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Flow Cytometric Analysis of IL-6 Receptors on Peripheral Lymphocytes in Patients With Primary Biliary Cirrhosis

Masayoshi Yamashiki,[1]* Yoshitane Kosaka,[1] Junji Nishioka,[1] Yukihiko Tameda,[1] Kojiro Takase,[2] Shozo Watanabe,[3] Masahiko Kaito,[3] Akira Nishimura,[4] Hiroyuki Suzuki,[5] and Minoru Nomoto[6]

[1]Department of Laboratory Medicine; [2]1st Department of Internal Medicine; and [3]3rd Department of Internal Medicine, Mie University School of Medicine, Tsu, Japan

[4]Department of Internal Medicine, Suzuka Central General Hospital, Suzuka, Japan

[5]Division of Gastroenterology, University of Maryland, Baltimore, Maryland

[6]Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania

Interleukin-6 receptors (IL-6R) and interleukin-1 receptors (IL-1R) on lymphocyte surfaces were analyzed, using flow cytometry and dye-labeled IL-6 and IL-1β, to examine the clinical and immunological significance of these receptors. Incubation of peripheral blood mononuclear cells in the presence of mitogen resulted in a remarkable increase of lymphocytes expressing the IL-6 and IL-1β receptors on the cell surface. The increase in lymphocytes bearing these cytokine receptors may reflect an increase in stimulated lymphocytes. When peripheral blood from patients with primary biliary cirrhosis (PBC) was examined for these receptors, the percentage of IL-6R positive cells was significantly higher in the patients than in healthy controls (P<0.01). The increase in IL-6R positive cells was only significant for the T lymphocyte fraction (P<0.01). No significant change in IL-1R was observed. There was a significant positive correlation between the percentage of IL-6R positive T lymphocytes and the titer of antimitochondrial antibody in patients with PBC. These findings concerning IL-6R may be noteworthy elucidating autoimmune etiological features of PBC. J. Clin. Lab. Anal. 12:83–87,1998.

Key words: IL-6 receptor; IL-1 receptor; primary biliary cirrhosis; flow cytometry; peripheral lymphocyte; cytokine

INTRODUCTION

Interleukin-6 (IL-6) and IL-1 are known to play a central role in many systems involved in host defense such as the neurological, endocrine, and hemopoietic systems (1,2). A decrease in these cytokines can reduce resistance to infection. Excessive production of these cytokines can manifest unfavorable effects in patients with autoimmune disease (such as rheumatoid arthritis) septic shock or malignant disease.

IL-6 is produced by monocytes macrophages and Th2 lymphocytes and stimulates B lymphocytes to produce antibody or activates T lymphocytes. IL-6 binds to the IL-6 receptor α chain (CD126) to yield IL-6/IL-6R complexes that then bind to IL-6Rβ (gp130, CD130) which is involved in signal transduction (3,4).

IL-1 is produced by monocytes and macrophages and induces activation of lymphocytes. IL-1 can be divided into IL-1α and IL-1β depending on the amino acid sequence. In humans, IL-1β production is predominant over IL-1α production. There are however few differences in the biological actions of IL-1α and IL-1β (5). Target cells of IL-1 express IL-1 receptors (IL-1R). IL-1R can be divided into type I (CDw121a) and type II (CDw121b) (6). Type II IL-1R is mainly involved in IL-1 signal transduction (7).

Analysis of peripheral blood lymphocyte surface markers using flow cytometry and dye-labeled monoclonal antibodies has been routinely conducted in laboratory tests for the past 10 years. This technique has been primarily used for the analysis of T lymphocyte subsets (8–10). Analysis of cytokine receptors on lymphocyte surfaces may provide valuable clinical and immunological information, but there have been few studies concerning the analysis of cytokine receptors other than the IL-2 receptor α chain (CD25).

In the present study, we evaluated IL-6R and IL-1R expression on lymphocyte surfaces, using IL-6 and IL-1β la-

*Correspondence to: Masayoshi Yamashiki, MD, Ph.D., Department of Laboratory Medicine, Mie University School of Medicine, 2-174 Edobashi, Tsu 514, Japan.

