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Semi-Quantitative Analysis of Cytokine mRNA Expression Induced by the Herbal Medicine Sho-saiko-to (TJ-9) Using a Gel Doc System

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The RT-PCR method was employed to determine the cytokine mRNA expression of human peripheral lymphocytes induced by the Japanese herbal medicine Sho-saiko-to (TJ-9). The results showed that the mRNA expression of IL-12, IL-1β, IL-10, TNF-α, G-CSF, and IFN-γ increased after 6 hr in culture. This is the first reported finding that TJ-9 is an IFN-γ inducer. Next, cytokine mRNA expression was semi-quantitatively measured using the Gel Doc system with a CCD camera and then statistically analyzed in order to determine which component of TJ-9 was the true cytokine inducer. The results showed that the scutellaria root is the main component inducing the cytokines, while the glycyrrhiza root is the secondary component. When the cytokine concentrations in the supernatants of cell cultures were measured by ELISA, the levels of IL-12, IL-1β, IL-10, TNF-α, and G-CSF reflected mRNA expression levels in the cell fraction. However, the level of IFN-γ was below the detectable limit. The effects of various reagents on many different kinds of cytokine mRNA expression could be analyzed objectively in a short time using the Gel Doc system. Many important findings could be demonstrated by this simple, easy, sensitive, and cheap method. After the clinical significance of cytokine analysis is confirmed, this method may become a useful clinical examination tool. J. Clin. Lab. Anal. 15:199–209, 2001.

Key words: cytokine mRNA; RT-PCR method; semi-quantitative analysis; Gel Doc system; ELISA; Sho-saiko-to (TJ-9); cytokine inducer

Cytokine research has become one of the most important parts in the clinical research field. Measurements of cytokine production capability are expected to become new markers for diagnosis or treatment in clinical practice. The enzyme immunoassay (EIA) is a popular method for cytokine analysis. Researchers can quantitatively analyze cytokine protein using this method. The EIA is applied mainly to two analytical methods of cytokine network. One is analysis of cytokine production level by measuring the cytokine concentration in the supernatant after cell culture for several days (1); the other is analysis of the cytokine level in serum obtained from peripheral blood (2). As a routine clinical examination method, the analysis of serum cytokine levels are higher than expected. However, unfortunately, most of the time the cytokine concentration in serum is very small and undetectable. Even using highly sensitive ELISA (enzyme-linked immunosorbent assay) kits with long reaction times that are currently on the market, the results of cytokine concentration as a clinical laboratory marker are still not satisfactory. Another commonly used method for analysis of cytokines is flow cytometry (FCM), which can be used to measure the amount of cytokine receptor on the cell surface or the amount of intracellular cytokine expression induced by stimulants (3). However, the positive cell ratios expressing cytokine receptors or intracellular cytokine analyzed by FCM are insufficient. Distinctions between the positive cells and negative cells are not as clear as the analysis of CD3, CD4, and CD8, although it is possible to compare slight differences in density. In addition, manufactured monoclonal antibodies are very expensive and their varieties are limited. The FCM methods need more in depth studies before they can be used in routine clinical examination.

An additional method for analyzing cytokine expression involves the detection of cytokine mRNA (4–18). This method is usually applied to the immunological research field. In recent years, analyses of RNA virus in peripheral blood using

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biological molecular techniques have become a routine clinical examination method. Most clinical examination departments already possess the instruments required to carry out RT-PCR (reverse transcriptase polymerase chain reaction), and so it is possible to analyze cytokine mRNA using these instruments. In the future it may become a routine laboratory examination method for new immunological markers. Quantitative measurement of cytokine mRNA expression is necessary in order to establish it as a useful tool for analyzing cytokine networks. However, more complicated techniques and very expensive instruments are needed in this method.

Sho-saiko-to (TJ-9, Xiao-Chai-Hu-Tang in Chinese) has been most frequently used to treat approximately one million patients with chronic viral liver diseases to improve liver dysfunction as well as to prolong their life expectancy (19–25). TJ-9 consists of seven crude drugs (bupleurum root, pinellia tuber, scutellaria root, jujube fruit, ginger rhizome, ginseng root, and glycyrrhiza root). As one of its major pharmaceutical effects, TJ-9 is presumed to improve generally and gradually the biological defense mechanism via its effects on the immune and nervous systems. However, its mode of action has not yet been fully elucidated. We have reported that TJ-9 in vitro could improve the low levels of interleukin (IL-) 1β, IL-10, and IL-12 production of peripheral blood mononuclear cells in patients with chronic viral liver disease and that TJ-9 could strongly induce useful cytokines, such as tumor necrosis factor (TNF) α and granulocyte colony-stimulating factor (G-CSF), in HCC patients (1,26–32).

However, the above report showing the effects of TJ-9 on the cytokine production capability was analyzed by a method that measured protein levels. If TJ-9 is truly a "cytokine inducer," then we must at least demonstrate which herb components contained in TJ-9 can increase cytokine mRNA expression. For this purpose, it may be useful to compare the semi-quantitative values for cytokine mRNA expression in cells cultured with each of the seven herb components.

In the present study, we investigated the expression levels of various cytokine mRNAs induced by each of the seven herb components in human peripheral lymphocytes in vitro using RT-PCR and a Gel Doc system with a CCD camera that allow the semi-quantitative analysis of the PCR products.

MATERIALS AND METHODS

Samples

Peripheral blood was collected from 14 healthy volunteers (university students or employees) who had consented to the blood sampling and its use in this study.

