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IFN-γ-STIMULATED TRYPTOPHAN DEGRADATION BY HUMAN HEMATOPOIETIC CD34+ PROGENITOR CELLS

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ABSTRACT. The addition of IFN-γ to cultured in vitro – purified and enriched human hematopoietic CD34+ progenitor cells, stimulated tryptophan degradation. Low but frequently added doses of IFN-γ stimulated trytophan degradation more than when the cytokine was added once and in high concentrations at the time of cell culturing.

IFN-γ-stimulated tryptophan degradation corresponded to enhanced cell proliferation and hematopoietic (erythroid-, mixed granulocyte/macrophage- and pure macrophage-) colony formation. Besides the effects of IFN-γ, the culture media also enhanced trytophan degradation and hematopoietic cell proliferation: cells cultured in *recombinant cocktail* (RC) used higher amounts of trytophan than those cultured in *agar-leukocyte conditioned medium* (Agar-LCM). In our experiments trytophan depletion didn't stimulate apoptosis.

KEY WORDS. IFN-γ, *purified* and *enriched* human CD34+ progenitor cells, *in vitro* trytophan degradation, hematopoietic cell proliferation, colony formation

INTRODUCTION

L-tryptophan is an essential amino acid required for protein biosynthesis and a precursor for different neurotransmitters. Its normal serum concentrations vary between 40-100 μmol/l and depend on the diet. Two enzymes, discovered in mammals are capable of tryptophan degradation – *indoleamine 2,3-dioxygenase (IDO)* and *tryptophan dioxygenase (TDO)*[15].

IDO is a *42 kDa heme-containing enzyme* – activated in cases of inflammation and expressed on the surface of dendritic cells (DC) [2], in the epydidimis, thymus, spleen, lungs, placenta [4]. One of the growth factors activating IDO is the inflammatory cytokine IFN-γ – produced by activated monocytes/macrophages [1].

It's determined that tryptophan depletion from the culture medium blocks the cell cycle of activated T-lymphocytes and natural killer cells (NK) in G_o-S phase [6]. Bone marrow stromal cells also expressed IDO upon stimulation with IFN-γ which blocks the allogeneic T-cell responses [3]. Tryptophan degradation represents a defense mechanism because it inhibits intracellular replication of some patogens (Toxoplasma gondii and Chlamydia psittaci) and stimulates formation of secondary metabolites that are toxic for T-cells [4, 15]. Decreased tryptophan concentrations are also measured during pregnancy [10]. Munn et al. [5] suggest that this can possibly lead to suppression of maternal T-cell response. Similar results are also reported [8, 13, 14] in cases of some neurodegenerative such as Alzheimer's, Huntington's and Parkinson's diseases.

There are two hypotheses in the scientific literature: tryptophan degradation stimulated by IFN-γ activates apoptosis [7] and inhibits proliferation and differentiation of the erythroid cells [11,15].

In cases of inflammation IFN-γ induces IDO-mediated tryptophan degradation leading to increased serum concentrations of *kynurenine. Kynurenine* and its circulating metabolites can cause uremic symptoms (resembling nephropathy), lipid disturbancies, hypertension, anemia, heart diseases, etc. [9, 11, 16].

The *kynurenine/tryptophan ratio (K/T)* is used for determining the activity of IDO, regardless of the tryptophan concentrations [12].

MATERIAL AND METHODS

Cell cultures. *Purified* (92% purity) and *enriched* (5%) *human hematopoietic CD 34+ progenitor cells* (kindly provided by the Laboratory for Immune Biology, Internal Medicine, Innsbruck), were thawed, washed with *Hanks Balanced Salt Solution – HBSS* (PAA Laboratories, Austria) and centrifuged for 15 min at 1050 rpm. After the first centrifugation the cells were washed in the same solution, centrifuged again (10 min at 1000 rpm.), resuspended in *Iscove's Modified Dulbecco Medium* (*IMDM*), counted (after trypan blue staining for cell viability) and plated in *4-well Nunc Petri dishes* at a density of $2.5x10^3$ / well – for *purified*, and $1x10^4$ cells / well – for *enriched* cells.