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beled with a dye (phycoerythrin, PE), in order to examine the clinical and immunological usefulness of their expression. Furthermore, we analyzed the frequency of IL-6R and IL-1R expression on peripheral blood lymphocytes from patients with primary biliary cirrhosis (PBC; an autoimmune disease of the liver presenting with diverse immunological abnormalities), to examine the clinical and immunological significance of their expression.

MATERIALS AND METHODS

Subjects

Blood samples were collected from 20 patients with PBC (Table 1) and 24 healthy employees at our department (Table 2). PBC was diagnosed histologically on liver biopsy specimens. Antimitochondrial antibody (AMA) was performed by indirect immunofluorescence on mouse kidney substrate.

White Blood Cell Sample Preparations

Peripheral blood was placed in a 5-ml blood collection tube containing EDTA-2Na powder (Terumo, Tokyo, Japan). The tube was turned upside down to mix the blood and powder. Then, a 30-fold volume of hemolysis reagent (ammonium chloride) prepared in the laboratory, was added to the tube, mixed for 10 min, and centrifuged. The collected precipitation was washed twice with a phosphate buffer solution (PBS) containing bovine serum albumin (BSA), and then suspended in 2 ml of 1% BSA-added PBS. This suspension was used as the WBC suspension.

TABLE 1. Clinical Findings in Patients with PBC

No.	Age	Sex	Clinical features	AMA (titer)
1	55	Male	Asymptomatic	Positive (unknown)
2	42	Female	Asymptomatic	Positive (×640)
3	43	Female	Asymptomatic	Positive (×320)
4	50	Female	Asymptomatic	Positive (×1280)
5	51	Female	Asymptomatic	Positive (×40)
6	55	Female	Asymptomatic	Positive (×160)
7	56	Female	Asymptomatic	Positive (×1280)
8	57	Female	Asymptomatic	Positive (×40)
9	60	Female	Asymptomatic	Positive (×1280)
10	60	Female	Asymptomatic	Positive (×320)
11	60	Female	Asymptomatic	Positive (×320)
12	62	Female	Asymptomatic	Positive (×1280)
13	62	Female	Itching	Positive (×320)
14	62	Female	Asymptomatic	Positive (×80)
15	62	Female	Asymptomatic	Positive (×40)
16	63	Female	Asymptomatic	Positive (×40)
17	66	Female	Asymptomatic	Positive (unknown)
18	68	Female	Asymptomatic	Positive (×40)
19	69	Female	Asymptomatic	Positive (×80)
20	75	Female	Asymptomatic	Positive (×20)

PBC, primary biliary cirrhosis; AMA, antimitochondrial antibody examined using the indirect immunofluorescence method.

TABLE 2. Number of Healthy Subjects by Age and Gender

Age group (years)	Male	Female
20~29	3	4
30~39	4	5
40~49	3	2
50~58	2	1

Mononuclear Cell Culture

Twenty ml of blood was taken into heparinized tubes from the cubic vein of each of 5 healthy students. Whole blood was then layered onto a lymphocyte separation fluid (Muto Pure Chemicals, Tokyo, Japan). The mixture was centrifuged and isolated lymphocytes were washed 3 times in Roswell Park Memorial Institute (RPMI) medium 1640 (Gibco Laboratories, Grand Island, NY). The mononuclear cell (MNC) fraction was then suspended in RPMI medium containing 15% heat-inactivated fetal bovine serum (Gibco Laboratories, Grand Island, NY) and two antibiotics, penicillin and streptomycin (Flow Laboratories, Irvine, Scotland). The final concentrations of the two antibiotics were 50 IU/ml (penicillin) and 50 μg/ml (streptomycin). The MNC suspension (1.6 ml), containing 1×10^6 cells/ml, combined either with 0.1 ml of the RPMI medium or with a solution of phytohemagglutinin (PHA, Sigma) at a final concentration of 8 μg/ml, was added to each well of the plate. The sample was then incubated for 24 hrs at 37°C and under 5% CO_2. The cellular fraction was harvested, centrifuged, and washed once in PBS. The cells were then suspended in 1.5 ml of PBS containing 1% BSA.

