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Two-color Flow Cytometric Analysis of Cytokine Receptors and Surface Markers on Blood Cells

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Summary. **In order to analyze the expression of cytokine receptors on a specified cell population, two-color flow cytometric analysis of cytokine receptors and surface markers using fluorescence labeled cytokines and monoclonal antibodies was performed. In normal peripheral blood cells, granulocytes expressed granulocyte colony-stimulating factor (G-CSF) receptor and interleukin-6 (IL-6) receptor, but mostly expressed interleukin-3 (IL-3) receptor weakly. Monocytes were positive for each receptor. In lymphocytes, CD3 positive T cells did not express any of these receptors; however, CD19 positive B cells expressed G-CSF receptor, IL-3 receptor, and partly expressed IL-6 receptor. In clinical cases, leukemic cells of acute myeloid leukemia (AML) patients tended to show the same characteristics as normal granulocytes and, in common acute lymphoblastic leukemia (ALL), B cell type chronic lymphocytic leukemia (CLL), leukemic cells tended to show the same characteristics as normal B cells. This shows that G-CSF receptors are expressed not only on myeloid cells but also on normal and leukemic B cells. Results show that two-color flow cytometry accompanied with surface marker analysis allows for the easy analysis of the expression of cytokine receptors on the specified cell population and the obtaining of useful information for clinical cytokine therapy.**

Key words—two-color flow cytometry, cytokine receptors, fluorescence labeled cytokines, surface markers.

INTRODUCTION

In recent years, the characteristics and the actions of various cytokines have been ascertained and their clinical applications are now in progress. Since the cytokines are considered to act through their specific receptors, analysis of cytokine receptors is very important for cytokine therapy. Traditionally, analysis of cytokine receptors has been done by radioreceptor assay using radiolabeled cytokines.[1-8] This, however, requires many samples, much time, and can be used only for analyzing bulky populations. Therefore, analysis of cytokine receptor expression on a specified cell population such as B cells is difficult. Recently, flow cytometric analyses using biotinylated cytokines have been reported.[9-16] By this method, a more sensitive analysis can be easily done. More recently, cytokines directly labeled by fluorochrome have become available. The expression of these cytokine receptors on the specified cell populations was therefore studied by two-color flow cytometry using monoclonal antibodies and these labeled cytokines.

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MATERIALS AND METHODS

Preparation of cells

Peripheral blood cells were obtained from 10 healthy volunteers in our department. White blood cells were obtained by hemolysing using a hemolysis buffer containing ammonium chloride and washing twice with RDF1 buffer (R & D Systems, Minneapolis, MN). In clinical cases, peripheral blood cells or bone marrow cells were used.

Fluorescence staining of cells

Detection of cytokine receptors was performed using phycoerythrin (PE) labeled granulocyte colony-stimulating factor (G-CSF), interleukin-3 (IL-3), and interleukin-6 (IL-6) (R & D Systems, Minneapolis, MN).

To detect cytokine receptor expression, typically 100 μl of cell suspension (4×10^6 cells/ml) were incubated with PE-labeled cytokines and fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies for 60 min at 4℃. After incubation, cells were washed twice with RDF1 buffer and resuspended in 500 μl of PBS. As negative control, cells incubated with streptavidin-PE alone were used.

Flow cytometry

Receptor expression and surface marker analysis were performed by single or two-color flow cytometry using FACScan (Becton Dickinson, San Jose, CA). The data were analyzed using LYSIS II software (Becton Dickinson).

RESULTS

Cytokine receptor expression on normal peripheral blood cells

Cytokine receptor expression was analyzed on granulocytes, monocytes, and lymphocytes identified by their characteristics on scattergram. The results are shown in Table 1. Most granulocytes expressed G-CSF receptor and IL-6 receptor, but the expression of IL-3 receptor was weak (Fig. 1). Monocytes were positive for the three receptors (Fig. 2). Most lymphocytes were negative for each receptor but there were small numbers of cells which were positive (Fig. 3). By two-color analysis, though CD3 positive T cells hardly expressed any of these receptors, there were cells which were positive for each receptor in CD3 negative fraction (Fig. 4), and CD19 positive B cells expressed G-CSF receptor, IL-3 receptor, and partly expressed IL-6 receptor (Fig. 5). Specificities of labeled cytokines were confirmed by competitive blocking assay using a 100-fold molar excess of unlabeled cytokines which resulted in the shift of the fluorescence histogram to the negative control peak (data not shown).