The *semi-solid agar cultures* were prepared from the *purified* and *enriched cells* in the same Petri dishes: 0.3% agar, supplemented with IMDM, 10% fetal calf serum (FCS – Gibco), 2% bovine serum albumin (BSA-Sigma), 6 U/ml erythropoietin (Erypo-Janssen-Cilag Pharma), $2x10^{-4}$M mercaptoethanol, 4 mM glutamine and recombinant cocktail – RC [consisting of *recombinant human interleukin-3 (rhIL-3)* – 50 U/ml and *recombinant human stem cell factor (rhSCF)* – 10 U/ml (Chemicon)].

Purified and *enriched cells* from the same populations were also cultured in *20% agar leukocyte conditioned medium – Agar-LCM* (CellSystems Biotechnologie

Vertrieb GmbH), supplemented with 10% FCS, 2% BSA, 6 U/ml Epo, $2x10^{-4}$M mercaptoethanol and 4 mM glutamine.

rhIFN-γ (Rentschler Biotechnologie GmbH & Co.KG) was added to both experimental systems at different doses (5000 U/ml – once, at the time of cell cultures' preparing; 200 U/ml and/or 400 U/ml – every second day of CD34+ cell cultivation).

Tryptophan measurement. The amount of *tryptophan* (μM) and *kynurenine concentrations* (μM) were measured in the liquid layer of agar cultures after 14 days of cell incubation *by High Pressure Liquid Chromatography (HPLC).* In brief, *to* 100 μl of each sample's supernatant, 100 μl of *Internal standard 3-nitro-L-tyrosine* and 25 μl of *Trichloroacetic acid (TCA)* were added *(12).*

Kynurenine/Tryptophan ratio was automatically calculated by the system's software.

Statistical analysis. To investigate whether the applied doses of *IFN-γ* influence *tryptophan degradation* in different ways, the *Student's t-test* was used. A difference is assumed to be significantly large if the corresponding $t \geq t_{\alpha,f}$ where $t_{\alpha,f}$ is the critical value at **α** level of significance and ***f –*** degrees of freedom.

RESULTS

The results for *tryptophan degradation* showed that this amino acid was used more by *hematopoietic CD34+ progenitor cells – purified and enriched,* when they were *cultured in RC*. The lower *tryptophan concentrations* measured in the culture medium corresponded to a higher number of *hematopoietic colonies* formed (determined by morphological analysis in the *agar cultures*).

In the *Agar-LCM,* where less colonies were formed, higher *tryptophan concentrations* were measured (**Fig.1, 2**).

On the other hand it was observed that when added to the cell cultures *IFN-γ* stimulated the degradation of *tryptophan*. A clear reduction of *tryptophan* and a simultaneous increase of *kynurenine* were detected in the presence of this cytokine – used frequently and in small portions (200 U/ml and/or 400 U/ml/culture medium) rather than when it was applied once at a dose of 5 000 U/ml. The lowest *tryptophan* concentrations were measured in *RC* when IFN-γ was added at 400 U/ml/culture medium, every second day. Significant statistical difference ($\acute{\alpha}<0.005$) was determined when the latter dose of IFN-γ was compared to the other two cytokine concentrations (200 U/ml and 5000 U/ml) in the culture media and for both – *purified* and *enriched hematopoietic CD34+ progenitor cells*. The low *trytophan* concentrations led to an increase in the amount of the synthesized by these cells *kynurenine* (**Fig. 3, 4**), resulting in a high *kynurenine/tryptophan ratio* (**Fig. 5, 6).**

To investigate the affect of the culture media – RC and Agar-LCM, on trytophan degradation, the concentrations of this amino acid in both experimental systems were compared. The results from the statistical analysis showed that there

was a significant difference ($\alpha < 0.001$) between the tryptophan concentrations with smaller values in the RC than those in Agar-LCM. The addition of IFN-γ further increased the previously stimulated by the culture media tryptophan degradation (**Tables 1 and 2**).