Cell Preparation

As described in our previous studies, heparinized peripheral venous blood was collected, and the mononuclear cell (MNC) fraction obtained using a lymphocyte separation solution (Muto Pure Chemicals, Tokyo, Japan). The MNC fraction was washed three times with Roswell Park Memorial Institute (RPMI) Medium 1640 (Gibco Laboratories, Grand Island, NY), and the cells were resuspended at a density of 5×10^6 cell/ml in the RPMI culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco Laboratories), 50 IU/ml penicillin (final concentration), and 50 μg/ml streptomycin (Flow Laboratories, Irvine, Scotland). In all of the following experiments, except for when washing, we used this MNC suspended RPMI medium (MNC suspension).

To obtain the lymphocyte fractions, the wells of a 24-well culture plate (Becton Dickinson Labware, Lincoln Park, NJ) were coated with heat-inactivated human serum type AB, and then 1 ml of the MNC suspension was added to each well, the plate was incubated in 5% CO_2-in-air for 60 minutes at 37°C. The nonadhered cells were obtained as the lymphocyte fraction. Cell surface markers were examined using a flow cytometer (FACScan, Beckton Dickinson, San Jose, CA), and CD14-positive cells were found to be 1% or less in the lymphocyte fraction.

Reagents

The bulk powder of TJ-9 and its seven herbal components was supplied by Tsumura & Co. (Tokyo, Japan). Each drug was dissolved into distilled water by gently shaking the tube for 2 hr at 37°C, and centrifuging twice to remove any precipitates. For sterilization, the solution was passed through a filter unit (Millipore Products Division, Bedford, MA), it was then diluted to the final concentration required in the RPMI-1640 solution supplemented with antibiotics.

Cell Culture

The lymphocytes were resuspended at 5×10^6 cells/ml with the previously mentioned culture medium. To each well of a 24-well culture plate, 980 μl of either cell suspension together with 20 μl of a single test substance or RPMI-1640 medium was added. The final concentrations were as follows: 200 μg/ml for TJ-9 and 100 μg/ml for each of the seven herb components. The plate was cultured with 5% CO_2-in-air at 37°C for 6 hr.

After culture, the lymphocyte suspensions were transferred to 1.5-ml tubes (Eppendorf, Hamburg). Next, 1 ml of TRIzol reagent for RNA extraction (Life Technologies, Gaithersburg, MD) was added to each well of the 24-well culture plate, which was then maintained at room temperature for 5 minutes. The tubes containing the lymphocyte suspensions were centrifuged, and the supernatants were transferred to fresh tubes before being stored at –80°C for future use. The lymphocyte fraction pellets were then mixed with the TRIzol reagent transferred from the culture plate.

RNA Isolation and RT-PCR

Total RNA was extracted from the peripheral blood lymphocytes (5×10^6) of 14 healthy volunteers by the acid guani-

dine thiocyanate–phenol–chloroform method of Chomczynski and Sacchi using the TRIzol Reagent kit according to the manufacturer's instructions (32). RNA pellets were resuspended in 20 µl of DEPC-treated water and then used in a reverse-transcriptase (RT) reaction to synthesize first-strand cDNA. A total of 2 µl (about 1 µg RNA) RNA was used as a template for the RT. It was added to a mixture containing 5 mM $MgCl_2$, 1 mM of each dNTP, 20 U recombinant RNasin® ribonuclease inhibitor, 15 U AMV reverse transcriptase (high conc.), 0.5 µg oligo $(dT)_{15}$ primer, and 10× RT-Buffer (10 mM Tris-HCl [pH 9.0 @ 25°C], 50 mM KCl, 0.1% Triton® X-100) (Promega, Madison, WI), and then the total volume of the reaction mixture was adjusted to 20 µl by adding DEPC-treated water. The mixture was incubated at 42°C for 60 min, heated to 99°C for 5 min, and then incubated at 4°C for 5 min.

The cytokine cDNAs were amplified by polymerase chain reaction (PCR) as follows: 6 µl of the produced cDNA (for use as a template) were added to a reaction mixture containing 200 µM dNTPs, 2 mM $MgCl_2$ (with a contribution from the first-strand cDNA reaction), 1.25 U Taq DNA polymerase, 10× PCR Buffer (Promega), and 6 pmol of both sense and antisense primers, which amplify individual cytokine sequences. Between 25 and 35 cycles of PCR were carried out [25 cycles for β-actin; 30 cycles for IL-6, IL-8, IFN-γ, transforming growth factor (TGF) β, and granulocyte-macrophage (GM) CSF; 32 cycles for IL-2, IL-10, IL-15, and TNF-α; and 35 cycles for IL-1β, IL-4, G-CSF, and macrophage (M) CSF] in a DNA Thermal Cycler 480 (Perkin-Elmer, Norwalk, CT) with the following time and temperature conditions: 94°C for 45 sec; 60°C for 45 sec; and 72°C for 60 sec. The β-actin amplifications were included as a positive control. Amplified PCR productions were detected by electrophoresis of the solutions in 2% agarose gels, which were then stained with ethidium bromide. The gels were observed and photographed under ultraviolet light. The nucleotide sequences of the primers used are shown in Table 1 (4–18).

