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Quantitative Comparative Analysis of the Influence of IFN-γ on the Erythroid (BFU-E and CFU-E) and Myeloid (CFU-GM) Progenitors *in vitro*

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The addition of different doses of IFN-r (5000 U/ml once or 200/400 U/ml — every second day) to cultured *in vitro* purified and enriched human hematopoietic CD34+ progenitor cells stimulated significantly their proliferation and differentiation to erythroid and myeloid lineages. The cytokine enhanced the formation of primitive erythroid colonies (burst-forming unit-erythroid — BFU-E) and myeloid colony-forming units (granulocyte/macrophage — CFU-GM). IFN-γ exhibited weaker stimulatory affect on colony-forming unit — erythroid (CFU-E). Compared to the CFU-GM colony formation, BFU-E were more sensitive to the effect of IFN-r. CD34+ hematopoietic colony formation *in vitro* was quantitatively influenced not only by IFN-r but also by culture medium.

Key words: interferon-gamma (IFN-γ), human hematopoietic CD34+ progenitor cells - purified and enriched, BFU-E, CFU-E, CFU-GM.

Introduction

CD34+ hematopoietic progenitor cells are the most commonly used cell population for *in vitro* assays. Recent reports suggest that CD34 antigen is involved in the onset of hematopoietic cell proliferation and adhesion [4, 7]. The antigen expression decreases when maturing hematopoietic cells loose their capacity to form colonies *in vitro*.

Interferon-gamma (IFN-γ) is an inflammatory cytokine known to act *in vitro* (when added to bone marrow cultures) as inhibitor of erythroid cell proliferation [8, 9, 14]. The results demonstrating the inhibitory effect of IFN-γ on human hematopoietic progenitor cells suggest that it is mediated by accessory cells as T-lymphocytes and macrophages [8, 12]. On the other hand there are reports showing the synergistic effects of IFN-γ with SCF and IL-3 and thus IFN-γ stimulates hematopoiesis [10]. The cytokine switches monocyte differentiation to macrophages instead of dendritic cells [3]. Due to the bipotent activities of this inflammatory cytokine [2, 5, 9, 10, 12, 13], special attention must be paid to its affects on the hematopoietic progenitor cells — both *in vitro* and *in vivo*.

When added to *in vitro* cultures of erythroid/myeloid cells the affects of IFN-γ depend on the cytokine's doses, duration time, the other growth factors present in the culture medium as well as on the degree of cell maturity (differentiation).

The aim of the present work is to investigate the affects of different doses of IFN-γ on hematopoietic (erythroid and myeloid) colony formation *in vitro* (in semi-solid agar cultures of purified and enriched human hematopoietic CD34+ progenitor cells).

Materials and Methods

1. Cell cultures

Purified (92% purity) and enriched (5%) human hematopoietic CD34+ progenitor cells (kindly provided by the Laboratory for Immune Biology, Internal Medicine, Innsbruck, Austria), 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, centrifuged again (10 min at 1000 rpm), resuspended in Iscove's Modified Dulbecco Medium (IMDM), counted and plated in 4-well Nunc Petri dishes at a density of 2.5×10^3/well for the purified and 1×10^4 cells/well — for the enriched cells. Each sample was performed in three parallels. The two phase *semi-solid agar cell cultures* were prepared in the same Petri dishes with 0.3% agar.

The superficial liquid layer of the cultures contained: IMDM, 10% fetal calf serum (FCS — Gibco), 2% bovine serum albumin (BSA-Sigma), 6 U/ml erythropoietin (Erypo-Janssen-Cilag Pharma), 2×10^{-4}M mercaptoethanol, 4 mM glutamine and recombinant cocktail — RC [consisting of recombinant human IL-3 — 50 U/ml and recombinant human stem cell factor (SCF) — 10 U/ml (Chemicon)].

Besides in RC, purified and enriched human CD34+ hematopoietic progenitor cells 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, 2×10^{-4}M mercaptoethanol and 4 mM glutamine.

IFN-γ (Rentschler Biotechnologie GmbH & Co.KG) was added at different doses (5000 U/ml — once; 200 U/ml and/or 400 U/ml — every second day) to the liquid phase of both experimental systems at time of hematopoietic cell cultivation.

The *agar cell cultures* prepared were incubated at 37°C in humidified air of 5% CO_2 for 14 days. After incubation cell colonies were scored on inverted microscope. The agar layers were fixed in glutaraldehyde, mounted on glass slides and stained with May-Grünwald/Giemsa to confirm colony identity and their cytological characteristics [6].

2. Scoring criteria for hematopoietic colonies' identification

In order to classify the colonies as erythroid burst-forming-unit (BFU-E) and colony-forming unit-erythroid (CFU-E) as well as colony-forming unit-granulocyte/macrophage (CFU-GM), the following scoring criteria [1, 6] were used:

— CFU-E consists of less that 65 cells which are fully hemoglobinized after 14 days of incubation;

— BFU-E consists of more than 65 cells which form colonies with at least two clusters or bursts. They could or could not be fully hemoglobinized after 14 days of incubation;

— CFU-GM colonies are morphologically identified as having a dense central core of not well distinguished undifferentiated cells, surrounded by a less dense halo of more differentiated myeloid cells (granulocyte/macrophages).

