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Influence of immunosuppressive agents on tryptophan degradation and neopterin production in human peripheral blood mononuclear cells☆,☆☆

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ABSTRACT

The anti-proliferative and immunomodulatory enzyme indoleamine 2,3-dioxygenase (IDO) degrades the essential amino acid tryptophan via the kynurenine pathway. IDO is stimulated during cellular immune responses preferentially by Th1-type cytokine interferon-γ (IFN-γ). IDO activity is estimated by calculating the kynurenine to tryptophan ratio (Kyn/Trp). In human monocyte-derived macrophages and dendritic cells, GTP-cyclohydrolase I is induced in parallel to IDO and produces neopterin.

This study investigated the effects of common immunosuppressants on freshly isolated human peripheral blood mononuclear cells (PBMC) *in vitro*. PBMC were incubated with compounds for 30 min and then either left unstimulated or stimulated with mitogen phytohaemagglutinin (PHA). Concentrations of tryptophan, kynurenine and neopterin were measured in supernatants after 48 h.

Kyn/Trp, neopterin and IFN-γ concentrations were significantly higher in PHA-stimulated *vs.* unstimulated PBMC. Tacrolimus (FK506), cyclosporine A (CsA), sirolimus and methylprednisolone dose-dependently inhibited tryptophan degradation and neopterin production. FK506, CsA and sirolimus showed significant inhibition at concentrations as low as 0.1 μg/ml, whereas prednisolone and methylprednisolone required higher doses to suppress tryptophan degradation. Mycophenolate-mofetil suppressed neopterin formation more efficiently than Kyn/Trp. All tested drugs also strongly decreased mitogen-induced IFN-γ concentrations. Overall the investigated immunosuppressants are effective to inhibit IDO activity and neopterin production in a similar and dose-dependent manner, however with some differences in IC50s when comparing individual compounds. The corresponding changes of IFN-γ concentrations are in line with its role as a trigger of both biochemical changes.

1. Introduction

Immunosuppressive agents are clinically widely used for the treatment of diseases that involve hyperactive immune responses. Most of the compounds that are currently available influence both the innate and adaptive immune system and interact with pathways of lymphocyte activation and proliferation. Immunosuppressants are therefore applied after allograft transplantation and for the treatment of autoimmune diseases [1–3]. The risk of frequent and serious side effects from immunosuppressive agents including but not limited to metabolic side effects, opportunistic infections complications and malignancies is well established and a limiting factor for their clinical use [4].

The most commonly available immunosuppressants are calcineurin inhibitors such as tacrolimus (FK506) and cyclosporine A (CsA), inhibitors of the mammalian target of rapamycin (mTOR) such as sirolimus, anti-metabolites like mycophenolate-mofetil (MMF), and steroids prednisolone and methylprednisolone. The active compounds dampen the immune response and counteract with lymphocyte proliferation at different target positions: MMF interacts with the purine biosynthesis in lymphocytes, prednisolone and methylprednisolone act by binding to glucocorticoid receptors, whereas FK506, sirolimus and CsA interfere with the signaling cascade of Th1-type cytokines like interleukin-2.

During cell-mediated (=Th1-type) immune response pro-inflammatory cytokines especially interferon-γ (IFN-γ) activate indoleamine 2,3-dioxygenase (IDO) and GTP-cyclohydrolase I in human macrophages and dendritic cells (DC) [5–9]. IDO degrades the essential amino acid tryptophan (Trp) to kynurenine (Kyn), and

Abbreviations: CsA, cyclosporine A; DC, dendritic cells; FK506, tacrolimus; GTP, guanosinetriphosphate; mTOR, inhibitors such as sirolimus; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon-γ; Kyn/Trp, kynurenine to tryptophan ratio; MMF, mycophenolate-mofetil; mTOR, mammalian target of rapamycin; PBMC, peripheral blood mononuclear cells; PHA, phytohaemagglutinin.

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enzyme activity can be estimated by calculating the ratio of Kyn to Trp concentrations (Kyn/Trp) [10]. Ongoing conversion of Trp leads to deprivation of the essential amino acid below certain thresholds for T-cell activation and proliferation and thereby also acts antibacterial, antiviral and anti-tumoral. Therefore, activating IDO during immune responses is an important counter balancing mechanism [11,12] that also represents a negative feedback loop of IFN-γ and is a tool to down-regulate overwhelming immune activation. In a murine model successful pregnancy was only possible, if activation of IDO prevented rejection of the fetus by inhibiting the proliferation of maternal T-cells [13]. In this regard, IDO expression in macrophages and DC may also enable to develop and maintain peripheral immune tolerance by preventing the proliferation of autoreactive T cells. Interestingly, not only the deprivation of Trp seems to be important, but also the accumulation of specific Trp catabolites like Kyn and anthranilic acid appears to be involved in such potent immunoregulatory mechanisms [14].

In parallel to IDO, IFN-γ induces the enzyme GTP-cyclohydrolase I in monocyte-derived macrophages and DC, which forms neopterin during Th1-type immune responses [5,6,9]. Thereby neopterin formation coincides with increased IDO activity in various human diseases [15,16]. In patients with, *e.g.*, HIV infection, malignancy and autoimmune syndromes enhanced Trp degradation and in parallel elevated neopterin production reflect the clinical course of disease and are predictive for an unfavorable outcome [17–19].