Cytokine receptor expression on leukemic cells

In clinical cases, cytokine receptor expression was analyzed on leukemic cells by gated analysis using a scattergram. The results are shown in Table 2. Though the patient number was small, leukemic cells in acute myeloid leukemia (AML) and myeloid crisis of chronic myelogenous leukemia (CML) tended to show the same characteristics as normal granulocytes, and in common acute lymphoblastic leukemia (ALL), B cell type chronic lymphocytic leukemia (CLL), leukemic cells tended to show the same characteristics as normal B cells. Fig. 6 shows the result of the two-color analysis in one B-CLL case.

Table 1. Cytokine receptor expression on normal peripheral blood cells

Sample No.	Cytokine receptor positivity (%)								
	Granulocytes			Monocytes			Lymphocytes		
	G-CSFR	IL-3R	IL-6R	G-CSFR	IL-3R	IL-6R	G-CSFR	IL-3R	IL-6R
1	96.2	28.9	91.9	91.2	87.0	93.4	9.7	11.0	15.0
2	99.4	45.3	96.8	84.3	86.6	95.1	11.1	14.8	25.4
3	87.6	38.4	70.7	88.4	91.6	80.6	21.3	21.6	12.0
4	96.3	53.7	52.7	94.3	93.8	83.4	9.1	12.5	12.8
5	97.3	60.9	66.5	94.9	94.0	91.3	10.8	17.7	7.0
6	87.0	7.9	64.1	96.9	91.2	94.1	11.1	12.7	6.4
7	88.5	40.3	84.0	89.8	93.4	92.5	18.7	20.7	19.6
8	93.3	40.6	73.4	88.1	93.2	75.2	14.2	19.6	11.1
9	91.3	33.3	58.4	93.6	90.3	83.1	8.8	10.9	10.0
10	93.5	42.8	86.8	91.3	91.6	87.4	10.2	11.7	8.3
Mean	93.0	39.2	74.5	91.3	91.3	87.6	12.5	15.3	12.8
SD	4.3	14.6	14.8	3.8	2.7	6.8	4.3	4.2	5.9

G-CSFR, G-CSF receptor; IL-3R, IL-3 receptor; IL-6R, IL-6 receptor.

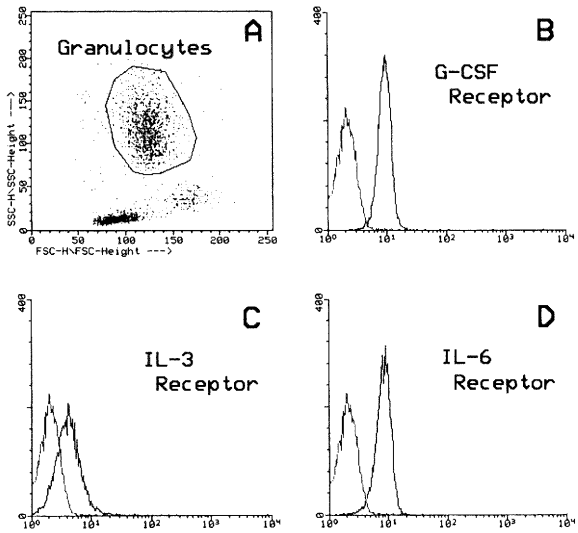

Fig. 1. Cytokine receptor expression on normal granulocytes. Typical fluorescence profiles of normal granulocytes stained for G-CSF, IL-3, and IL-6 receptor expression (**B, C, D** respectively). Normal peripheral white blood cells were incubated with PE-labeled G-CSF, IL-3, and IL-6 respectively. Granulocytes were identified by their light scattering property (**A**). The thin line histograms are negative controls incubated with streptavidin-PE alone. Horizontal axis: forward light scatter (**A**). Fluorescence intensity (**B, C, D**). Vertical axis: right angle light scatter (**A**). Relative number of cells (**B, C, D**).

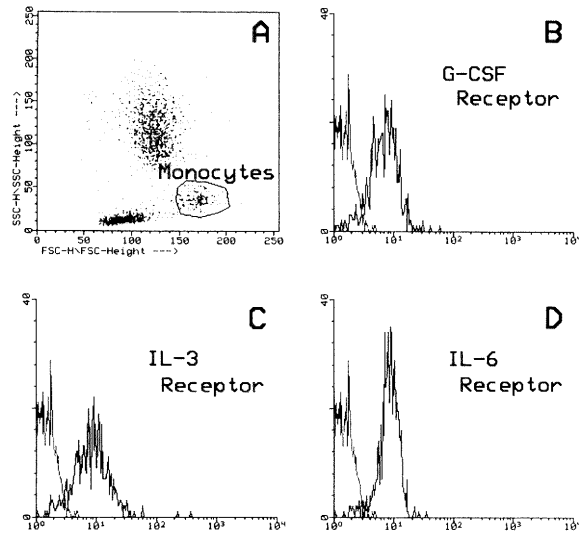

Fig. 2. Cytokine receptor expression on normal monocytes. Typical fluorescence profiles of normal monocytes stained for G-CSF, IL-3, and IL-6 receptor expression (**B, C, D** respectively). Methods of cell staining, cell identification, and histograms of negative controls are as indicated in the legend for Fig. 1. Annotation of the horizontal and the vertical axes are also as indicated in the legend for Fig. 1.