TABLE 1. Primer sequences for PCR amplification

Target	Sense primer (5′-3′) and antisense primer (5′-3′)
β-Actin	GAGAAGATGACCCAGATCATGT
	ACTCCATGC AGGAAGGAAGG
IL-1β	AAACAGATGAAGTGCTCCAG G
	TGGAGAACACCACTTGTTGCTCCA
IL-2	ATGTACAGGATGCAACTCCTGTCTT
	TGGAGAACACCACTTGTTGCTCCA
IL-4	CTGCTTCCCCC TCTGTTCTTC
	ACTCTGGTTGGCTTCCTTCAC
IL-6	ATGAACTCCTTCTCCACAAGCGC
	GAAG AGCCCTCAGGCTGGACTG
IL-8	CTTGGCAGCCTTCCT GATTT
	CTCAGCCCTCTTCAAAAACT
IL-10	CTGCTCTGTCTGGTCCTC
	GTTTCGTATCTTC ATTGTCAT
IL-12	CCACCTGCCGAGAATTCTTTAA
	ACCCCTGAAGAAGATGGTATCA
IL-15	CTCGTCTAGAGCCAACTGGGTGAATGTAATAAG
	TACTTAGGATCCGAATCAATTGCAATCAAGAAGTG
TNF-α	CGGGACGTGGAGCTGGCCGAGGAG
	CACCAGCTGGTTATCTCTCAGCTC
G-CSF	GTGCAGG AAGCCACCCCCCTGGGC
	CCCTCCTGCCCGGCGCTG GAAAGC
M-CSF	GGCCATGAGAGGCAG TCCGAGGG
	CACTGGCAGTTCCACCTGTCTGTC
GM-CSF	GCTGCTGAGATGAATG AAAC
	AGTCAAAGGGGATGACAAG
TGF-β	TACAGCAACAATTCCTGG
	GTTATGCTGGTTGTACAGGGC
IFN-γ	TTGGCTTTTCAGCTCTGC
	CTGGGATGC TCTTCGACC

Quantitative Determination of the PCR Products

The mRNA expression levels of some cytokines, on which TJ-9 was confirmed to have effects, were measured using a Gel Doc system (Bio-Rad Laboratories, Hercules, CA) after addition of the different herb components to the culture. The Gel Doc system is an image-analyzing computer system with a highly sensitive CCD camera (34,35). A standard curve was made using a Precision Molecular Mass Standard reagent kit containing standard DNA concentrations of 100, 70, 50, 20, and 10 ng (Bio-Rad Laboratories). The mass standard was loaded onto each of the 2% agarose gels. Furthermore, to determine whether the concentration volumes of the PCR products obtained by this method would reliably reflect the cytokine mRNA expression level, we measured the PCR product concentration of β-actin with various amounts cDNA.

Cytokine Production Level

For the cytokines on which the effects of TJ-9 on mRNA expression had been confirmed, the levels of cytokine production in supernatant were measured using an ELISA kit (BioSource Europe S.A., Nivelles, Belgium).

Statistical Analysis

Statistical analysis was performed using Stat View software for statistical analysis. All values were expressed as the mean ± standard error (SE). Data for cytokine mRNA expression levels in the cell fraction and cytokine production levels in the supernatant were tested by one-way ANOVA and Fisher's protected LSD test. Levels of significance that were less than 5% were considered significant.

RESULTS

Standard Curve

A standard curve was obtained from the precision molecular mass standard marker containing 100, 70, 50, 20, and 10 ng of DNA. Estimates of the amount of DNA showed a good linear correlation with the concentration of mass standard marker added to the electrophoresed gel.

Correlation of PCR Products and the Original Template

The optical density volumes for the PCR products of β-actin measured after 25 amplification cycles using a spectrophotometer with ultraviolet light were 0.195, 0.170, 0.140, 0.101, 0.069, and 0.022, respectively. This indicated a linear correlation with the different amounts of cDNA (0, 2, 4, 6, 8, and 10 μl) added as the original template.

Effects of TJ-9 on the Cytokine mRNA Expression Level

The level of β-actin mRNA expression in the RNA fraction of peripheral blood lymphocytes collected from five healthy volunteers was measured by the method described above. After no effects on the PCR production levels were confirmed, the mRNA expression levels of 14 cytokines were measured. The results indicated that the levels of cytokine mRNA expression for IL-12, IL-1β, IL-10, TNF-α, G-CSF, and IFN-γ were increased by TJ-9 (Fig. 1).

Effects of the Seven Individual Herb Components on Cytokine mRNA Expression

The effects of each herb component on the production levels of the above-mentioned six induced cytokines were measured using peripheral blood lymphocytes collected from nine healthy volunteers.

The increased level of IL-12 mRNA expression was greatest with scutellaria root, and this was followed by glycyrrhiza root and then bupleurum root (Fig. 2). An analysis of the IL-12 concentrations expressed indicated that they were significantly higher in the cultures with scutellaria root and glycyrrhiza root than compared with the control culture solution (Fig. 3, $P < 0.0001$, $P < 0.001$). The levels of production in the supernatants reflected well the mRNA expression levels in the cell fractions. The mean production levels induced by scutellaria root and glycyrrhiza root were about seven-fold and three-fold higher, respectively, than in the control culture solution. The increase induced by scutellaria root was statistically significant (Fig. 4, $P < 0.01$).

An increased IL-1β mRNA expression was also induced by scutellaria root and glycyrrhiza root. The expressed concentrations of IL-1β induced by scutellaria root and glycyrrhiza root were significantly higher than in the control culture solution (Fig. 3, $P < 0.05$). The levels of cytokine production in the supernatant were more remarkable than the mRNA expression in the cell fraction, and a significant increase was induced by scutellaria root (Fig. 4, $P < 0.0001$).