Determination of neopterin production and Trp degradation in cell culture systems has proven to be an elegant method to measure the influence of cytokines, drugs and compounds on the interaction between T-cells and macrophages [20]. Stimulation of cultured PBMC can be standardized by exposure to specific mitogens like phytohaemaglutinin A (PHA) or concanavalin A to induce IDO and neopterin formation. In this study, the influence of various immunosuppressants widely in use to treat patients after allotransplantation was investigated in particular with respect to their impact on IFN-γ mediated biochemical pathways.

2. Materials and methods

2.1. Chemicals

Tacrolimus (FK506) was purchased from Fujisawa (Vienna, Austria), cyclosporine A (CsA) was obtained from Novartis Pharma (Vienna, Austria), sirolimus (Rapamune) from Wyeth Europa Ltd. (Taplow, Maidenhead, United Kingdom), mycophenolate-mofetil (MMF) was from Roche Registration Ltd. (Vienna, Austria), prednisolone from Nycomed Austria (Vienna, Austria) and methylprednisolone (Urbason) from Aventis Pharma, (Vienna, Austria).

The compounds were dissolved in RPMI-1640 obtained from MedPro (Vienna, Austria), sterile filtered after 15 min sonification and stored at −20 °C until use. PHA was obtained by Sigma-Aldrich, (Vienna, Austria) dissolved in phosphate buffered saline (PBS) and stored at −20 °C until use.

2.2. Cell culture

For cell culture peripheral blood mononuclear cells were isolated from the blood of healthy voluntary donors following written informed consent for the use of blood for scientific purposes was obtained.

After PBS was added, the separation of blood cells was performed via density centrifugation using Biocoll solution (MedPro, Vienna, Austria). The isolated cells were washed for three times with PBS supplemented with 0.3% EDTA (0.5 mmol/l) (Merck, Darmstadt, Germany). Cells were then incubated in RPMI 1640 (MedPro, Vienna, Austria) containing 10% heat inactivated calf serum, 2 mM L-glutamin (Serva, Heidelberg, Germany) and 50 μg/ml gentamicin (Serva, Heidelberg, Germany). For experiments cells were plated at a final density of 1.5×10^6 cells/ml in the medium and were incubated in moist atmosphere at 37 °C and 5% CO_2. At first PBMC were preincubated for 30 min with or without immunosuppressant, then they were stimulated with 10 μg/ml of PHA or not. The concentration of 10 μg/ml PHA has been documented earlier to allow optimal stimulation of cells before reaching a plateau [20]. Cell viability was proven by trypan-blue test (Sigma-Aldrich, Vienna, Austria). Every experiment was performed in duplicates for at least three times using cells of different donors. After 48 h, experiments were stopped and culture supernatants were harvested by centrifugation.

2.3. Determination of tryptophan, kynurenine, IFN-γ and neopterin concentrations

Concentrations of Trp and Kyn were measured using HPLC (ProStar Varian, Palo Alto, CA) with 3-nitro-L-tyrosine (Sigma-Aldrich, Vienna, Austria) as internal standard. Kyn and 3-nitro-L-tyrosine concentrations were determined by means of their UV absorption at 360 nm. Fluorescence emitted by Trp at 366 nm wavelength was measured under exposure to light at 286 nm wavelength (ProStar). Kyn/Trp was calculated to estimate IDO activity and expressed in μmol Kyn/mmol Trp. Concentrations of neopterin and IFN-γ were determined by ELISA (neopterin: BRAHMS, Hennigsdorf, Germany; IFN-γ: R&D Systems Europe, Abingdon, UK) following the manufacturer's instructions. Sensitivity of the tests was 2 nmol/l neopterin and 8.0 pg/ml IFN-γ.

2.4. Statistics

Results were expressed as percent of unstimulated and PHA-stimulated control and were shown as means ± standard error of the mean (S.E.M.). For statistical evaluation of the data the program SPSS 15.0 was used. Group comparisons were performed using non-parametric tests (Kruskal–Wallis-, Mann–Whitney-test), p-values <0.05 were considered as statistically significant. IC50 concentrations were calculated using CalcuSyn software from Biosoft, Cambridge, UK [21].

3. Results

Concentrations of Trp, Kyn and neopterin measured in supernatants of unstimulated cells as well as the mean Kyn/Trp are shown in Table 1. Stimulation of PBMC with PHA-induced Trp degradation was reflected by higher Kyn/Trp and also neopterin production increased significantly ($p<0.001$, see Table 1). IFN-γ concentrations in the supernatants of PBMC were 26.4 ± 3.8 pg/ml and increased about 21-fold to 561.4 ± 129.4 pg/ml upon stimulation with PHA ($p<0.05$).

Table 1
Concentrations of tryptophan, kynurenine and neopterin (black bars) in supernatants of unstimulated PBMC and cells stimulated with 10 μg/ml of mitogen phytohaemagglutinin (PHA). Indoleamine 2,3-dioxygenase (IDO) activity is assessed by calculation of the kynurenine to tryptophan ratio (Kyn/Trp). Mean values ± S.E.M. of three independent experiments run in duplicates are shown.

	Neopterin [nmol/l]	Tryptophan [μmol/l]	Kynurenine [μmol/l]	Kyn/Trp [μmol/mmol]
Absolute concentrations				
Control	3.3 ± 0.1	31.4 ± 0.6	0.86 ± 0.04	27.4 ± 1.1
PHA	13.4 ± 0.8	6.7 ± 0.9	10.8 ± 0.4	2330 ± 445
Percent change related to unstimulated control = 100%				
Control	100 ± 0.9	100 ± 0.9	100 ± 1.5	100 ± 1.4
PHA	407 ± 21	21.6 ± 3.0	1294 ± 81	9231 ± 2032