Reagents

Phycoerythrin (PE)-conjugated IL-6 and PE-conjugated IL-1β were purchased from R & D Systems (Minneapolis, MN) for use in the analysis of IL-6R and IL-1R expression. Fluorescein isothiocyanate (FITC)-conjugated CD3 monoclonal antibody and FITC-conjugated CD19 monoclonal antibody were purchased from Beckton Dickinson Immunocytometry Systems (B-D, Mountain View, CA) to analyze T and B lymphocytes. Simultest LeukoGATE was purchased from B-D for identification of lymphocyte fraction. Simultest Control was also purchased as a negative control reagent from B-D.

Sample Staining

Six Falcon 2052 tubes (B-D) were used. The first tube contained 20 μl of Simultest LeukoGATE, the second contained 20 μl of Simultest Control, the third and fourth contained 20 μl of FITC-conjugated monoclonal antibody, and the fifth and sixth contained 20 μl of FITC-conjugated CD19 monoclonal antibody. PE-conjugated IL-1β (10 μl) was added to the third and fifth tubes. PE-conjugated IL-6 (10 μl) was added to the fourth and sixth tubes. The leukocyte suspension (0.1 ml) or a cultured MNC suspension (0.2 ml) was added to each of

these 6 tubes, which were then incubated at 4°C for 30 mins, while being protected from light. The cells were then washed once in PBS and suspended in PBS containing 1% BSA.

Flow Cytometer Analysis

The flow cytometer used in this study was a FACSCAN (B-D), and the software used for collection and analysis of the results was the Consort 30 program.

Statistical Analysis

The positive rates of each cell group in the PBC group and the control group were expressed as mean ± SD. The significance of differences was tested using each student's unpaired t-test.

RESULTS

Figure 1 shows IL-1R expression on lymphocyte surfaces following incubation with or without PHA. When stimulated with PHA (Fig. 1, right), the number of IL-1R positive cells increased markedly. Similar results were also obtained for IL-6R expression (Fig. 2). The number of IL-6R positive cells was higher in PHA-stimulated cultures, although the increase was less marked than that of IL-1R positive cells (Fig. 2, right).

The percentage of CD3 positive cells (T lymphocytes) among all lymphocytes did not differ between the PBC group (mean ± SD = 56.1 ± 9.4%) and the normal control group (59.8 ± 18.0%), while the percentage of CD19 positive cells (B lymphocytes) was significantly higher in the PBC group (22.1 ± 8.1%) than in the normal control group (14.9 ± 14.2%) ($P<0.05$).

The percentage of IL-1R positive cells among all lymphocytes did not differ significantly between the PBC group (12.0 ± 5.9%) and the control group (10.2 ± 3.8%). There was no significant intergroup difference in the percentage of $CD3^+$-$IL\text{-}1R^+$ cells among $CD3^+$ lymphocytes or the percentage of $CD19^+$-$IL\text{-}1R^+$ cells among $CD19^+$ lymphocytes.

The percentage of IL-6R positive cells among all lymphocytes was significantly higher in the PBC group (9.1 ± 5.%) than in the normal control group (4.4 ± 2.3%) ($P<0.01$, Fig. 3). Furthermore, the percentage of $CD3^+$-$IL\text{-}6R^+$ cells among all lymphocytes was significantly higher in the PBC group (3.6 ± 1.8% vs. 1.8 ± 1.1%; $P<0.01$) and the percentage of these cells among $CD3^+$ lymphocytes was also significantly higher in the PBC group (6.4 ± 3.1% vs. 3.2 ± 2.0%, $P<0.01$, Fig. 4). The percentage of $CD19^+$-$IL\text{-}6R^+$ cells among all lymphocytes was significantly higher in the PBC group (1.3 ± 0.8% vs. 0.6 ± 0.6%, $P<0.01$), while the percentage of these cells among $CD19^+$ lymphocytes did not differ significantly between the two groups.

When the data concerning IL-1R and IL-6R expression was analyzed in relation to laboratory test data, there was a significant positive correlation between the percentage of $CD3^+$-$IL\text{-}6R^+$ cells among all lymphocytes or $CD3^+$ lymphocytes and the AMA titer. No other significant correlations were observed.

