



Effect of Granulocyte-Macrophage Colony-Stimulating Growth Factor on Interferon and Tumor Necrosis Factor Production in Whole Blood Cell Cultures of Patients with Acute Myelogenous Leukemia

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Abstract. The effect of recombinant human granulocyte-macrophage colony-stimulating growth factor (rHuGM-CSF) treatment on *in vitro* interferon (IFN) and tumor necrosis factor (TNF) production in peripheral blood cells of 46 patients with acute myelogenous leukemia (AML) was examined. GM-CSF significantly enhanced virus-induced IFN- α production in blood cells (containing 68% of blasts) of 28 patients with M4-M5 AML according to the French-American-British (FAB) classification and also phytohemagglutinin (PHA)-induced IFN- γ production in blood cells (containing 70% of blasts) of 18 patients with AML M0-M3 type. In control blood cells (25 healthy persons) GM-CSF enhanced PHA-induced IFN- γ but did not influence IFN- α production. In the presence of GM-CSF, TNF- α titers induced with lipopolysaccharide were also higher in control blood cells but not in cells of patients with M0-M3 or M4-M5 type of AML. The significance of GM-CSF-enhanced IFN- α and IFN- γ production in antimicrobial and anti-leukemic immune reactions which can develop during GM-CSF therapy is discussed.

Key words: acute myelogenous leukemia; GM-CSF; interferon; tumor necrosis factor.

Introduction

Over the past decade numerous studies have attempted to approach of granulocyte-macrophage colony-stimulating growth factor (GM-CSF) therapy in acute myelogenous leukemia (AML). In most of these studies, GM-CSF was used before or during induction therapy of the patients, as well as following completion of chemotherapy until neutrophil recovery. Ran-

domized studies showed that GM-CSF applied after induction therapy was useful in augmenting neutrophil recovery in patients with AML^{8, 9, 11, 17, 20}. However, the application of GM-CSF with AML is still controversial, as leukemia cells from many AML patients have receptors for GM-CSF and may proliferate in response to GM-CSF treatment⁶. Moreover, it has been shown that GM-CSF induces IL-1 β and TNF- α production in blood leukocytes from healthy persons^{4, 10} as

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well as in blast cells isolated from the blood of AML patients. Both these cytokines may stimulate the growth of AML blasts³. On the other hand, GM-CSF exhibits several properties which can be beneficial for AML patients. Besides of supporting the growth expansion of granulocytic and monocytic colony forming units (GFU-GM) and myeloid or megakaryocytic cell colonies, especially in combination with erythropoietin (EPO), GM-CSF also enhances chemotactic responses of granulocytes and macrophages, their surface expression of adhesion molecules and activates the response to microorganisms^{2, 21}. GM-CSF as an inducer of IL-1 β and TNF- α production can also influence the T cells recognition of leukemia specific peptide sequences and modulate anti-leukemia T cell activity¹.

In contrast to GM-CSF, IL-1 and TNF- α , which stimulate AML blast proliferation, interferon α (IFN- α) was found to be a potent inhibitor of it, and IFN- β and IFN- γ were also recognized as such as well as inducers of their differentiation^{3, 5, 18}. However, GM-CSF was found not to influence IFN- α and IFN- γ production in whole blood cell cultures of healthy persons¹⁰.

Therefore, it was of interest to examine the influence of recombinant human (rHu) GM-CSF on the ability of whole blood cells of AML patients to produce IFN- α and IFN- γ and TNF- α *in vitro* after stimulation with the cytokine inducers: virus, phytohemagglutinin (PHA) and bacterial lipopolysaccharide (LPS), which can *in vitro* imitate the viral and bacterial infection of patients with AML during GM-CSF therapy.

Materials and Methods

Patients. The groups studied consisted of 46 newly diagnosed, untreated patients with AML (27 females, 19 males; mean age 45.7 ± 16.2 ; range: 17–82 years) and a control group of 25 age- and sex-matched healthy subjects. The diagnosis of leukemia was based on clinical, morphological and cytochemical criteria according to the French-American-British (FAB) criteria. The distribution of FAB types were: M0 = 1, M1 = 10, M2 = 5, M3 = 2, M4 = 19 and M5 = 9. Two groups of pa-

tients were formed: group I of 18 patients with M0-M3 types, and group II of 28 patients with leukemia types M4-M5. Group III was the control group. The clinical characteristics of the patients and the results of immunophenotyping of the peripheral blood leukocytes are presented in Table 1.