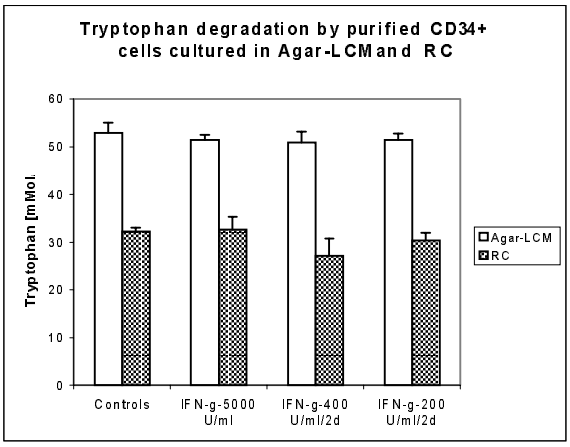


Fig. 1.

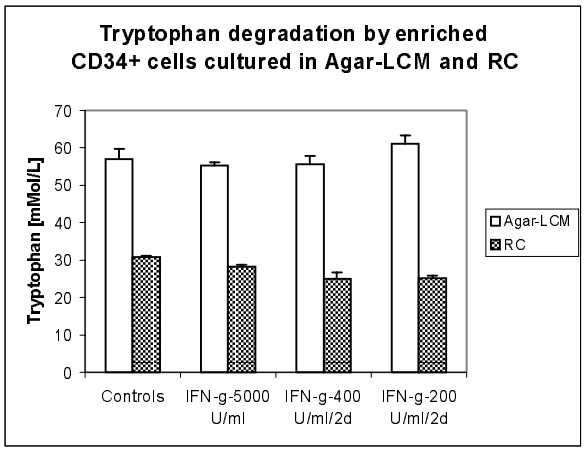


Fig. 2.

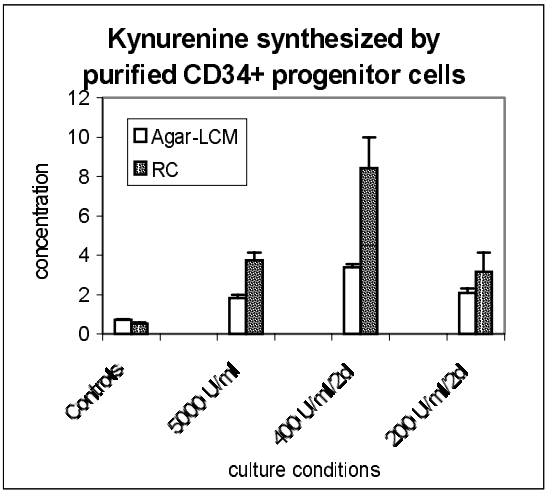
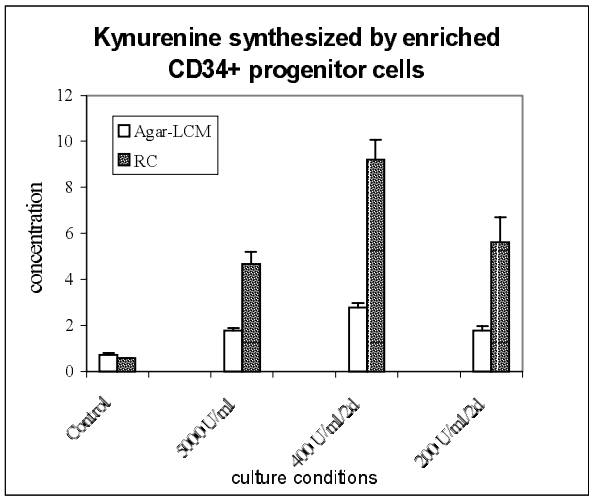


Fig. 3.



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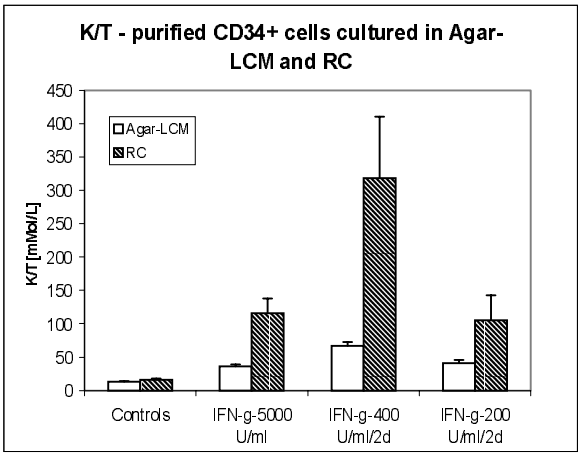

