaceuticals, Munich, Ger-

Table 1. Graft Survival, Adverse Events, and Graft Histology in a Model of DA × LEW Cardiac Allografting[a]

Groups	Treatment[b] (dose in mg/kg)	Survival (median)	P[c]	Comments	Histology
Prophylaxis of rejection therapy					
1 (n = 6)	Control (none)	5			Severe AR
2 (n = 8)	BQR 3	11	0.003		Moderate AR
3 (n = 5)	BQR 12	6 (5–12)	0.075	3/5 aortic graft rupture 1/5 toxicity, 1/5 > 12 days	Mild AR 4/5
4 (n = 5)	BQR 3 + Lef 5	14 (13–15)	0.009		Severe AR 5/5
5 (n = 4)	BQR 3 + FK 0.5	14 (14–18)	0.014		Severe AR 4/4
Rescue therapy of established rejection					
6 (n = 7)	BQR 12	14	0.0118	3/7 aortic graft rupture	Mild AR
7 (n = 5)	BQR 3 + Lef 5	14	0.009		Moderate AR
8 (n = 5)	BQR 3 + FK 0.5	14	0.009		Moderate AR

[a]*Employing brequinar in combination with leflunomide or tacrolimus.*
[b]*All drugs given orally by gavage; BQR given thrice weekly as mono- dual therapy. The above doses are in mg/kg.*
[c]*All comparisons made to group 1.*

many) was dissolved in normal saline. Leflunomide (HWA 486) (a gift from Hoechst AG, Wiesbaden, Germany) was suspended by agitation in 1% carboxymethyl cellulose (CMC). All drugs were given orally by gavage under light halothane anesthesia.

Previous database from this laboratory had treatment course for 10 days. We stuck to the same schedule treatment to obtain results comparable to our previous studies. Because BQR has long half-life, it was recommended to be given thrice weekly, as were FK and Lef. The decision was made to exclude any additional immunosuppressive effect from FK or Lef, in case they were given daily.

In this study, BQR was administered alone (monotherapy schedule) in low (3 mg/kg) and effective (12 mg/kg) doses thrice weekly over a total period of 10 days. Treatment began on the day of grafting (day 0) and was withdrawn on posttransplant day 9. This schedule delivered four doses in 10 days. The latter schedule was also observed when BQR was applied in combination schedule protocols, i.e., BQR + FK or BQR + Lef. For the latter studies, a dose of 3 mg/kg of BQR was used in conjunction with FK (0.5 mg/kg) or Lef (5 mg/kg). This combination treatment strategy employing four treatments over a 10-day period postgrafting was studied for the PRT experiments. Grafts were monitored for acute rejection throughout the 10-day treatment period and after the withdrawal of immunosuppression. An identical combination treatment protocol was applied to RT studies. However, in these experiments, pharmacological treatment was given from the fourth posttransplant day onward for a total of 10 days thereafter, with a view to reversing ongoing rejection. In these experiments, recipients were intentionally sacrificed on posttransplant day 15.

RESULTS

Prophylaxis of Rejection Therapy

Median allograft survival for untreated DA × LEW controls was 5 days (range, 4–5), demonstrating histological features of severe acute cellular rejection (Table 1). The application of BQR in a dose of 3 mg/kg for the first 10 days posttransplantation improved DA allograft survival in LEW recipients to 11 days (range, 10–13 days; $P = 0.003$); cardiac histology showed moderate acute cellular rejection. Under BQR 12 mg/kg monotherapy, acute rejection was averted so long as immunosuppressive treatment continued. However, a high incidence of spontaneous rupture of the aortic anastomosis (3/5 grafts) was noticed 4–5 days after treatment commenced. Histology showed bacterial infection with complete destruction of the graft aortic wall. Although the majority of recipients receiving 12 mg/kg of BQR demonstrated clinical and biochemical signs of toxicity, one animal lost more than 20% of its pretransplant body weight and was euthanized on POD 6. Only one recipient remained alive for more than 12 days with a grade 4+ graft heart beat. All the cardiac grafts derived from recipients that received 12 mg/kg of BQR showed mild acute cellular rejection.

Graft survival was significantly prolonged under BQR 3 mg/kg plus Lef 5 mg/kg or FK 0.5 mg/kg combination treatment. Median survival time was identical for both treatment series (14 days) and proved statistically significant for the combination of BQR + Lef ($P = 0.009$) and BQR + FK ($P = 0.014$) compared to untreated controls.

Rescue Therapy of Established Rejection

Since LEW grafted recipients were intentionally sacrificed after the completion of the observation period lasting 10 days following the institution of immunosuppressive treatment on POD 4 onward, all animals in these experiments achieved a median graft survival of 14 days. Since DA × LEW untreated controls reject the transplanted heart by POD 5, it is clear that graft survival is enhanced under BQR 12 mg/kg monotherapy. Interestingly, three cases of exsanguination resulted owing to rupture of the aortic graft anastomotic site; a similar histological picture was obtained here, as in the series of experiments addressing the PRT design.

