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Mizoribine—An inosine monophosphate dehydrogenase inhibitor—acts synergistically with cyclosporine A in prolonging survival of murine islet cell and heart transplants across major histocompatibility barrier☆,☆☆

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ABSTRACT

Introduction: Mizoribine (MZR) is an inosine monophosphate dehydrogenase inhibitor. It has been widely used in Japan in the treatment of autoimmune diseases and is known to inhibit T and B cell proliferation. The aim of this study was to evaluate the efficacy of MZR as an immunosuppressive agent and determine its ability to synergize with a commonly used calcineurin inhibitor Cyclosporine A (CsA) in prolonging survival of murine islet cells and heart transplanted across major histocompatibility barrier.

Methods: Murine allogeneic islet cell transplantation between Balb/c donor mice and C57BL/6 recipient mice and heterotopic heart transplantation was done between C3H/He donor mice and Balb/c recipient mice. Recipients were divided into groups based on immunosuppression: Group 1—No immunosuppression, Group 2—MZR alone (20 mg/kg/day), Group 3—CsA alone (20 mg/kg/day), Group 4—MZR + CsA (20 mg/kg/day). Donor specific IFN-γ, IL-10, IL-2, IL-4 secreting cells were enumerated by ELISpot. Serum cytokine and chemokine concentration was measured by Luminex.

Results: Islet cell allograft recipients treated with CsA and MZR had prolonged islet function compared to other groups [normoglycemia (blood glucose <200 mg/dL) up to 32 ± 4 days, $p<0.05$]. Similarly, heart allograft survival was significantly improved in mice treated with CsA and MZR compared to other groups (50% 30-day survival, $p=0.04$). Donor specific IFN-γ, IL-4, IL-2 secreting cells were significantly decreased in recipients treated with CsA and MZR with marked increase in IL-10 secreting cells ($p<0.05$). There was also an increase in serum IL-10 with decrease in IFN-γ, IL-4, IL-2, MCP-1, and IL-6 in mice treated with CsA and MZR

Conclusion: MZR and CsA when used in combination are potent immunosuppressive agents in murine islet cell and heart transplantation models. These agents lead to a decrease in donor specific IFN-γ with increase in IL-10 secreting cells leading to improved allograft survival and function.

1. Introduction

Mizoribine (MZR) is an immunosuppressive agent that was initially isolated as an antibiotic from the mold *Eupenicillium brefeldianum* [1]. In its active form—mizoribine-5′-monophosphate, MZR acts as a competitive inhibitor of Inosine monophosphate dehydrogenase (IMPDH). IMPDH plays a role in the de novo synthesis of guanosine nucleotides especially in lymphocytes. Due to this MZR has a potent immunosuppressive effect on both humoral and cellular immunity [2-4]. MZR was approved for clinical use in 1984 in Japan and has been used as a immunosuppressive agent in rheumatoid arthritis [5] and a variety of autoimmune kidney diseases like lupus nephritis [6], IgA childhood nephropathy [7], steroid resistant nephritis [8] and even in renal transplantation [9]. It is also known to have anti-viral properties especially against respiratory syncytial, influenza and parainfluenza, measles and hepatitis C virus [10-12].

Cyclosporine A (CsA) is a widely used immunosuppressant that is used in both autoimmune diseases as well as in the setting of transplantation. As an inhibitor of calcineurin, it prevents the activation of both T-cells and B-cells [13]. Due to the different modes of action of MZR and CsA, we postulated that immunosuppressive properties of these two agents may synergize leading to better islet and heart allograft survival across MHC mismatches.

Abbreviations: MZR, Mizoribine; MMF, Mycophenolate mofetil; CsA, Cyclosporine; IMPDH, Inosine monophosphate dehydrogenase; NFGB, Non-fasting blood glucose.

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2. Objective

The aim of this study was to investigate the immunosuppressive effects of MZR and to determine its effect in combination with CsA in vivo models of murine allogeneic islet cell transplantation and heterotopic heart transplantation. We hypothesized that MZR can synergize with CsA thus acting as an efficacious immunosuppressive combination to promote allograft survival and function.

3. Materials and methods

3.1. Mice

Inbred 6–8 week old C3H/He ($H2^k$), male Balb/c ($H2^d$) and C57BL/6 ($H2^b$) were obtained from Jackson Laboratories, Bar Harbour, ME. All animal studies were performed in accordance with the Animal Studies Committee, Washington University, St. Louis, MO guidelines. The procedures described were done under aseptic techniques.

3.2. Induction of diabetes

Diabetes was chemically induced in C57BL/6 recipient mice using Streptozotocin (Sigma, St. Louis, MO). A single intraperitoneal injection of Streptozotocin freshly dissolved in citrate buffer at 200 mg/kg body weight dose was administered 7 days prior to transplantation. Mice with diabetes defined as two consecutive non-fasting blood glucose (NFBG) levels of greater than 300 mg/dL were used as recipients.

