Cytokine Production in Whole Blood Cell Cultures of Patients with B-Lineage Acute Lymphoblastic Leukemia. The Influence of Granulocyte-Macrophage Colony-Stimulating Factor

Teresa Kamińska¹, Iwona Hus², Anna Dmoszyńska² and Martyna Kandefer-Szerszeń¹*

¹Department of Virology and Immunology, Institute of Microbiology and Biotechnology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland, ²Department of Hematology, University School of Medicine, Jacekswkiego 8, 20-950, Lublin, Poland

Abstract. We investigated the levels of 6 different cytokines in the sera of 10 newly diagnosed patients with B cell lineage acute lymphoblastic leukemia (ALL) and detected a significant increase in IL-6 and IFN-α serum levels in comparison to that of healthy controls. Whole blood cell cultures of 10 ALL patients and 20 control individuals were induced with classical cytokine inducers, such as virus, PHA and LPS, and their ability to produce 9 different cytokines was compared. Blood cells of ALL patients produced significantly less IL-1α, IL-1β, IL-10 and TNF-α than control cells and not significantly lower levels of IL-6, but comparable with control levels of IL-2, IL-4. rHuGM-CSF added to cell cultures 24 h before induction significantly enhanced the production of IL-1α, IL-1β and TNF-α in controls, but only IL-1α and IL-1β in the blood cell cultures of patients with ALL. GM-CSF did not significantly influence the production of IFN-α, IFN-γ, IL-2, IL-4 and IL-10 in the control cells and the cells of ALL patients. The patients examined differed not only in the expression of CD10 and CD34 antigens on blast cells, but also in the reaction to GM-CSF treatment, which was found as very high standard deviation values. We suppose that these differences can partially explain the different effects of GM-CSF when used to ameliorate neutropenia of ALL patients after chemotherapy and to reduce the incidence of microbial infections.

Key words: GM-CSF; acute lymphoblastic leukemia; IL-1; IL-2; IL-4; IL-6; IL-10; TNF-α; IFN-α; IFN-γ.

Introduction

Acute lymphoblastic leukemia (ALL) is a heterologous disease with distinct biologic and clinical features displayed by various subtypes of leukemic cells. Roughly 75% of cases of adult ALL are of B cell lineage which can be identified by expression of CD19 antigen. Most are also CD10 positive (the common ALL (cALL) antigen) and some express CD34, the stem cell-associated antigen. Acute B-lineage ALL is

Author’s fees were financed by the Association for the Joint Administration of Copyright KOPIPOL (Kielce, Poland) from funds collected on the basis of the Law on Author’s Rights.

* Correspondence to: Prof. Martyna Kandefer-Szerszeń, Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland, tel.: +48 81 537 59 36, e-mail: KANDEM@BIOTOP.UMCS.LUBLIN.PL
defined as an uncontrolled proliferation of B cell precursors that lack the capacity to differentiate to mature cells.

Management of ALL patients may include the growth factors (GFs) such as G-CSF and GM-CSF to reduce post-chemotherapy aplasia. A potent risk of their administration is a stimulatory signal on the leukemic population. The results of some studies have suggested that GFs may affect both myeloid and lymphoid leukemic cells. ALL blast cells were detected to have receptors for G-CSF and GM-CSF and can exhibit autocrine or paracrine proliferative response to them. On the other hand, no stimulation of ALL cell lines or ALL blasts by GM-CSF or PIXY-321 (GM-CSF/IL-3 fusion protein) was observed by other authors. It seems likely that ALL blasts, which proliferate in response to GM-SCF express myeloid antigens (My+ALL) or are Ph1 positive. In addition to GM-CSF and G-CSF, other cytokines, such as IL-6, have a growth-promoting activity on My+ALL blasts. Also, IL-1 and TNF-α can support survival and stimulate DNA synthesis in adult cALL blasts in vitro. In contrast to the cytokines mentioned above, IL-4 was detected to suppress the growth of most ALL blasts by blocking the production of autocrine or paracrine growth-promoting cytokines as well as by direct inhibition of the growth of neoplastic cells.