DISCUSSION

Analysis of the amounts of IL-6 and IL-1 receptor expression on lymphocyte surfaces following incubation of peripheral mononuclear cells with or without a mitogen revealed that the number of receptor positive cells was markedly higher in the presence of a mitogen than in the absence of the mitogen. The increase in IL-6R and IL-1R positive cells among peripheral blood lymphocytes may reflect an immune state excessively stimulated by some factors in vivo.

When these receptors were analyzed using peripheral blood lymphocytes from patients with PBC, IL-1R expression did not differ from that in healthy controls, while IL-6R expression was significantly increased in the PBC group ($P<0.01$). Thus, we analyzed IL-6R positive cells in T and B lymphocytes. IL-6R expression on both T and B lymphocytes was significantly higher in the PBC group than in the control group. The percentage of T lymphocytes among all lymphocytes did

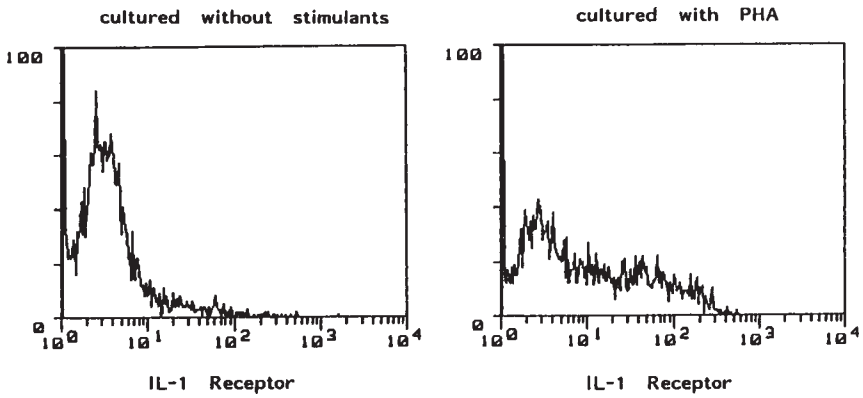

Fig. 1. Fluorescence intensity histogram of IL-1 receptor on cultured lymphocytes. **Left:** Without stimulants. **Right:** Stimulated with PHA.

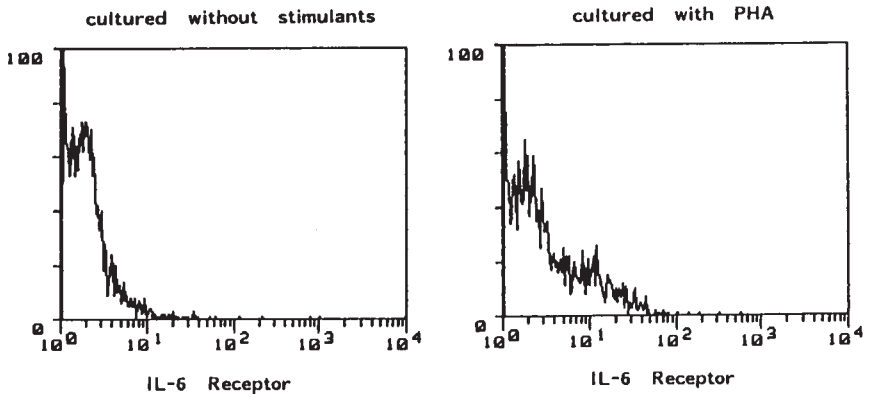

Fig. 2. Fluorescence intensity histogram of IL-6 receptor on cultured lymphocytes. **Left:** Without stimulants. **Right:** Stimulated with PHA.

not differ between the PBC group and the control group, while the percentage of B lymphocytes was significantly higher in the PBC group ($P<0.05$). Therefore, we could not rule out that the increase in IL-6R positive B lymphocytes in the PBC group results from an increase in the percentage of B lymphocytes in this group. Thus, we then analyzed the percentage of IL-6R positive cells among T and B lymphocytes. The percentage of IL-6R positive cells among T lymphocytes was significantly high in the PBC group ($P<0.01$), but that among B lymphocytes did not differ between the two groups. Therefore, the increase in IL-6R positive B lymphocytes among all lymphocytes from PBC patients cannot be regarded as a valuable finding. On the other hand, the increase in IL-6R positive T lymphocytes in PBC patients suggests that the T lymphocytes of these patients were excessively stimulated by some unknown factors.