Cytokine production *in vitro*. Blood samples collected in tubes with heparin (20 U/ml Heparinum, Polfa) were mixed with Eagle's minimum essential medium (MEM, Gibco) supplemented with 2 mM L-glutamine, penicillin 100 U/ml and streptomycin 100 μ g/ml, to obtain a density of leukocytes 1×10^6 cells/ml, supplemented with autologous serum to obtain a 10% serum concentration, and distributed into 24-well plastic plates. Recombinant human GM-CSF (Sandoz Pharma Ltd, Basle, Switzerland) was added to the blood cell cultures to obtain a final concentration of 50 ng/ml and the cultures were incubated at 37°C for 24 h. The classical IFN inducers Newcastle disease virus (NDV), 5 TCID₅₀/leukocyte, and PHA (Sigma), 50 μ g/ml, were added and the cultures were incubated at 37°C (5% CO₂) for 24 h (virus) or 72 h (PHA). TNF production was induced by adding 10 μ g/ml LPS from *E. coli*, serotype 0111: B4 (Sigma), to the blood cell cultures. After 24 h of incubation at 37°C, supernatants were collected by centrifugation and TNF activity was measured.

Assays for cytokines. Interferon activity in blood cell culture supernatants was measured in A549 cells (ATCC CCL 185) with encephalomyocarditis virus (EMC) as the challenge. The IFN titer was defined (international unit) as the reciprocal of IFN dilution that reduced the virus's cytopathic effect by 50%. The laboratory standards of IFN calibrated against International Standard 69/19 were included in each assay. IFN type was identified in neutralization tests with the anti-IFN antibodies: canine polyclonal anti-HuIFN- α (gift of Dr. E. Lundgren, Umea, Sweden), mouse monoclonal NK2, anti-HuIFN- α (Celltech Ltd) and mouse monoclonal E-4-18 anti-HuIFN- γ (gift of Dr. Y. Yamamoto, Japan). Briefly, polyclonal and monoclonal antibodies were diluted to neutralize about 200 U/ml of IFN and mixed with an equal volume of IFN samples (diluted

Table 1. Clinical characteristics of patients and the results of immunophenotyping of blood leukocytes

Group	Age (years)	Leukocytes ($\times 10^6$ /ml)	Blast cells in blood (%)	Immunophenotyping of blood cells (%)				FAB classification of AML
				CD34	CD33	CD13	CD14	
I	49 $\pm$ 15	51.4 $\pm$ 65.0	70.9 $\pm$ 17.0	34.4 $\pm$ 33.7	57.2 $\pm$ 34.8	52.0 $\pm$ 33.0	7.9 $\pm$ 12.5*	M0-M3
II	45 $\pm$ 17	64.4 $\pm$ 76.9	68.4 $\pm$ 28.0	25.8 $\pm$ 28.6	63.0 $\pm$ 25.0	52.0 $\pm$ 25.7	40.7 $\pm$ 33.0	M4-M5

* Statistically significant difference compared with M4-M5 group at $p < 0.05$.

to have less than 100 U/ml of IFN) and incubated at 37°C for 1 h. Residual IFN activity was assayed in parallel with the original sample diluted with the medium.

TNF activity present in cell culture supernatants was measured in L929 cells (ECACC 85011425). Briefly, L929 cells were grown in 96-well microtiter plates for 24 h. The medium was removed and replaced by 50 µl/well of fresh medium with actinomycin D (final concentration: 1 µg/ml). Fifty µl of serial 3-fold dilutions of the samples examined and standard rHuTNF-α (received from the Department of Bioorganic Chemistry, Center for Molecular and Macromolecular Research, Polish Academy of Sciences, Łódź) were added in triplicate to the wells and incubated for 24 h. The cytotoxic effect of TNF was determined by using the MTT method¹⁴. The reciprocal of the highest dilution causing a destruction of cells of 50% compared with the standard was defined as 1 U of TNF. Identification of TNF type was done by neutralization with polyclonal anti-HuTNF-α antibodies (Genzyme) according to the method described above for IFN neutralization.