To date, several studies have examined the role of GM-CSF and G-CSF in the proliferation of ALL blasts, but only limited data is available regarding the production of cytokines by ALL blasts or blood cells of patients with ALL before chemotherapy or after GM-CSF treatment to ameliorate chemotherapy-induced neutropenia.

The main purpose of the present study was to elucidate the influence of GM-CSF on the cytokine production ability of peripheral blood cells of patients with B-lineage ALL, especially cytokines which can be involved in stimulation of peripheral ALL growth, such as IL-1, IL-6 and TNF-α, or such which can inhibit ALL growth, such as IL-4. Peripheral blood cell cultures in vitro were treated with strong cytokine inducers such as virus, PHA or bacterial LPS in order to mimic microbiological infections or antigenic stimulation, which are very common in patients with ALL. The cytokine levels produced by the peripheral blood cells of patients with ALL were then compared with those produced by blood cells of healthy persons. Additionally, serum levels of some cytokine was examined in ALL patients and compared with those detected in control subjects.

Materials and Methods

Patients. Peripheral blood specimens from 10 patients with B-lineage ALL were obtained at diagnosis after written informed consent according to institutional guidelines. Leukemia was diagnosed and classified according to the criteria of the French-American-British (FAB) Cooperative Group. Normal peripheral blood specimens were obtained from 20 healthy persons after informed consent.

Immuno phenotyping. Cell surface antigens were detected by flow cytometry following staining with monoclonal antibodies to B-lineage-associated antigens (CD10, CD19) and stem-cell-associated antigen (CD34). Antigen expression was calculated on a gated population based on light-scattering characteristics (blast cell gate). Double color immunofluorescence studies were performed using a combination of phycoerythrin (PE) and fluorescein isothiocyanate (FITC) conjugated monoclonal antibodies (Dako). As a negative control, IgG, FITC/IgG,PE and IgG,FITC were used. Cells (10⁶) were incubated with antibodies for 30 min at 4°C and washed twice with PBS and 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 Cytomix flow cytometer (Ortho Diagnostic Systems). Ten thousand cells were tested in each measurement. The clinical characteristics of the patients and the results of phenotyping are presented in Table 1.

Cytokine production in vitro. Blood samples collected in tubes containing heparin (20 U/ml Heparinum Polfa) were mixed with Eagle’s Minimum Essential Medium (MEM) supplemented with 2 mM L-glu-

Table 1. Clinical characteristics of patients and the results of blast cells immunophenotyping

<table>
<thead>
<tr>
<th>Number of patients</th>
<th>Age (years)</th>
<th>Leukocyte number x10⁹/l</th>
<th>Blast cells in blood (%)</th>
<th>Immunophenotyping</th>
<th>Leukemia type (FAB)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>33.1±6.30</td>
<td>53.8±25.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>17–78</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6 females, 4 males)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* An antigen was considered to be positive if more than 20% of cells had fluorescence intensity above the isotypic control.
tamine, penicillin 100 U/ml and streptomycin 100 µg/ml to obtain a leukocyte density of 10⁶ cells/ml, supplemented with autologous serum to obtain a 10% concentration and distributed into 24-well plastic plates. Recombinant human GM-CSF (Sandoz Pharma Ltd, Basle, Switzerland) was added to blood cell cultures to obtain a final concentration of 50 ng/ml. The cultures were incubated at 37°C for 24 h. The classical cytokine inducers Newcastle disease virus (NDV), 5 TCID₅₀/leukocyte (inducer of IFN-α); phytohemagglutinin (PHA Sigma), 50 µg/ml (inducer of IFN-γ), IL-2, IL-4 and lipopolysaccharide from E. coli, serotype 0111: B4 (LPS, Sigma), 10 µg/ml (inducer of IL-1α, IL-1β, IL-6, IL-10 and TNF-α) were added and the cultures were incubated at 37°C (5% CO₂) for 24 h (virus, LPS) or 72 h (PHA). Supernatants were collected by centrifugation and cytokine activity was measured.