3. Statistical analysis

To investigate quantitatively whether the applied doses of IFN-γ influence significantly and in different ways hematopoietic colony formation, the *Student's t-test* was used. A difference is assumed to be significantly large if $p<0.05$.

Results and Discussion

a) Influence of IFN-γ on the formation of erythroid - BFU-E and CFU-E colonies

The erythropoietic effect of the IFN-γ was better expressed when the cytokine was added *in vitro* in small portions (200 and/or 400 U/ml - every second day); weaker stimulatory effect was observed at high single dose — 5000 U/ml.

The stimulatory effect of IFN-γ was well defined quantitatively in both culturing conditions: at low doses — $p<0.005$, at high dose — $p<0.05$ for purified and $p<0.001$ for enriched cells (Table 1).

Table 1. Quantitative analysis of the influence of IFN-γ on erythroid and myeloid colony formation

Type of colonies	Cell type	Culture medium	Control samples	IFN-γ concentration		
				5000 U/ml once	400 U/ml/2 d	200 U/ml/2 d
	CD34+		Number of hematopoietic colonies / 0.5 ml			
BFU-E	— purified	Agar-LCM	27	49.25***	47.75*	47.7*
		RC	59	70.25	83.5**	79.75**
	— enriched	Agar-LCM	37.75	50.5*	62**	59.5****
		RC	95	141****	144.5****	143***
CFU-GM	— purified	Agar-LCM	44.25	52.25	43.5	41.5
		RC	23.5	27.25	36**	34.5*
	— enriched	Agar-LCM	19.7	30.5*	34*	35.75***
		RC	17.5	30.5**	27**	23

* $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.005$.

BFU-E showed higher proliferation and colony formation activities (2.2 fold increase for purified and 2.5 — for enriched cells) when the erythroid progenitors were cultured in RC compared to Agar-LCM ($p<0.005$ for purified and $p<0.001$ for enriched). When the cytokine was added to cultured in Agar-LCM purified cells a 1.8 fold increase of colony formation was registered (at dose 5000 U/ml $p<0.001$ and $p<0.05$ for the low doses respectively). When the same population was cultured in RC a 1.2 to 1.4 increase (5000 U/ml IFN-γ; 200 and 400 U/ml/2 days respectively) was observed.

The results from the quantitative analysis (presented in Fig. 1a, b) have shown that the addition of IFN-γ to the semi-solid agar cultures stimulated the formation of the immature erythroid colonies by BFU-E progenitors.

Unlike high cytokine concentrations, low doses of IFN-γ have more significant synergistic effects with SCF and IL-3 in the RC culture medium. The quantitative analysis for the enriched CD34+ hematopoietic cells has shown that when they were cultured in Agar-LCM, the addition of IFN-γ resulted in 1.3 (5000 U/ml IFN-γ) and 1.6 (200/400 U/ml/2 days) fold increase in the number of erythroid colonies. In RC culture conditions, a 1.5 increased colony formation was seen.

The results presented in Fig. 2a, b show that CFU-E colonies develop better in Agar-LCM. In case of the enriched cells a clear stimulatory affect of the cytokine is observed. For the purified cells only the high dose IFN-γ (5000 U/ml) stimulated insignificantly

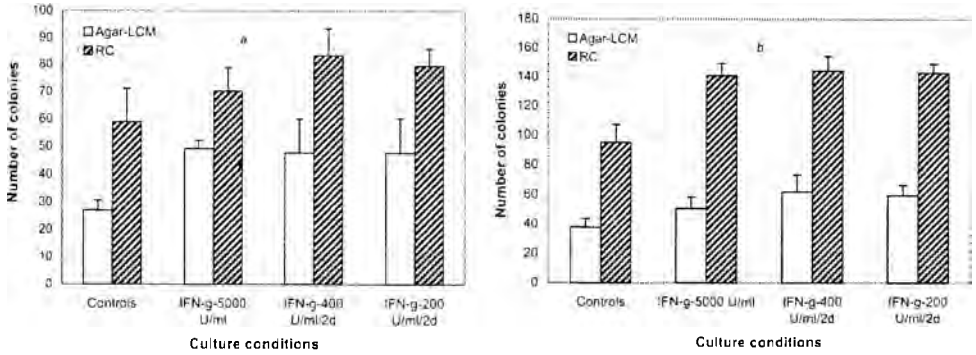

Fig. 1. BFU-E colonies formed by cultured in Agar-LCM and RC
a — purified and *b* — enriched CD34+ hematopoietic progenitor cells

colony formation in Agar-LCM. Therefore, the effect of IFN-γ is possibly inhibitory for both cell populations cultured in RC. This means that the culture conditions are important for the stimulatory or inhibitory effect of the cytokine.