When the data concerning cytokine receptors was analyzed in relation to laboratory test data, we found a significant positive correlation between the percentage of IL-6R positive T lymphocytes among all lymphocytes or T lymphocytes and the titer of antimitochondrial antibody (AMA) detected specifically in patients with PBC. It is difficult at present to determine the immunological importance of this finding. However, it suggests that IL-6 is a cytokine which promotes the proliferation and differentiation of B lymphocytes and thus induces the production of antibodies. Clarification of the mechanism by which AMA appears is now considered as the most important issue when elucidating the etiology of PBC (11). Our finding of an increase in T lymphocytes with IL-6 receptors expressed on their surfaces may be noteworthy when discussing the etiology of PBC.

Our flow cytometric analysis of IL-6R and IL-1R expression on the peripheral blood lymphocytes in patients with PBC yielded interesting findings concerning IL-6R. Analysis of

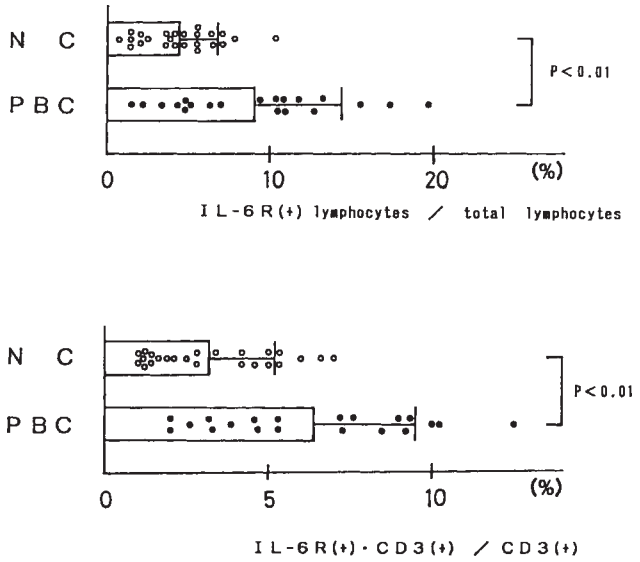

Fig. 3. IL-6 receptor positive cell ratios in lymphocyte (upper) and T lymphocyte (lower) fraction. **PBC:** patients with primary biliary cirrhosis. **NC:** normal healthy controls. Mean ± SD.

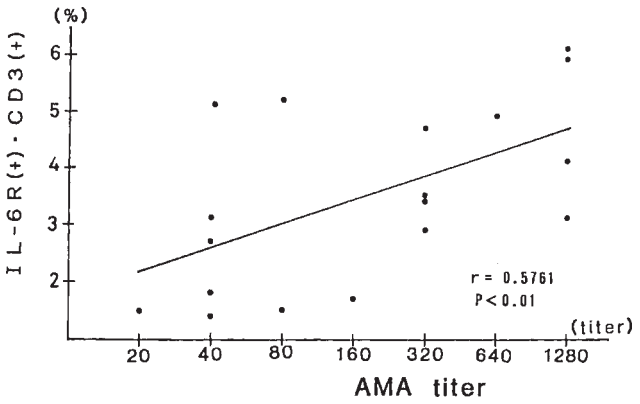

Fig. 4. Comparison of serum antimitochondrial antibody titer and IL-6 receptor positive T lymphocyte ratio in the lymphocyte fraction of patients with primary biliary cirrhosis.

cytokine receptors on lymphocyte surfaces, except for such an analysis of IL-2R expression, has not yet been included in routine laboratory tests. Few basic studies have been performed concerning the clinical and immunological significance of this kind of analysis. Cytokine receptors deserve additional analyses because they may provide valuable clinical, immunological, and etiological information.

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