**Assay for cytokines.** Levels of cytokines were measured in supernatants from the whole blood cell cultures by the enzyme-linked immunosorbent test (ELISA) utilizing microtiter plate technology according to the instructions provided by the manufacturers. The IL-1β, IL-2, IL-4, IL-6, IL-10 and TNF-α Predicta Kits were obtained from Genzyme (Cambridge, MA, USA). The IL-1α and IFN-α ELISA kits were from Endogen (Woburn, MA, USA). The detection limits were: IL-1α 2 pg/ml, IL-1β 3 pg/ml, IL-2 4 pg/ml, IL-4 6 pg/ml, IL-6 2 pg/ml, IL-10 5 pg/ml, IFN-α 3 pg/ml, IFN-γ 3 pg/ml and TNF-α 3 pg/ml. Optical densities were measured and the amounts of cytokines were calculated from standard curves using the SOFT max program on the Molecular Devices ELISA Reader (Menlo Park, CA, USA).

**Statistics.** The statistical significance of the differences between the cytokine titers produced with or without the presence of GM-CSF as well as in comparison with the control were tested using Student’s t-test. Significance was reported at p<0.05.

### Results

Of the 10 patients diagnosed as suffering from B-lineage ALL, 5 were classified as L1 and 5 as L2 type of ALL according to the FAB classification. Peripheral blood blast cells of 4 patients expressed CD10 antigen and 6 were CD10 negative. Three patients had blast cells expressing CD34 antigen. The mean results from phenotyping are presented in Table 1.

We measured cytokine levels present in the sera of 10 newly diagnosed patients with ALL and 20 healthy control subjects and detected significantly higher levels of IL-6 and IFN-α in comparison with the controls. No significant differences in the levels of IL-1α, IL-2, IL-10 and TNF-α were detected (Table 2).

The whole blood cell cultures were also induced for cytokine production with virus, PHA or LPS. As can be seen from Table 3, the blood cells of ALL patients produced normal levels of IFN-α, IFN-γ, IL-2 and IL-4 in response to cytokine inducers, but significantly lower levels of IL-1α, IL-1β, IL-10 and TNF-α than those produced by the blood cells of the control subjects, and IL-6 levels which were statistically insignificantly lower.

A part of the blood cell cultures was also treated with GM-CSF and, after 24 h of incubation, induced with virus, LPS or PHA. GM-CSF significantly enhanced the production of some cytokines in control cells: the levels of IL-1α, IL-1β and TNF-α produced by cells treated with GM-CSF were higher than those of cells not treated. GM-CSF also enhanced cytokine production in blood cells of ALL patients. The levels of IL-1α and IL-1β were significantly higher in the presence of GM-CSF, as well as those of IL-2, IL-4, IL-6, IL-10 and TNF-α, but the differences in comparison to cells not treated with GM-CSF or to the controls were not statistically significant because of the very high individual differences in the sensitivity to GM-CSF and the individual differences in response to the cytokine inducers. It should be noted that the levels of all cytokines which were enhanced by GM-CSF in the blood cells of ALL patients never reached the titers observed in the control blood cells induced in the presence of GM-CSF.