The concentrations of IL-10 mRNA expressed in the cell

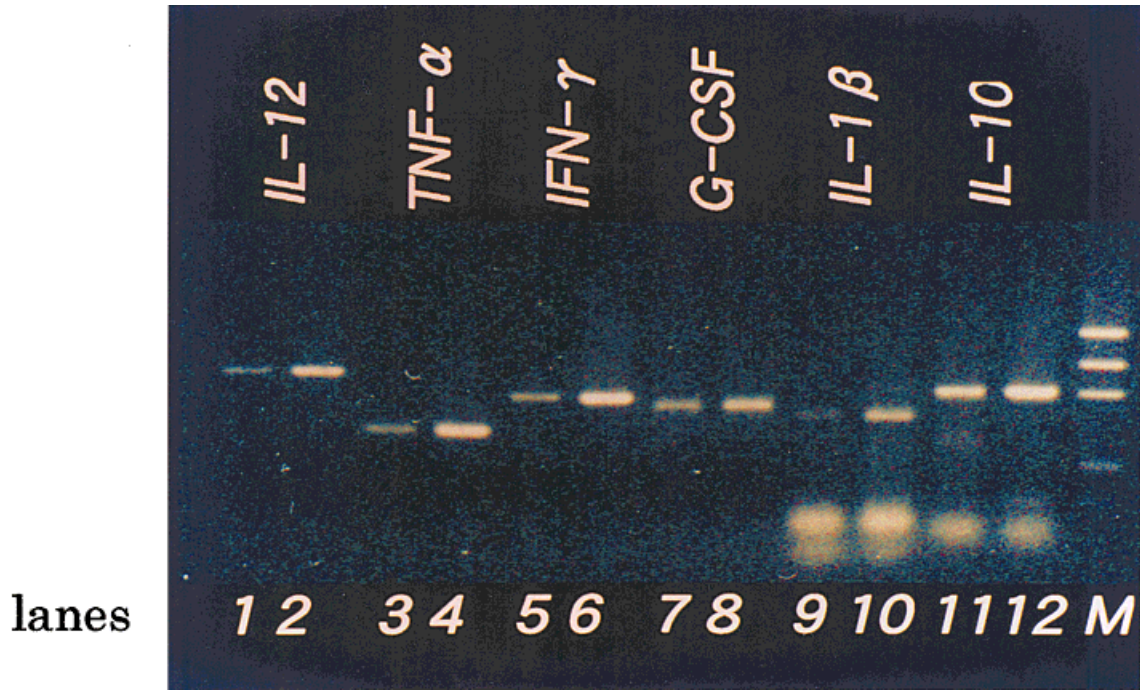

Fig. 1. Effect of TJ-9 on cytokine mRNA expression by peripheral blood lymphocytes. Lanes 1 and 2 are IL-12 mRNA; lanes 3 and 4 are TNF-α mRNA; lanes 5 and 6 are IFN-γ mRNA; lanes 7 and 8 are G-CSF mRNA; lanes 9 and 10 are IL-1β mRNA; and lanes 11 and 12 are IL-10 mRNA. M is the DNA markers. Lanes with odd numbers show the culture solution only; lanes with even numbers show when TJ-9 was added to the culture solution. The mRNA expression level was increased by the addition of TJ-9. Bands of the DNA markers (from top) are 1,000 base pairs (bp, 100 ng), 700 bp (70 ng), 500 bp (50 ng), 200 bp (20 ng), and 100 bp (10 ng). Distinction of the 100-bp band in the picture is rather difficult.

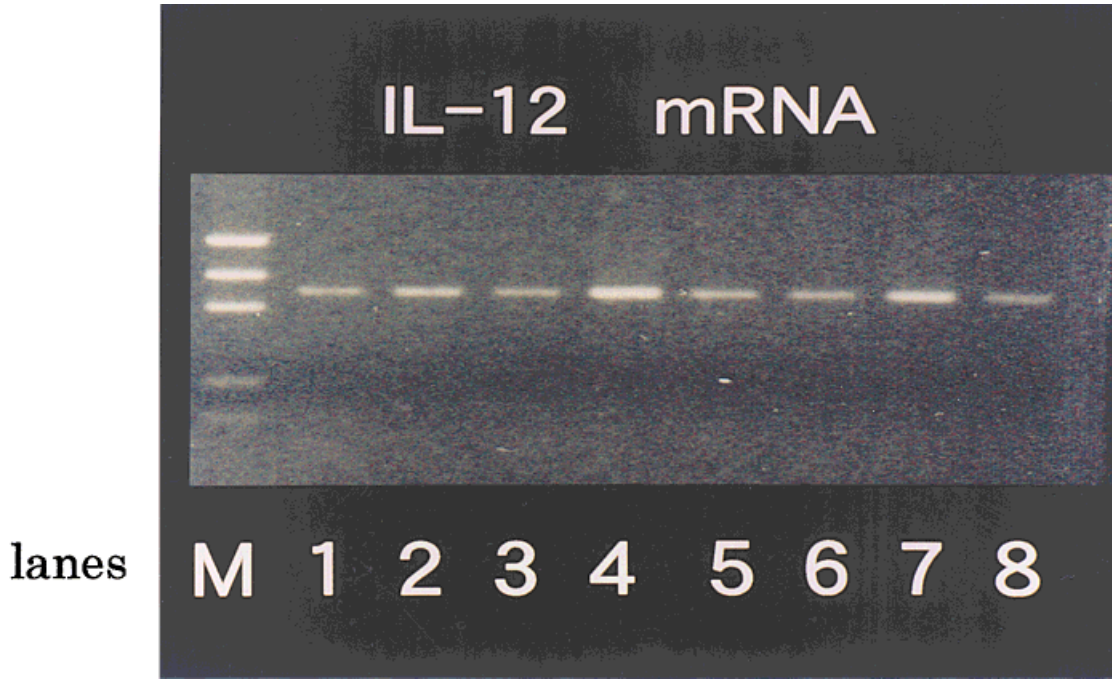

Fig. 2. Effects of the seven individual herb components in Sho-saiko-to on IL-12 mRNA expression. Lanes, from the left, show (lane M) the DNA markers, (lane 1) control culture solution, (lane 2) bupleurum root, (lane 3) pinellia tuber, (lane 4) scutellaria root, (lane 5) jujube fruit, (lane 6) ginger rhizome, (lane 7) glycyrrhiza root, and (lane 8) ginseng root. Scutellaria root and glycyrrhiza root increased the mRNA expression.

fraction induced by scutellaria root and glycyrrhiza root were significantly higher than in the control culture solution (Fig. 3, $P < 0.01$, $P < 0.05$). In the supernatants, the production level induced by scutellaria root was also significantly higher (Fig. 4, $P < 0.001$).

