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ScienceDirect
Transplant Immunology 19 (2008) 25–29
Transplant Immunology
www.elsevier.com/locate/trim

Efficacy of tacrolimus in inhibiting inflammation caused by carrageenan in a murine model of air pouch

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Received 22 October 2007; received in revised form 2 January 2008; accepted 8 January 2008

Abstract

Background: Tacrolimus (Tac) is a macrolide immunosuppressant drug isolated from *Streptomyces tsukubaensis*, widely used in organ transplantation.
Objective: This study examined the effect of tacrolimus administered by oral route (p.o.) on inflammation in mouse subcutaneous air pouch triggered by carrageenan (Cg 1%).
Methods: The air pouch was induced as described by Benincá et al. [Benincá JP, Montanher AB, Zucolotto SM, Schenkel EP, FrödeTS. Anti-inflammatory effects of the *Passiflora edulis*: forma *flavicarpa* Degener inhibition of leukocytes, enzymes and pro-inflammatory cytokine levels in the air pouch model, in mice. Food Chem 2007; 104(3); 1097–1105.]. The inflammatory parameters (leukocytes, exudation, myeloperoxidase (MPO) and adenosine-deaminase (ADA) activities, as well as nitrate/nitrate concentrations (NO^x), interleukin-1 beta (IL-1β), chemokine to neutrophil (KC) and tumor necrosis factor-alpha (TNF-α) levels were analysed 24 h after injection of carrageenan.
Results: Tacrolimus, indomethacin and dexamethasone significantly inhibited leukocytes, neutrophils and exudation ($P<0.05$) when they were administered 0.5 h before inflammation. These drugs, under the same conditions, decreased MPO and ADA activities ($P<0.05$), NO^x and IL-1β levels ($P<0.01$). Tacrolimus and indomethacin, but not dexamethasone, inhibited KC levels ($P<0.01$). On the other hand, tacrolimus and dexamethasone, but not indomethacin, decreased TNF-α levels ($P<0.01$).
Conclusions: Results of this study indicate that tacrolimus has an important anti-inflammatory property, showing not only inhibition of pro-inflammatory mediators release, but also inhibition of activated leukocyte infiltration into the site of inflammation. Furthermore, these results showed that most of the anti-inflammatory actions of tacrolimus were similar to those observed in animals treated with either indomethacin or dexamethasone.

Keywords: Tacrolimus; Anti-inflammatory activity; Cytokines; Enzymes; Leukocytes; Exudation

1. Introduction

Tacrolimus formerly FK506 is a natural anti-fungal macrolide immunosuppressant drug isolated from *Streptomyces tsukubaensis* [1], which is widely used in organ transplantation [2–4], and to treat atopic dermatitis [5], psoriasis [6] and bowel disease [7]. Clinical studies have also demonstrated the efficacy of tacrolimus in the treatment of rheumatoid arthritis (RA) [8,9].

Tacrolimus exerts its immunosuppressive effects primarily by interfering with the activation of T cells [10,11]. Its mechanism of action is linked to the binding of intra-cellular proteins called immunophilins (FKBP-12) forming a tacrolimus–FKBP-12 complex. This complex promotes the inhibition of calcineurin phosphatase, an enzyme involved in activation of the nuclear factor of activated T cells (NF-AT), a transcription factor required for expression of cytokine genes in T cells. This results

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doi:10.1016/j.trim.2008.01.003

in a complete blockage of the production of T cell-derived cytokines such as tumor necrosis factor alpha (TNF-α), interleukin-2 (IL-2) and interferon-γ [10]. Another calcineurin inhibitor, cyclosporine A (CsA), exerts similar inhibitory effects on inflammatory cytokine, although the inhibitory effect of CsA is less potent than that of tacrolimus [12]. Additionally, inflammatory cytokines such as tumor necrosis factor, interleukin-1 beta (IL-1β), and interleukin-8 (IL-8), and other mediators such as nitric oxide (NO), as well as pro-inflammatory enzymes such as myeloperoxidase (MPO) and adenosine-deaminase (ADA) are, in part, involved in the inflammatory response and they are the target of studies on drugs that have anti-inflammatory properties, such tacrolimus. Previous data obtained in our laboratory [13] revealed that tacrolimus administered systemically exerted important anti-inflammatory effects by inhibiting leukocytes and pro-inflammatory mediators in a murine model of pleurisy induced by carrageenan.

2. Objectives

This study was designed to examine the effect of tacrolimus administered by oral route (p.o.) on acute inflammation in a murine model compared to those obtained with classical non-steroidal and steroidal anti-inflammatory drugs (indomethacin and dexamethasone). Specifically, we evaluated the effect of this drug on inflammation induced by carrageenan, in the air pouch model.

3. Materials and methods

3.1. Animals

Swiss mice, weighing 18–22 g, were housed under standardized conditions (room at constant temperature (22±2 °C) with alternating 12 h periods of light and darkness and humidity (50–60%)) and fed a standard mouse diet with water *ad libitum* before use. All experiments were in agreement with guidelines on ethical standards of investigation of experimental procedures in animals [14]. This study was approved by the Committee for Ethics in Animal Research (CEUA of our university, protocol number 23080.007040/2006-39) and performed in accordance with norms of the Brazilian College of Animal Experimentation (COBEA).