### Discussion

We examined the levels of some cytokines in the circulation of patients with newly diagnosed, untreated B-lineage ALL in order to find the abnormalities present in ALL with regard to the secretion of T cell and monocyte-derived cytokines. The results demonstrated...
significantly increased levels of IL-6 and IFN-α. The presence of elevated levels of IFN in the sera of patients with ALL and other hematological malignancies has also been described by other authors, but the origin and the endogenous inducer of this serum IFN in leukemia patients is still unknown. Regarding IL-6, which is a pro-inflammatory cytokine secreted normally by monocytes/macrophages and activated T cells, the enhancement of its serum level has been described in various hematological malignancies as well as in ALL patients after cytoreductive therapy. However, very high IL-6 levels correlated mainly with fever and infections but not with the stage of disease nor blood parameters. In our study, the IL-6 levels detected in the sera of ALL patients were not high, so we can speculate that leukemia blasts can modulate T cell function and alter the cytokine profile and amounts produced by these cells.

In our study we also measured the ability of whole blood cell cultures of patients with ALL to produce cytokine after in vitro induction with virus, LPS and PHA and detected a significant defect in the production of IL-1α, IL-1β, IL-10 and TNF-α. Theoretically, the decrease of the cytokine levels in the supernatants during cell culture might be due to a short half-life of the protein, the binding to cell surface receptors, an elimination of cytokine-producing cells or an exhaustion of the cells to produce these cytokines, or to a combination of these factors. As serum overproduction concerned IL-2, IL-6 and IFN-α but not the cytokines mentioned above, we can suppose that an exhaustion of cells was not the reason for the defect observed. As leukemic B cell precursors constitutively express functional receptors for IL-1, it is possible that the defect in IL-1 levels in supernatants from blood cell cultures of ALL patients was partially caused by IL-1 binding by normal B, normal T and leukemic blasts. However, we cannot exclude that also the number of cytokine-producing cells or their ability to produce IL-1 was affected in ALL patients. As IL-1β, besides IL-2 and IFN-γ, is a very important cytokine which not only enhances NK cell activity but also activates T cells, a defect in its production can partially explain the low NK cell activity observed in ALL patients.

IL-10 is a cytokine produced by human T lymphocytes, B lymphocytes and monocytes. It has pleiotropic effects and can exert both immunosuppressive and immunostimulatory effects on different cell types. IL-10 increases the proliferation of immunoglobulin secretion from normal B lymphocytes and is an enhancing cytokine for the development of cytotoxic cells. The role of IL-10 in ALL was not intensively examined; however, as common ALL blast cells were shown to highly express IL-10, its role in some types of leukemia is considered. We suppose that the defect in IL-10 production observed in our study can contribute to at low T cell cytotoxicity.

The role of TNF-α in ALL is very complex. On the one hand, endogenous production of TNF-α in ALL blasts is connected with their resistance to TNF and chemotherapy. Moreover, a high serum level of TNF can be a predictor of early disease relapse. On the other hand, TNF-α is a very important cytokine in the regulation of B cell functions and in the antimicrobial activity of neutrophils, monocytes and macrophages. The defect in TNF-α production observed in our study can thus partially explain the high susceptibility of ALL patients to microbial infections.

Chemotherapy-induced neutropenia is a very serious problem in the treatment of leukemia. Recombinant GFs have been introduced into the treatment of myelo-suppression in leukemic patients. In controlled clinical trials, not only enhancement of neutrophil recovery, but also improvement of the median duration of fever in patients with ALL, reduction of incidence of mucositis, fungal and bacterial infections have been observed. However, there are several controversies regarding the stimulatory effects of GM-CSF on leukemic blast growth. In some studies GM-CSF did not stimulate ALL blast proliferation, or it stimulated only a part of the bone marrow samples from ALL patients. Probably only My+ALL blasts expressing myeloid antigens or Ph1 positive can be stimulated by such cytokines as GM-CSF, IL-3 or IL-6. It seems likely that IL-1 and TNF-α can also stimulate B cell lineage ALL blast growth, and IL-4 can inhibit it in vitro and in vivo or induce apoptosis of human B cell precursors.