An increased TNF-α mRNA expression was induced by scutellaria root. Analysis of the expressed concentrations indicated a significant difference (Fig. 5, $P < 0.05$). The production level in the supernatant induced by scutellaria root was also significantly higher than the control (Fig. 6, $P < 0.01$). In contrast, TNF-α expression was inhibited by ginseng root. This effect was more marked for the production level in the supernatant than for that of the mRNA expression in the cell fraction.

G-CSF mRNA expression was induced by scutellaria root and glycyrrhiza root. The concentrations expressed were significantly higher in the culture of scutellaria root and glycyrrhiza root than in the control culture solution (Fig. 5, $P < 0.01$, $P < 0.05$). The results of the production levels in the supernatant were more remarkable than those of the mRNA expression in the cell fraction. The increased levels of production induced by scutellaria root and glycyrrhiza root were both significant (Fig. 6, $P < 0.01$, $P < 0.05$).

IFN-γ mRNA expression was increased in the culture with scutellaria root. An analysis of the concentration expressed indicated that the effect of scutellaria root was significant (Fig. 5, $P < 0.05$). However, the levels produced in the supernatant of all groups were below the level of detection.

DISCUSSION

We compared the level of PCR products from cultures with and without TJ-9 and included β-actin as the control. In total, we measured 14 cytokines, including IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-15, TNF-α, G-CSF, M-CSF, GM-CSF, TGF-β, and IFN-γ. For the PCR reaction the differences in production between cultures with and without TJ-9 were most marked between 20 and 35 cycles for all cytokines. The results indicated that the PCR products of six cytokines including IL-1β, IL-10, IL-12, TNF-α, G-CSF, and IFN-γ were significantly increased when TJ-9 was added to the cultures, while the PCR production of β-actin showed no difference. In the present study, PCR cycle numbers were studied by gradually decreasing the number of PCR cycles and specifically deciding the point at which the PCR product was at its lowest detectable limit, so it was not thought to be influenced by the plateau phenomenon.

For the first time we were able to show that TJ-9 induced IFN-γ mRNA expression directly, because previously the level of IFN-γ produced in the supernatant of cultures with TJ-9 had been below the detectable limit (25). The new finding obtained from this molecular analysis indicates that TJ-9 has the possibility to induce very small amounts of IFN-γ and therefore may be an IFN-γ inducer. At the same time, it also suggests that for immunopharmacological studies of cytokine production systems the molecular analysis of cytokine mRNA is an equivalent and potentially more useful method than the