3.2. Experimental protocol

In this experimental protocol, different groups of animals received air injection (1.5 ml) on three alternate days to induce the air pouch. On the sixth day animals received carrageenan (Cg 1%) administered by subcutaneous route (s.c.) and 24 h later animals were killed by an overdose of ether [15]. Animals were fixed on a surgical table and an incision in the dorsal skin was made perforating the air pouch. The cavity was washed with 1.0 ml of sterile PBS (pH 7.6, composition mmol: NaCl 137, KCl 2.7 and phosphate buffer salts 10) containing heparin (20 IU/ml) [15]. Cell migration, exudation, MPO and ADA activities, as well as NO^x, IL-1β, KC and TNF-α levels were evaluated 24 h after phlogogen administration. Indomethacin (5.0 mg/kg) and dexamethasone (0.5 mg/kg) administered by intra-peritoneal route (i.p.) 0.5 h before inflammation were used as reference drugs [13,15].

Initially, to establish a standard for the tacrolimus, together with doses and time periods to be used in the experiments, different groups of animals were treated (0.5 h before Cg) with different doses of tacrolimus (1.0 to 10.0 mg/kg) administered 0.5 h (p.o.) before Cg or treated with sterile saline (NaCl 0.9 %) administrated by subcutaneous route (s.c.), and the inflammatory parameters (cell migration and exudation) were analysed 24 h after carrageenan injection. In another set of experiments, animals were pre-treated with tacrolimus (2.0 mg/kg, p.o.), at different time points (0.5–4 h) and the same inflammatory parameters were evaluated 24 h after carrageenan-induced inflammation. According to this protocol, 2.0 mg/kg of tacrolimus was also selected in the experiments to analyse its effects on MPO and ADA activities, as well as on NO^x, IL-1β, KC and TNF-α levels.

3.3. Quantification of cell migration and exudation

After killing animals with an overdose of ether, samples from the air pouch exudate were collected for determinations of total and differential leukocytes. Total leukocyte counts were performed in a Neubauer chamber diluting the exudate in Türk solution (1:20), and cytospin preparations of exudate were stained with May Grünwald–Giemsa for the differential leukocyte counts, which were performed under an oil immersion objective [15].

To evaluate the degree of exudation, animals were previously challenged with a solution of Evans blue dye (25.0 mg/kg) administered by intravenous route (i.v.), 5 min after treatment with carrageenan [15]. The amount of dye was estimated by colourimetry using an Elisa plate reader (Organon Teknika, Roseland, NJ, USA) at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50.0 μg/ml.

3.4. Quantification of nitrate/nitrite concentrations

Nitric oxide was measured as its breakdown product of nitrite (NO_2^-) and nitrate (NO_3^-) using the Griess method [16]. Samples of the air pouch cavity lavage obtained from control and treated animals that did not receive Evans blue dye injection were separated and stocked at −70 °C. On the day of the experiments, the samples were thawed and deproteinized by the addition of 6 mM sodium hydroxide and 0.6% of zinc sulfate. Afterwards, 250 μl of air pouch cavity lavage was diluted in 30 μl of ammonium format, 30 μl of hydrated disodium hydrogen phosphate-12 and 30 μl of *Escherichia coli* (EC ATCC 25922: diluted (1:10) in PBS), and then the mixture was incubated for 2 h at 37 °C. After centrifugation at 50 × g for 5 min, 250 μl of the supernatant was transferred to cuvettes and the same volume of fresh Griess reagent (5% vol/vol) of H_3PO_4, 1% of sulfanilic acid and 0.1% of *N*-(1-naphythyl)-ethylenediamine) was added and incubated for 10 min at room temperature. The reaction of NO_2^- with this reagent produces a pink colour, which was quantified at 543 nm against standards (0–150 μM) on a spectrophotometer Hitachi U2001, model 121-0031 (Tokyo, Japan) [17]. Results were expressed as μM.

3.5. Quantification of myeloperoxidase and adenosine-deaminase activities

In-house assays of both myeloperoxidase and adenosine-deaminase activities were employed according to the methods described in the literature [18,19]. Using conventional reagents, the concentration of each enzyme was estimated by means of colourimetric measurements (absorbances of 450 and 620 nm, respectively) with an ELISA plate reader (Organon Teknika, Roseland, NJ, USA). Results were expressed as mU/ml (MPO) and U/l (ADA).

3.6. Quantification of IL-1β, TNF-α and KC levels

Samples of exudate were collected and immediately prepared for the analysis of cytokine levels. In this protocol, commercially available kits were used with specific antibodies for each cytokine. The cytokine levels were measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. When necessary the fluid exudate samples were diluted 1:3 or 1:10 with specific diluents (buffered with 0.09% sodium azide for TNF-α or buffer diluent with 1% bovine serum albumin (BSA) plus 0.05% Tween 20 in PBS for KC) to determine the TNF-α and KC levels, respectively. The range of values detected by these assays was: IL-1β (100.0–6400.0 pg/ml), KC (23.4–1500.0 pg/ml) and TNF-α (5.0–2000.0 pg/ml). The intra- and inter-assay coefficients of variation (CV) for IL-1β, KC and TNF-α were: intra-CV: IL-1β:=6.2±0.4%, KC=9.7±0.9% and TNF-α=7.8±0.9%, inter-CV: IL-1β=5.1±0.6%, KC=4.1±0.9% and TNF-α=9.6±2.2%, with sensitivity values of IL-1β=1.7 pg/ml, KC=23.4 pg/ml and TNF-α=5.0 pg/ml. All cytokine