3.3. Murine islet cell isolation and transplantation

Islets were isolated from murine pancreata by collagenase digestion and transplanted under the kidney capsule as described previously [14,15]. Briefly, donor (Balb/c) pancreas was digested using collagenase-P (Roche, Indianapolis, IN, 2 mg/mL). Islets were purified on a Ficoll gradient and handpicked. They were cultured for 24 hours at 37 °C prior to transplantation. Approximately 500 islets were transplanted per diabetic recipient mice (C57BL/6) under the left kidney capsule. NFGB was measured daily with a cut-off of less than 200 mg/dL as a sign of graft function and rejection defined as a value greater than 250 mg/dL on 2 consecutive days.

3.4. Murine heterotopic cardiac transplantation

C3H/He ($H2^k$) cardiac allografts were transplanted heterotopically into male Balb/c ($H2^d$) mice as described previously [16]. Briefly, donor ascending aorta and the pulmonary trunk from the heart graft was anastomosed end-to-side to the recipient infrarenal abdominal aorta and inferior vena cava, respectively, using 10-0 sutures. Cold ischemic times were less than 30 min. Graft survival was checked by palpation for heart beat, direct visualization under a microscope and electrocardiogram.

3.5. Immunosuppression

CsA (Neoral, Novartis Pharmaceuticals, USA), MZR (Bredinin, Asahi Kasei Corp, Japan) and MMF (CellCept, Roche, USA) were obtained. Immunosuppressants were started immediately following transplantation as a once daily dose. CsA was diluted in normal saline and MZR and MMF in 0.2% hydroxypropyl methylcellulose and administered orally by gavage needle. The mice that were islet transplantation recipients were divided into four groups (15 islet transplantations per group): Group 1—no immunosuppression ($n=15$), Group 2 ($n=15$)—MZR (20 mg/kg/day), Group 3 ($n=15$)—CsA (20 mg/kg/day), Group 4 ($n=15$)—MZR (20 mg/kg/day) + CsA (20 mg/kg/day). Five animals from each group were sacrificed on day 7 after islet transplantation to study histology, serum for cytokine analysis and splenocytes for Enzyme Linked Immunospot Assay (ELISpot).

The immunosuppressive effect of varying doses of MZR along with CsA as well as MMF and CsA was tested in the murine heterotopic heart transplantation model. The mice were divided into seven groups (10 heart transplantations per group): Group I ($n=10$)—No immunosuppression, Group II ($n=10$)—CsA (20 mg/kg/day), Group III ($n=10$)—CsA (20 mg/kg/day) + MZR (20 mg/kg/day), Group IV ($n=10$)—CsA (20 mg/kg/day) + MZR (10 mg/kg/day), Group V ($n=10$)—CsA (20 mg/kg/day) + MMF (20 mg/kg/day), Group VI ($n=10$)—CsA (20 mg/kg/day) + MMF (10 mg/kg/day), Group VII ($n=10$)—MZR (20 mg/kg/day).

3.6. Analysis of donor specific secretion of IFN-γ, IL-2, IL-4 and IL-10 by Enzyme Linked Immunospot (ELISpot) Assay

In order to determine the frequency of cells secreting IFN-γ, IL-2, IL-4 and IL-10 in response to donor antigens, splenocytes were isolated from recipient mice (C57BL/6) on day 7 after islet transplantation. The cells were cultured in the presence irradiated donor splenocytes (Balb/c) as antigen presenting cells (APC) at 37 °C in 5% CO_2. IFN-γ, IL-2, IL-4, IL-10 ELISpot was performed as per the manufacturer's instructions (BD Biosciences, CA) with recipient splenocytes cultured in triplicate (3×10^5 cells/well) in the presence of donor APC in a ratio of 1:1. Negative control were cells cultured in medium alone (RPMI-1640 supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 2 mM L-glutamine, 25 mM HEPES buffer, 1 mM sodium pyruvate, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 50 μM 2-mercaptoethanol (Gibco BRL, Life Technologies)) and phytohemagglutinin was positive controls. The spots were analyzed in an Immunospot Image Analyzer (CTL, Cleveland, OH). Spots greater than +2 standard deviations of the mean obtained in the negative control wells were considered to be significantly positive and expressed as mean spots per million cells (spm).

3.7. Measurement of serum insulin by ELISA

Serum insulin concentration was measured in the islet graft recipients using Insulin quantification ELISA Kit (Mercodia Inc, Winston Salem, NC). Serum was obtained from blood collected by retro-orbital puncture on alternate days using an anticoagulant coated capillary tubes. ELISA was performed as per manufacturer's instructions. Briefly, serum samples were incubated for 2 hours at room temperature in ELISA plates precoated with monoclonal anti-insulin antibodies. The plates were washed, developed using tetramethyl benzidine substrate and read at 450 nm. Concentration of serum insulin was determined by a standard curve of the binding of a known concentration of insulin solution and expressed as pg/mL.

3.8. Measurement of serum cytokines and chemokines using luminex

Serum was obtained on day 7 after islet transplantation and the concentration of cytokines (IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL13, IL17) and chemokines (FGF, GM-CSF, IP-10, KC, MCP-1, MIG, MIP-1α, TNF-α and VEGF) were measured by multiplex bead assay using Luminex. Briefly, a mouse 20-plex cytokine and chemokine kit was used and assay performed as per manufacturer's instructions (Invitrogen, Carlsbad, CA). The mean fluorescence intensity of experimental and standard wells was determined on Luminex xMAP (Fischer, Pittsburgh, PA). Concentrations were obtained by a standard curve and expressed in pg/mL.