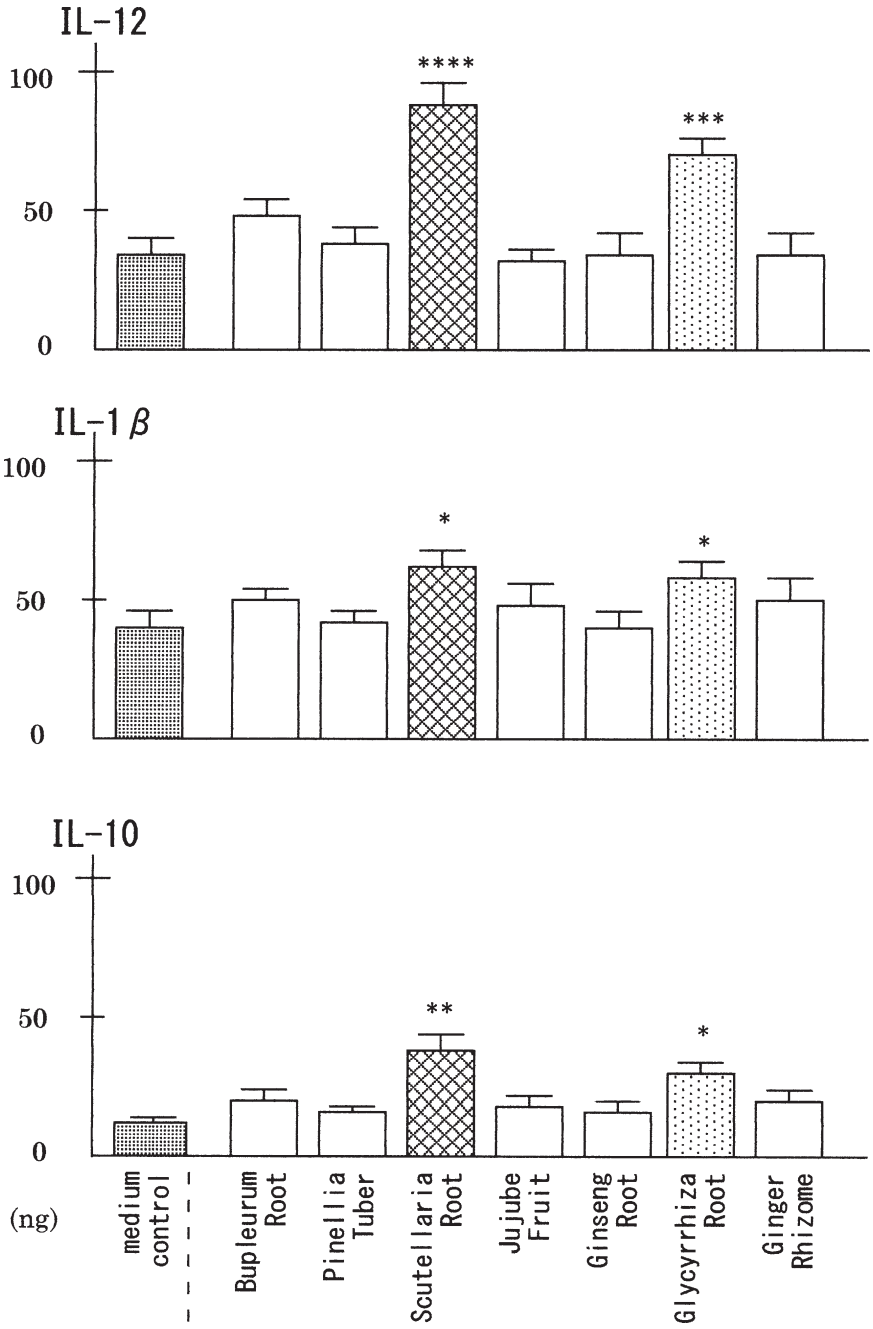

Fig. 3. mRNA expression levels of IL-12, IL-1β, and IL-10 induced by the seven individual herb components. The levels of mRNA expression were significantly increased by scutellaria root and glycyrrhiza root (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$, mean ± SE).

analysis of cytokine production levels by ELISA because the present molecular analysis yielded important information, previously missed. In addition, all of the cytokine primers were cheap and easy to prepare. Therefore, molecular analysis of cytokine mRNA may be more economical than analysis using ELISA kits.

The six cytokines in which the mRNA expression was induced by TJ-9 were next investigated with each of the herb components in TJ-9. The results of this examination were judged by the density and size of PCR products detected on agarose gel. However, objective findings shown by statistical analysis are needed for a more precise decision on the effects of the seven individual herb components. The amount of products represented by a numeric value was needed. Therefore, we used the Gel Doc system for semi-quantitative determination. This method does not require either the newest

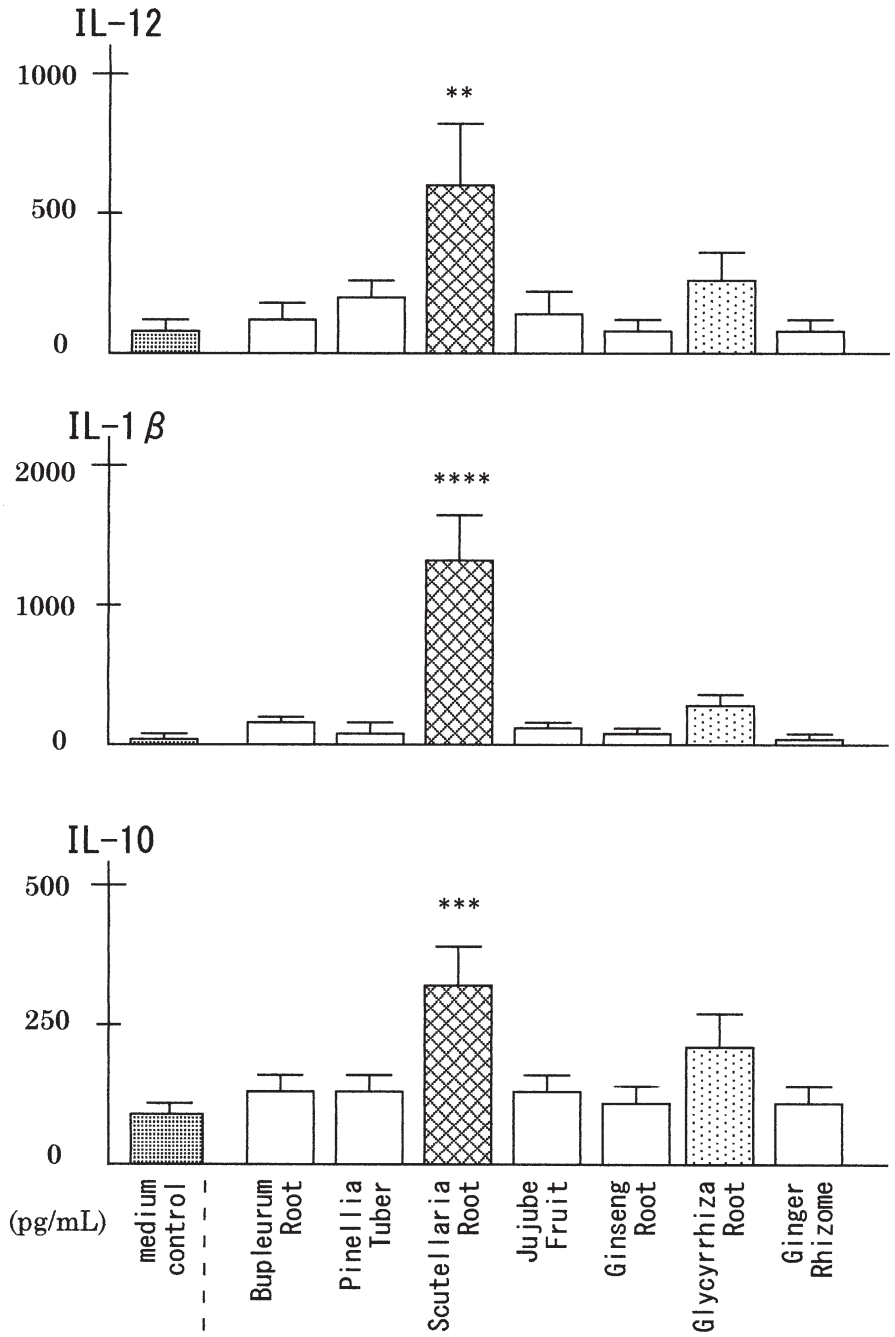

Fig. 4. Effects of the seven individual herb components on production levels of IL-12, IL-1β, and IL-10 in the cultural supernatant. The concentrations were measured by ELISA. The levels induced by added scutellaria root were significantly increased compared to the control culture (**P < 0.01, ***P < 0.001, ****P < 0.0001, mean ± SE).