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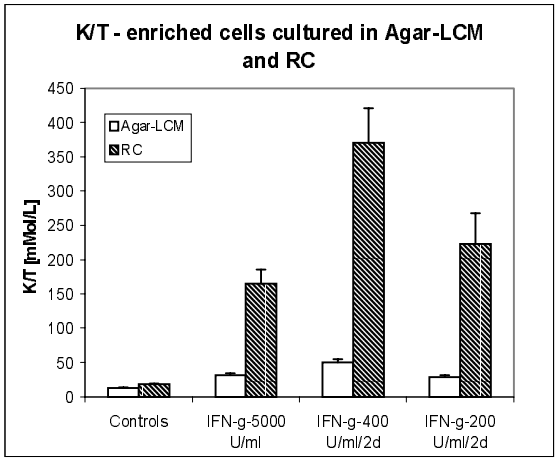

Fig. 6.

DISCUSSION

Tryptophan degradation represents a biological defense mechanism occuring in cases of immune activation [15]. Deprivation of this amino acid and IDO activation respectively correlate with the stage of some neurodegenerative diseases [8, 13, 14]. Interestingly, the changes in the tryptophan metabolism in these neurological disturbancies are more clearly expressed in peripheral blood than in the brain [15].

Addition of IFN-γ, in our experimental model, stimulates tryptophan degradation possibly via IDO activation. The depletion of the amino acid from the culture media (RC and Agar-LCM) corresponds to stimulated proliferation and colony formation of the *purified* and *enriched human CD34+ hematopoietic progenitor cells*.

The results obtained are in contrast with the current hypothesis [11, 15] for a possible inhibition of the cell cycle and a provoked apoptosis of the erythroid cells due to tryptophan degradation *in vitro*. Besides IFN-γ, the *culture media* also affects tryptophan metabolism. Lower concentrations of the amino acid were measured in RC than in Agar-LCM. The reason may be due to synergistic effects of this inflammatory cytokine with SCF and IL-3, present in the RC.

We suggest that the effects of trytophan degradation are different on immature (stem- and progenitor-) and mature cells (such as differentiated T-cells). Although exhibiting antitumor, antimicrobial and antiproliferative effects, tryptophan depletion could possibly affect progenitor cell growth in a different manner, stimulating *in vitro* their proliferation and colony formation.

CONCLUSIONS

IFN-γ stimulates *in vitro* tryptophan degradation. The culture media also enhances tryptophan metabolism due to synergistic effects with IFN. The effect of trytophan degradation depends on the degree of erythroid- and myeloid cell differentiation.

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REFERENCES

CARLIN, J. M., E. C. BORDEN, P. M. SONDEL, G. I. BYREN. 1987. Biologic-response-modifier-induced indoleamine 2,3-dioxygenase activity in human peripheral blood mononuclear cell cultures. J. Immunol., **139**, 7: 2414-2418.

HWU, P., M. X. DU, R. LAPOINTE, M. DO, M. W. TAYLOR, H. A. YOUNG. 2000. Indoleamine 2,3-Dioxygenase production by human dendritic cells results in the inhibition of T cell proliferation. J. Immunol., **164**, 3596-3599.

MEISEL, R., A. ZIBERT, M. LARYEA, U. GÖBEL, W. DÄUBENER, D. DILLOO. 2004. Human bone marrow stromal cells inhibit allogeneic T-cell responses by indoleamine

2,3-dioxygenase mediated tryptophan degradation. Blood, **103**, 12: 4619-4621.

MELLOR, A., D. MUNN. 2003. Tryptophan catabolism and regulation of adaptive immunity. J. Immunol., **170**, 5809-5813.

MUNN, D. H., M. ZHOU, J. T. ATTWOOD, I. BONDAREV, S. J. CONWAY, B. MARSHALL, C. BROWN, A. L. MELLOR. 1998. Prevention of allogeneic fetal rejection by tryptophan catabolism. Science, **281**, 5380, 1191-1193.