Expression of surface antigens. The surface antigens were assayed on fresh cells within 2 h after sampling. Unstimulated peripheral blood and bone marrow leukocytes were examined for expression of CD34, CD33, CD13 and CD14 antigens. Double color immunofluorescence studies were performed using the combination of phycoerythrin (PE) and fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (Dako, Denmark). As negative controls, IgG₂ FITC/IgG₂PE and IgG₁FITC were used. 1×10^6 cells were incubated with antibodies for 30 min at 4°C and washed twice with PBS, then incubated with 2 ml of Ortho-mune Lysing Reagent at room temperature until lysis was complete (10–15 min). All samples were measured on a Cytoron flow cytometer (Ortho Diagnostic Systems). Ten thousand cells were tested in each measurement.

Statistics. The statistical significance of the differences between the clinical data of patients, the surface antigen expression, or between the IFN and TNF titers produced in vitro with or without the presence of GM-CSF, were tested using Student's, *t* test. Significance was reported at $p < 0.05$.

Results

As can be seen from Table 1, there were no significant differences among the groups in the mean ages, the numbers of leukocytes and blasts in peripheral blood, the percentages of cells positive for CD34

antigen, which is characteristic of stem cells, and the percentages of cells bearing CD33 and CD13 antigens, which are characteristic of myeloid progenitors and committed granulocyte-macrophage progenitors, granulocytes and macrophages, respectively. A significant difference was observed only in the percentage of CD14-bearing cells, which antigen is functionally is LPS-binding protein and is characteristic for mature monocytes and granulocytes. AML patients with M4-M5 leukemia had more CD14 antigen positive cells than those with AML M0-M3 type according to the FAB classification.

Whole blood cells of patients with AML M0-M3 type (18 patients) and M4-M5 (28 patients) were cultured with GM-CSF for 24 h. Cytokine production was induced with NDV, PHA and LPS and the cultures were incubated for a further 24 h (NDV and LPS) or 72 h (PHA). The activity of IFN and TNF was measured in the supernatants and expressed in international units per 10^6 leukocytes. Neutralization tests revealed that about 80% of IFN activity present in the supernatants of control blood cells infected with virus was neutralized by anti-IFN-α antibodies. In contrast, about 90% of IFN activity present in the supernatants of control blood cell cultures was neutralized by anti-IFN-γ antibodies, but not neutralized by anti-IFN-α antibodies. Table 2 shows that GM-CSF did not change the ability of whole blood cells' cultures of the control subjects to produce IFN-α after NDV induction but significantly enhanced the ability of blood cells to produce IFN-γ after PHA induction. It is worth mentioning that GM-CSF did not induce IFN-α and IFN-γ production (data not shown). In contrast to this, the low (compared with control cells) ability of blood cells of M4-M5 AML patients to produce IFN-α was significantly enhanced by GM-CSF. In the M0-M3 group, these differences were not statistically significant because of the large individual differences in the sensitivity to GM-CSF. For example, blood cells of 5 of the 18 patients with AML M0-M3 produced less IFN-α after GM-CSF treatment than untreated cells (data not shown). GM-CSF also significantly enhanced the ability of blood cells of AML patients to produce IFN-γ in response to PHA, and this enhancement was statistically significant in the M0-M3 group and not significant in the M4-M5 group because of the large differences in the individual sensitivity of blood cells to GM-CSF treatment. When we analyzed the influence of GM-CSF on LPS-induced TNF-α production in control cells (Table 3), a significant increase was observed after GM-CSF treatment. The average titer of TNF-α produced by blood cells of patients with AML M0-M3 and the M4-M5 group was

Table 2. Effect of GM-CSF on AML patients' peripheral blood cells' ability to produce NDV-induced IFN- α and PHA-induced IFN- γ (*in vitro*)

Group	Inducers			
	NDV	NDV+GM-CSF	PHA	PHA+GM-CSF
I	480±260	668±531	263±87	403±155*
II	356±228	754±423*	284±184	406±222
Control	418±211	454±213	319±129	480±170*

Interferon U/10⁶ of leukocytes.

* Statistically significant difference compared with IFN production without GM-CSF at $p < 0.05$.

Table 3. Effect of GM-CSF on AML patient peripheral blood cells ability to produce LPS-induced TNF (*in vitro*)

Group	Inducers	
	LPS	LPS+GM-CSF
I	72±52	120±100
II	103±102	190±158
Control	58±38	113±39*

Tumor necrosis factor U/10⁶ of leukocytes.