and expensive precision machines or the preparation of miscellaneous and complicated reagents. It is a method in which the image of the ethidium bromide luminescence from the PCR products in an electrophoresed agarose gel on the UV illuminator is integrated into a computer memory directly by a CCD camera and then automatically analyzed. First, the DNA standard markers containing five gradually increasing DNA concentrations were loaded into each gel and then electrophoresed simultaneously with the PCR products to be measured. A quantitative standard curve was obtained by the Gel Doc system and a good linear correlation between the five quantitative concentration points and the size of the expression bands was demonstrated every time. After this, a preparatory experiment was performed to check that the con-

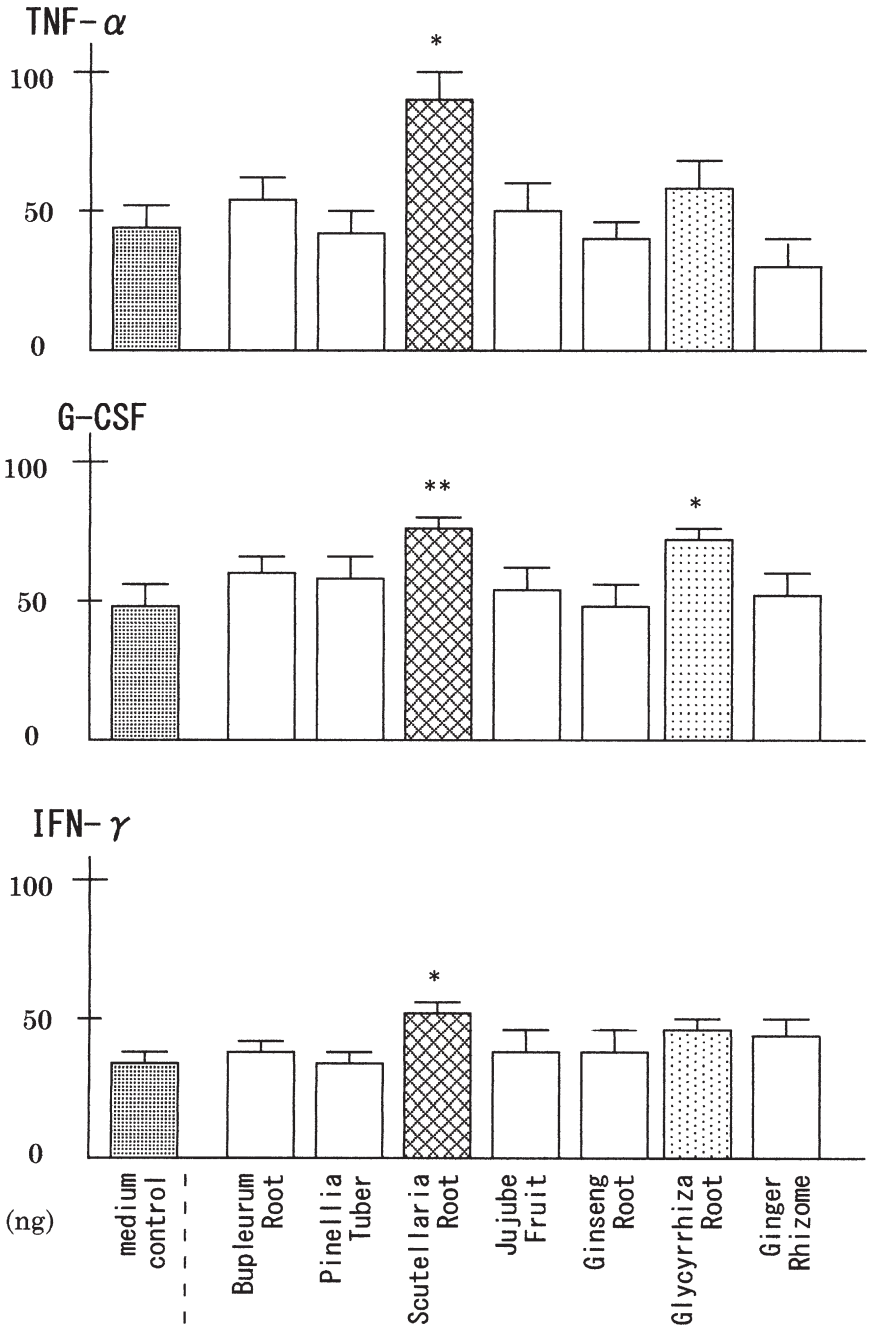

Fig. 5. mRNA expression levels of TNF-α, G-CSF, and IFN-γ induced by the seven individual herb components. The levels of mRNA expression for TNF-α and IFN-γ were significantly increased by scutellaria root, while the mRNA expression of G-CSF was significantly increased by scutellaria root and glycyrrhiza root (*$P < 0.05$, **$P < 0.01$, mean ± SE).

centration volume of the PCR products was in direct proportion to the cDNA concentration before PCR. The results suggested that the concentration of PCR products generally reflected the mRNA quantity.