MUNN, D. H., E. SHAFIZADEH, J. T. ATTWOOD, I. BONDAREV, A. PASHINE, A. L. MELLOR. 1999. Inhibition of T cell proliferation by macrophage tryptophan catabolism. J. Exp. Med., **189**, 9: 1363-1372.

KONAN, K. V., M. W. TAYLOR. 1996. Treatment of ME180 cells with interferon-γ causes apoptosis as a result of tryptophan degradation. J. Interferon Cytokine Res., **16**, 9: 751-756.

LEBLHUBER F., J. WALLI, K. JELLINGER, G. P. TILZ, B. WIDNER, F. LACCONE, D. FUCHS. 1998. Activated immune system in patients with Huntington's disease. Clin. Chem. Lab. Med., **36**, 10: 747-750.

PAWLAK, D., A. TANKIEWICZ, W. BUCZKO. 2001. Kynurenine and its metabolites in the rat with experimental renal insufficiency. J. Physiol. Pharmacol., **52**, 4: 755-766.

SCHROCKSNADEL, H., G. BAIER-BITTERLICH, O. DAPUNT, H. WACHTER, D. FUCHS. 1996. Decreased plasma tryptophan in pregnancy. Obstet. Gynecol., **88**, 1: 47-50.

WEISS, G., K. SCHROECKSNADEL, V. MATTLE, C. WINKLER, G. KONWALINKA, D. FUCHS. 2004. Possible role of cytokine-induced tryptophan degradation in anaemia of inflammation. Eur. J. Haematol., **72**, 139-134.

WIDNER, B., M. LEDOCHOWSKI, D. FUCHS. 2000. Interferon-γ-induced conversion of tryptophan: neuropsychiatric and immunological consequences. Curr. Drug Metab., 1: 193-204.

WIDNER, B., F. LEBLHUBER, J. WALLI, G. P. TILZ, U. DEMEL, D. FUCHS. 2000. Tryptophan degradation and immune activation in Alzheimer's disease. J. Neural. Transm., **107**, 2: 181-189.

WIDNER, B., F. LEBLHUBER, D. FUCHS. 2002. Increased neopterin production and tryptophan degradation in advanced Parkinson's disease. J. Neural. Transm., **109**, 2: 181-189.

WIRLEITNER, B., G. NEURAUTER, K. SCHRÖCKSNADEL, B. FRICK, D. FUCHS. 2003. Interferon-γ-induced conversion of tryptophan: immunologic and neuropsychiatric aspects. Curr. Med. Chem., **10**, 16: 1581-1591.

WIRLEITNER, B., V. RUDZITE, G. NEURAUTER, C. MURR, U. KALNIS, A. ERGLIS, K. TRUSINSKIS, D. FUCHS. 2003. Immune activation and degradation of tryptophan in coronary heart disease. Eur. J. Clin. Invest., **33**, 550-554.

Table 1. Effect of the culture media on tryptophan degradation by purified human CD34+ hematopoietic progenitor cells

Culture conditions	Agar-LCM – mean	***SD***	***RC – mean***	***SD***	ά – value
Agar-LCM / RC	52.925	2.235	32.275	0.842	< 0.001
IFN-γ 5000 U/ml	51.425	1.063	32.65	2.699	< 0.001
IFN-γ 400 U/ml/2 days	50.925	2.233	27.125	3.634	< 0.001
IFN-γ 200 U/ml/2 days	51.5	1.257	30.45	1.601	< 0.001

Table 2. Effect of the culture media on tryptophan degradation by enriched human CD34+ hematopoietic progenitor cells

Culture conditions	Agar-LCM – mean	***SD***	***RC – mean***	***SD***	ά – value
Agar-LCM / RC	57	2.699	30.75	0.387	< 0.001
IFN-γ 5000 U/ml	55.225	0.854	28.25	0.545	< 0.001
IFN-γ 400 U/ml/2 days	55.525	2.278	25.025	1.712	< 0.001
IFN-γ 200 U/ml/2 days	61.025	2.291	25.15	0.742	< 0.001