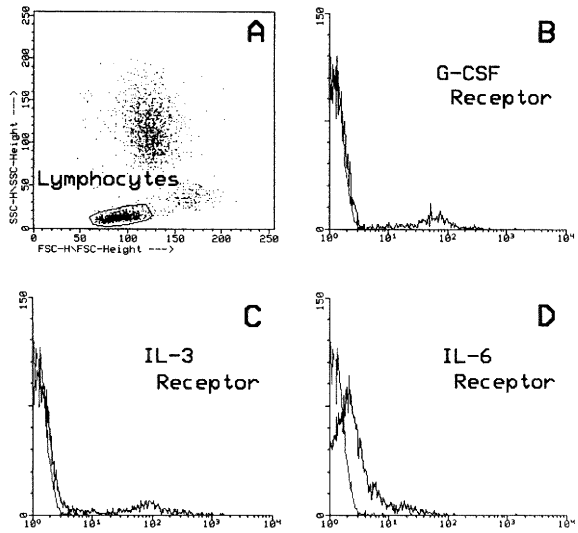

Fig. 3. Cytokine receptor expression on normal lymphocytes. Typical fluorescence profiles of normal lymphocytes stained for G-CSF, IL-3, and IL-6 receptor expression (**B, C, D** respectively). Methods of cell staining, cell identification, and histograms of negative controls are as indicated in the legend for Fig. 1. Annotation of the horizontal and the vertical axes are also as indicated in the legend for Fig. 1.

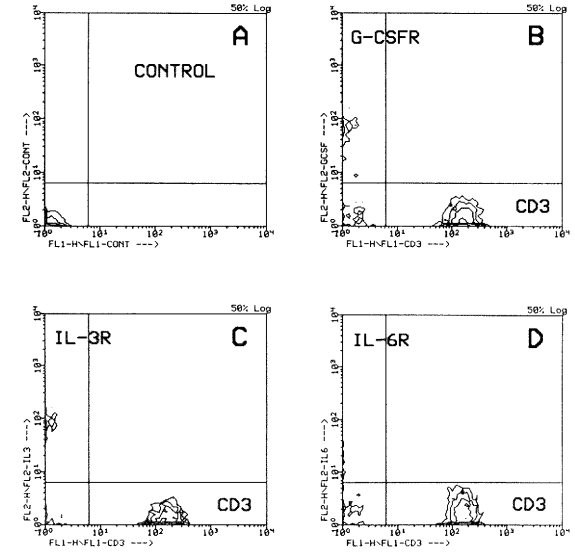

Fig. 4. Two-color analysis of CD3 and cytokine receptors on normal lymphocytes. Normal peropheral white blood cells were stimultaneuosly incubated with FITC-conjugated anti-CD3 monoclonal antibody and PE-labeled cytokine (G-CSF, IL-3, and IL-6 respectively). Lymphocytes were identified by their light scatter property. Horizontal axis: fluorescence intensity of FITC. Vertical axis: fluorescence intensity of PE.

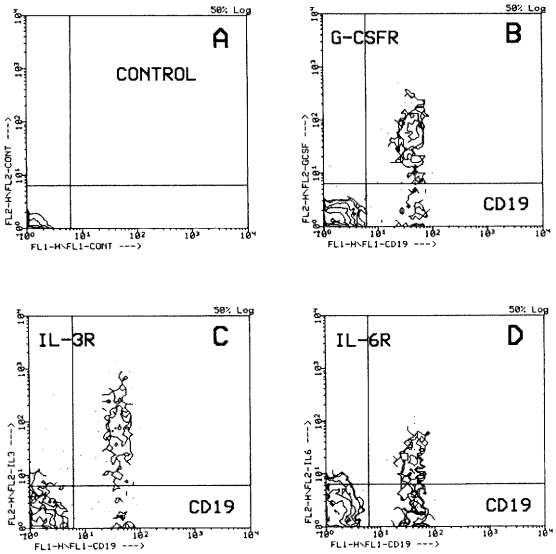

Fig. 5. Two-color analysis of CD19 and cytokine receptors on normal lymphocytes. Method of cell staining and cell identification are as indicated in the legend for Fig. 4. Annotation of the horizontal and the vertical axes are also as indicated in the legend for Fig. 4.