The semi-quantitative results measured by the Gel Doc system indicated that the most marked effect on IL-1β, IL-10, IL-12, TNF-α, G-CSF, and IFN- mRNA expression was exerted by scutellaria root, followed by glycyrrhiza root. The mRNA expression of these cytokines induced by scutellaria root was significantly increased compared to the control ($P < 0.05$, $P < 0.01$, $P < 0.0001$, $P < 0.05$, $P < 0.01$, $P < 0.05$, respectively). In addition, the mRNA expressions of IL-12 and G-CSF induced by glycyrrhiza root were also significantly increased ($P < 0.001$, $P < 0.05$, respectively). On the

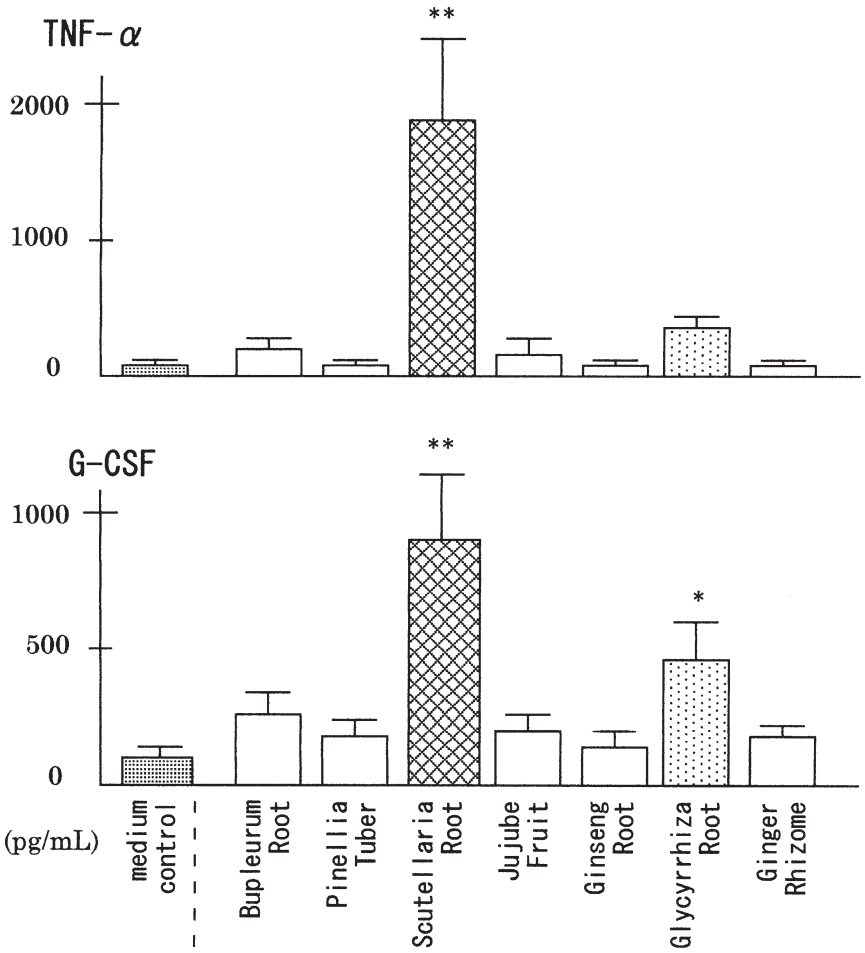

Fig. 6. Effects of the seven individual herb components on production levels of TNF-α and G-CSF in the cultural supernatant. The levels of TNF-α induced by added scutellaria root and G-CSF induced by added scutellaria root and glycyrrhiza root were significantly increased compared to the control culture (*$P < 0.05$, **$P < 0.01$, mean ± SE).

other hand, ginseng root slightly inhibited the TNF-α mRNA expression.

Measurement of the cytokine production in the cultural supernatants showed that the levels of IL-12, IL-1β, IL-10, TNF-α, and G-CSF induced by scutellaria root ($P < 0.0001$, $P < 0.001$, $P < 0.01$, $P < 0.01$, $P < 0.01$, respectively) and that of G-CSF induced by glycyrrhiza root were significantly increased compared to the control culture ($P < 0.05$). In the present study, as also found in previous reports, the levels of IFN-γ produced in the supernatants were below the detectable limit. However, the novel finding that scutellaria root was an IFN-γ inducer was obtained from the molecular analysis of cytokine mRNA expression.

The new information on TJ-9 as an IFN-γ inducer is important and could not be obtained from the analyses of production levels using ELISA kits. Thus, for examination of the effects of various medicines on cytokine production capacity, the semi-quantitative analysis of mRNA expression may attract the notice of clinical laboratory departments in the future, because the culture time for this molecular analysis is not so long. Such evidence is necessary to determine which cytokines may have adverse effects on the body and which may improve disease to act as a medicine. A semi-quantitative molecular analysis of cytokine-inducing allergic reactions using a patient's leukocytes cultured with various immunogens could become a useful tool for decisions on allergic diseases in the future.

Recently, a new quantitative method for nucleic acids, real-time PCR, has been used, and it is expected to become a practical method in the clinical hematology field (36). It is also possible to use this method in quantitative determination of cytokine mRNA expression. Several kinds of reagent kits for determination of cytokine mRNA can currently be found on the market. However, for cytokine determination, expensive instruments and high-priced specific reagents are necessary in order to use this method. If the clinical significance of cytokine measurement is confirmed in the future, then the quantitative determination of cytokine mRNA ex-

pression using this method could become a new clinical examination procedure.

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