GM-CSF alone is known to stimulate several immune functions, such as macrophage and monocyte

<table>
<thead>
<tr>
<th>Patients</th>
<th>GM-CSF</th>
<th>IFN-γ</th>
<th>GM-CSF</th>
<th>IL-1α</th>
<th>GM-CSF</th>
<th>IL-1β</th>
</tr>
</thead>
<tbody>
<tr>
<td>ALL</td>
<td>698±738</td>
<td>718±695</td>
<td>1101±648</td>
<td>1280±598</td>
<td>93±84</td>
<td>188±2120</td>
</tr>
<tr>
<td>Control</td>
<td>804±441</td>
<td>822±567</td>
<td>1636±203</td>
<td>1705±308</td>
<td>303±170</td>
<td>614±243</td>
</tr>
</tbody>
</table>

T. Kamińska et al.: GM-CSF Enhance IL-1 Production in Blood Cells of ALL Patients
chemotactic and cytotoxic activities, both \textit{in vitro} and \textit{in vivo}\textsuperscript{18}, but it also stimulates IL-1\(\alpha\), IL-1\(\beta\) and TNF-\(\alpha\) production in the blood cells of healthy persons\textsuperscript{23}, and these cytokines are known to stimulate pro-inflammatory reactions. In our study we measured the influence of GM-CSF on cytokine production induced with virus and bacterial LPS as models of microbial infections and PHA as a model of antigenic stimulation. We chose those cytokines which can be involved in the stimulation or inhibition of ALL blast growth, such as IL-1, TNF-\(\alpha\) and IL-4, and those which can influence the antimicrobial immune reactions, such as IL-2, IL-6, TNF-\(\alpha\) and IL-10.

In our study GM-CSF significantly enhanced the production of IL-1\(\alpha\) and IL-1\(\beta\) in ALL patients. It also increased TNF-\(\alpha\) production, but, because of the very high standard deviation, the differences in comparison to blood cells not treated with GM-CSF were not statistically significant. No significant influence of GM-CSF on the production of other cytokines, such as IFN-\(\alpha\), IFN-\(\gamma\), IL-4, IL-6, IL-10, was observed, mainly due to the very high standard deviations caused by the individual differences in response to GM-CSF and the cytokine inducers. It should be noted that these results are in agreement with those of other papers in which the stimulatory effect of GM-CSF on IL-1 and TNF production in blood cell cultures of healthy persons was described\textsuperscript{23}. Considering the role of cytokine production enhanced by GM-CSF in ALL blast growth, it seems likely that GM-CSF can potentiate the growth of some ALL blast cells by stimulation of IL-1 production, especially because the stimulation of IL-1 production was not accompanied by increased IL-4 production. On the other hand, GM-CSF-stimulated IL-1 production seems to be the main reason of the incidence reduction of fungal and bacterial infections observed in ALL patients treated with GM-CSF\textsuperscript{12, 27}.

Summing up, B cell lineage ALL patients differed in the type of leukemia according to FAB classification, CD10 and CD34 antigen expression, the number of leukocytes and blasts in their blood. Despite of these differences, a statistically significant elevation in plasma IFN-\(\alpha\) and IL-6 was detected. When blood cells of ALL patients were induced \textit{in vitro} to cytokine production, a defect in IL-1\(\alpha\), IL-1\(\beta\), IL-10 and TNF-\(\alpha\) was detected. GM-CSF significantly increased IL-1\(\alpha\) and IL-1\(\beta\) production, but both cytokine levels were still lower than those produced in control cell cultures and did not significantly influence the production of IL-4, IL-6, IL-10, IFN-\(\alpha\), IFN-\(\gamma\) and TNF-\(\alpha\). Very large differences in individual reactivity to GM-CSF treatment and to cytokine inducers such as virus, LPS and PHA were detected.

\textbf{References}


33. RAISBACH G., CAMP T., WELZI G., GEEZ C., ABEDI-NPOUR F., LODRI A., KAROTH W., DORMER P. and NERL C. (1996): Regu-


Received in February 2000
Accepted in April 2000