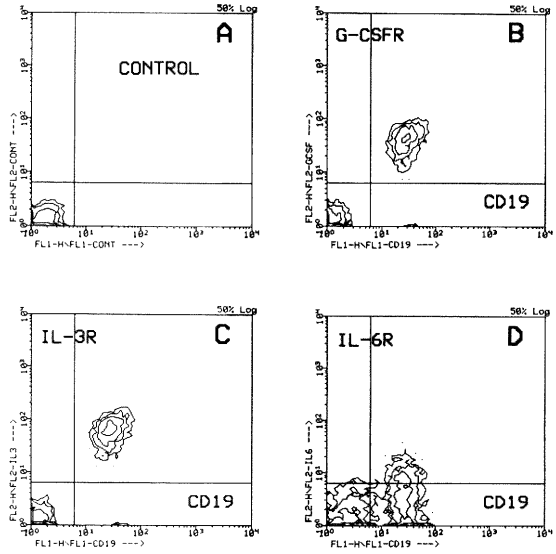

Fig. 6. Two-color analysis of CD19 and cytokine receptors on leukemic cells of one B-CLL patient. CD19 positive CLL cells expressed G-CSF receptor, IL-3 receptor, and partly expressed IL-6 receptor. Annotation of the horizontal and the vertical axes are as indicated in the legend for Fig. 4.

Table 2. Cytokine receptor expression on leukemic cells

Pt. No.	Age	Sex	Source	Diagnosis	G-CSFR(%)	IL-3R(%)	IL-6R(%)
1	27	M	BM	AML	45.4	3.4	ND
2	55	M	PB	CML-BC(My)	49.9	48.4	34.7
3	17	F	PB	C-ALL	72.3	28.0	7.7
4	57	M	PB	C-ALL	80.7	90.7	33.8
5	70	M	PB	B-CLL	54.2	54.2	22.0
6	53	M	PB	B-CLL	79.4	71.5	7.6

CML-BC (My), CML in myeloid crisis; C-ALL, common ALL; B-CLL, B cell type CLL; ND, not done.

DISCUSSION

Traditionally, cytokine receptors have been studied using the radioreceptor assay.[1-8] However, this method requires much time and many samples of single cell populations. Recently, flow cytometric analyses using biotinylated cytokines have been reported.[9-16] Most of these, however, have been about the receptor expression by single color analysis,[9,12,13] and there have been few reports on two-color analysis coupled with other parameters such as surface markers, except with CD34.[10,11,14-16] Previously, G-CSF receptor has been thought to be present only on myeloid cells and absent on lymphocytes.[11,17] However, a few recent reports suggesting the expression of G-CSF receptor on lymphoid cells have been published.[15,18-21] In our study, CD3 positive T cells were negative for G-CSF receptor, but CD19 positive B cells were considered to be positive for G-CSF receptor. Traditional radioreceptor assay cannot analyze the receptor expression on B cells unless B cells are purified fully enough for the analysis. On the other hand, single color flow cytometry can analyze the specified cell population by gated analysis, but it is impossible to distinguish B cell population using

only the light scatter property. If the sample of the rediorecеptor assay and the gated cell population of the single color flow cytometry were lymphocytes, the B cell comprises a minor population and therefore the result tends to be interpreted as negative. However, two-color flow cytometric analysis coupled with B cell marker clearly showed that B cells are positive for G-CSF receptor. Further, although only studied in a few clinical cases, neoplastic cells of B-cell malignancies expressed G-CSF receptor. Morikawa et al.[15] reported the possibility that G-CSF affects immunoglobulin synthesis of B cells. Further study is needed to clarify the significance of G-CSF receptor on B cells.

There have been few reports about the expression of IL-3 receptor on blood cells. In our study, IL-3 receptor expression on blood cells tended to show the same pattern as G-CSF receptor in both normal and leukemic subjects.

As to the IL-6 receptor, there is a report of two-color flow cytometric analysis using biotinylated IL-6.[14] According to the report, in normal peripheral blood cells, monocytes, granulocytes, and CD4 positive T lymphocytes are positive for IL-6 receptor, and CD19 positive B lymphocytes and CD56 positive NK cells are negative. In our study, monocytes and granulocytes expressed IL-6 receptor, T cells did not express IL-6 receptor, and B cells expressed IL-6 receptor partly. We have not examined NK cells but, since in lymphocytes, the IL-6 receptor positive population was limited to the CD19 positive fraction, NK cells were thought to be negative.

By two-color flow cytometric analysis with surface marker, cytokine receptor expression on the specified cell population can be easily analyzed and useful information for clinical cytokine therapy obtained.

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