* Statistically significant difference compared with TNF production without GM-CSF at $p < 0.05$.

also slightly higher after GM-CSF treatment, but the individual reactions were very different. TNF produced in control blood cells in response to LPS was neutralized to about 95% by anti-TNF- α antibodies.

Discussion

In the present study we investigated the influence of GM-CSF on the ability of blood cells of patients with AML to produce IFN and TNF after induction with virus, PHA and LPS in whole blood cell cultures. It is generally accepted that the concentrations of cytokines tested in such an *in vitro* experimental model depend on the balance between synthesis and consumption during the incubation time, and such a model may reflect the maximum capacity of the blood cells of certain patients to produce and consume cytokines during viral or microbial infection, making it very useful in the examination of the immune status of the organism.

Our results showed that, in the blood cells of controls, GM-CSF did not influence IFN- α production after viral induction, significantly enhanced PHA-induced IFN- γ production, but when used alone it did not induce IFN- α and IFN- γ production (data not shown). These observations confirm the results of other authors who also reported that GM-CSF alone in whole blood cells of healthy persons had no effect on IFN- α and IFN- γ secretion^{4, 10}.

When blood cells of patients with AML were exam-

ined *in vitro*, GM-CSF enhanced both IFN- α production after viral infection and IFN- γ production in response to PHA. It has been noted by other authors that not only normal blood leukocytes, but also AML blast cells can be stimulated *in vitro* by GM-CSF for increased TNF- α and IL-1 production¹, but the influence of GM-CSF on IFN production in AML blast was not examined. In our study, blast cells represented about 70% of the total leukocyte population in the blood of AML patients, so we can speculate that blast cells were also involved in the GM-CSF-stimulated IFN- α and IFN- γ production. Moreover, AML blast cells were shown to express IFN receptors, and these cytokines can influence blast proliferation and differentiation^{3, 5-7, 13, 16, 18}. Both types of IFNs are also known as inducers of MHC antigen expression on immune and tumor cells. Additionally, GM-CSF has also been described as increasing MHC class I and II antigen expression on monocytes of AML patients and increasing their cytotoxicity^{12, 22}. Therefore, we can speculate that GM-CSF may directly or indirectly induce antitumor immune reactions *in vivo* in patients with AML.

AML patients are extremely sensitive to viral infections, therefore, enhanced IFN- α and IFN- γ production after GM-CSF treatment can be considered as beneficial for them as both cytokines possess not only antiviral but also immunostimulatory activity^{11, 12}.

In our study, great differences between individual patients in their sensitivity to GM-CSF treatment were observed, and in the M0-M3 group, blood cells of 5 AML patients responded negatively to GM-CSF stimulation and produced less IFN- α than without treatment. Such large differences in individual reactions to GM-CSF and the heterogeneity of IL-1 and TNF- α secretion were also observed by others, who examined *in vitro* the sensitivity of leukemia blast cells to GM-CSF¹. We suppose that in our study not only the sensitivity of blast cells to GM-CSF but also differences in the number of granulocytes, monocytes and lymphocytes in the blood of certain patients resulted in the high variability of reaction to GM-CSF. However, we examined the GM-CSF effect on cytokine production in whole blood cell cultures at a fixed density of 1×10^6 leuko-

cytes/ml, and this method allowed us to measure the potency of the whole blood cell population of certain patients to produce cytokines.

The role of TNF- α in AML is complicated and controversial. In our study, GM-CSF significantly enhanced TNF- α production in control blood cells after LPS induction, consistent with observations by others^{4, 10}, but only slightly affected TNF- α production in the blood cells of AML patients. TNF- α was found to be a potent modulator of AML blast growth. Moreover, some AML blasts constitutively secrete TNF- α as an autocrine growth stimulator, especially in the presence of GM-CSF³. On the other hand, TNF- α can also inhibit AML blast proliferation, and rare cases of spontaneous remission in AML have been connected with a high endogenous TNF- α production^{3, 15, 19}. Therefore, the lack of influence of GM-CSF on TNF- α production can be considered as beneficial for the patient.

Summing up, the GM-CSF-enhanced IFN- α and IFN- γ production observed in our study can be considered as beneficial for AML patients, especially when GM-CSF is used as a supportive measure to abrogate therapy-induced cytopenias and to prevent patient mortality from viral or bacterial infections.

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