In the presence of combination treatment using BQR in the presence of Lef or FK, ongoing acute cellular rejection is intercepted, as demonstrated by the fact that all animals improved their heart beat score from POD 4 onward until POD 14, reaching the target time for elective sacrifice. No death or any sign of toxicity has been observed in these groups during the PRT course and all the grafts at the day of the intentional sacrifice (POD 14) presented with almost a normal beating heart (grade +3 or +4) without any clinical sign of rejection. Graft light microscopy showed evidence for reversible moderate acute rejection. These results were classified by pathologist and showed a small degree of infiltration, without any muscular tissue loss. Based on a previous database exploring monotherapy using immunosuppressive agents, such as Lef, FK 506, and CsA, the pathological classification was established as reversible the fact that we were able to rescue ongoing rejection successfully.

DISCUSSION

Two areas of concern in the management of patients posttransplantation continue to confront the transplant physician in the early phase following grafting. The first 3 months after transplantation also provide a window of opportunity for the patient to understand his or her immunosuppression regimen and learn to recognize and report adverse side effects. With the advent of newer immunosuppressive agents entering into phase 2/3 clinical trials and the postmarketing phase 4 pharmacovigilance of novel additions to transplantation pharmacotherapy, such as tacrolimus and mycophenolate mofetil, the posttransplantation outcomes within the first trimester are extremely important. Generally, the frequency of treatable graft acute rejection is high (by virtue of the failure of the primary immunosuppression regimen in the prophylaxis of acute rejection); where rescue therapy using conventional strategies (i.e., high-dose pulse steroids and/or OKT3) fails and threatens graft and patient survival, the patient may be switched to a tacrolimus-based or even mycophenolate mofetil-based immunosuppression protocol. The present study explores the possibility of achieving optimal immunosuppression with reduced doses of different drugs in combination to maintain desirable graft and recipient function without the adverse effects that are currently seen with contemporary agents in transplanted recipients, thus limiting their potential.

Our results obtained from a transplant model in a strongly MHC incompatible suggest that BQR can be given in conjunction with Lef of FK as prophylaxis of rejection therapy to achieve successful outcomes insofar as immunosuppressive efficacy is concerned. The need for continuous administration is highlighted by these studies and underscores the need to carry the preclinical investigation of such compounds through difficult models of transplantation, i.e., across strong MHC barriers. Immunosuppressive outcomes will predictably be superior across more closely matched donor-recipient strains. The occurrence of drug-related toxicity and infection-related sequelae seen in these experiments not only points to the potency of these agent when given in combination, but also bears a striking resemblance to the infection-based clinical adverse events seen with human transplantation. The infection screen applied to studies reported here were restricted to identifying and isolating bacterial-origin infections. Pseudomonas and enterococci were routinely cultured at the site of the graft aortic rupture and the lungs of transplanted animals that expressed adverse clinical signs. The rat model of transplantation offers the choice to explore more directly the propensity for both de novo or transplanted cytomegaloviral (CMV) infection, which is routinely encountered not only in renal, but liver and intestinal, allografts in humans.[14,15] These results collectively underscore the need for a rational paradigm for adjusting the individual doses of two promising drugs employed in combination.

Therefore, combination of BQR with Lef or FK506 using low or subtherapeutic doses may be important for controlling transplant rejection and deserves further attention. Despite the fact that both BQR and Lef act at the same receptor, we did not noted any antagonistic effect. It is more likely that these drugs may work through an additional pathway. That probably explains in part the additive effect of these two drugs we noted in our study. These outcomes may have relevance based on the kind of the immunosuppression schedule we used (administering two drugs in combination thrice weekly).

In summary, BQR 3 mg/kg was found to be subtherapeutic, whereas 12 mg/kg resulted in nonhealing of the graft aorta and spontaneous rupture under treatment in the majority of cases accompanied by clinical and biochemical signs of toxicity in both PRT and RT groups. Combination of BQR with Lef or FK was clinically therapeutic insofar as it was given in both study designs. Grafts were rejected 4 days after stopping immunosuppression in PRT protocol, whereas cardiac graft rejection was intercepted in RT protocol. Clinically normal heart-beating allografts under combination immunosuppression showed mild to moderate acute cellular cardiac and aortic rejection 1–2 days after stopping immunosuppression when employed in a mode of prophylaxis of rejection. Consequently, the combination of BQR with Lef or FK using low or subtherapeutic doses may be important for controlling transplant rejection and rescuing established acute rejection in cardiac allografts, as demonstrated in these studies, which employed a rigorous transplant model across a major histocompatibility strain barrier.

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