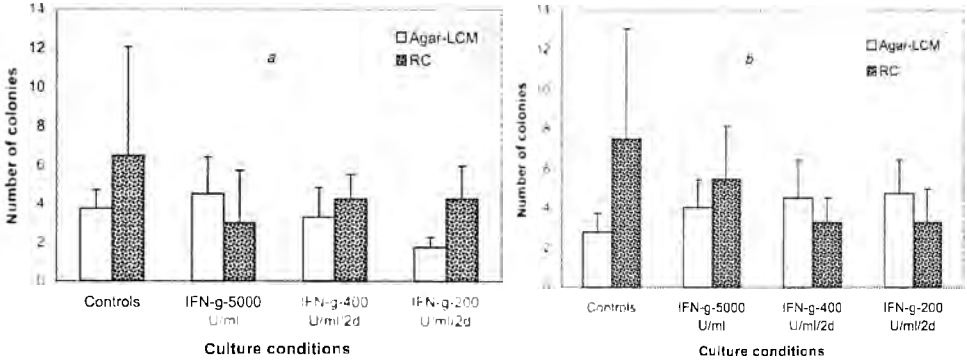

Fig. 2. CFU-E colonies formed by cultured in Agar-LCM and RC
a — purified and *b* — enriched CD34+ hematopoietic progenitor cells

The degree of cell maturity (differentiation) can be also a determining factor for the IFN-γ effect *in vitro*: more IFN-γ receptors are found on mature red blood progenitors [11].

b) Influence of IFN-γ on the formation of mixed-granulocyte/macrophage colonies (CFU-GM)

IFN-γ added *in vitro* at single high dose (5000 U/ml) stimulated significantly CFU-GM colony formation from purified CD34+ hematopoietic cells (1.2 fold increase in Agar-LCM). The same cytokine dose enhanced myeloid colony formation by enriched cells (1.5 fold, $p<0.05$) as well. The effect of IFN-γ was even stronger (1.7 to 1.8 fold increase) when the low concentrations were used (200 and/or 400 U/ml/2 days; $p<0.001$ and $p<0.05$, respectively — Table 1). In the latter case, a possible influence of the accessory mononuclear cells (in the buffy coat fraction) could be considered [9]. Surprisingly, no such stimulatory effect was observed for the purified CD34+ cells in the same culture conditions.

The addition of exogeneous IFN-γ at single dose 5000 U/ml stimulated 1.2 times myeloid colony formation by purified cells, cultured in RC. The same cytokine dose increased 1.7 times the number of CFU-GM colonies developing from enriched hematopoietic cells in the RC culture medium. In these experimental conditions, the low IFN-γ concentrations stimulated myeloid colony formation by the enriched cells: 1.3 times at dose 200 U/ml/2 days and 1.5 fold — at 400 U/ml/2 days. The same low cytokine concentrations increased 1.5 times the number of GM-colonies by purified CD34+ cells.

The quantitative analysis has shown that more CFU-GM colonies were formed when the hematopoietic cells (purified and enriched) were cultured in Agar-LCM than in RC ($p<0.05$ for purified and insignificant difference — for enriched). When the purified CD34+ cells were cultured in Agar-LCM a 1.9 fold increase in the number of myeloid colonies was observed (compared to that in RC). For the enriched cells in Agar-LCM a 1.1 fold increase was seen.

The results show (Fig. 3a, b) that the *in vitro* addition of high and low doses IFN-γ stimulated quantitatively but in a different manner mixed CFU-GM colony formation by human CD34+ hematopoietic progenitor cells.

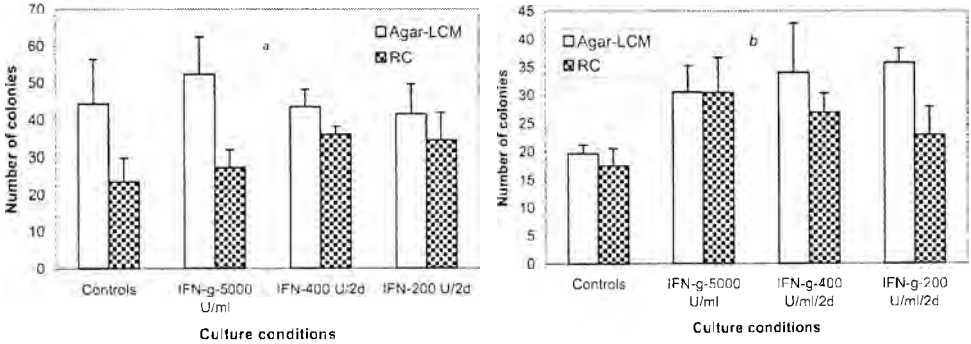

Fig. 3. CFU-GM colonies formed by cultured in Agar-LCM and RC
a — purified and *b* — enriched CD34+ hematopoietic progenitor cells

Conclusions

The effects of different doses IFN-γ exogeneously added to *in vitro* cultured (in semi-solid agar) human CD34+hematopoietic progenitor cells cannot be described as "only stimulatory or inhibitory". Our results are in agreement with the data that the influence of the cytokine on erythroid (BFU-E and CFU-E) and myeloid (CFU-GM) colony formation depends on IFN-γ doses, degree of hematopoietic cell maturity as well as on cell culture conditions.

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