The Relation between Serum Cytokine Levels and Common Laboratory Tests in Healthy Japanese Males

Masashi TSUNODA¹, Humio TSUNODA², Lucille GUEVARRA³ and David J. TOLLERUD³

¹Department of Public Health, School of Medicine, Fukushima Medical University, Fukushima

²Professor emeritus, Iwate Medical University, Morioka

³Center for Environmental and Occupational Health, School of Public Health, MCP Hahnemann University, USA

Abstract

Objectives: Cytokines are critical regulatory molecules that circulate in blood at measurable levels. However, the epidemiology of serum cytokine levels in healthy populations is still limited, particularly for Japanese populations. The objective of this study was to examine the relation between serum cytokine levels and common laboratory tests in a healthy Japanese population.

Methods: We studied serum levels of six cytokines (interleukin-4 (IL-4), IL-5, IL-6, IL-10, interferon- γ (IFN- γ), granulocyte-macrophage colony-stimulating factor (GM-CSF)) in a healthy Japanese population using a sensitive chemiluminescence ELISA (CL-ELISA). We examined the relations between serum cytokine levels and common laboratory tests using multiple regression analysis. We were particularly interested in potential relations between serum cytokine levels and C-reactive protein (CRP) in light of its relation to the risk of cardiovascular events. We also investigated the relation between cytokine levels, alcohol consumption and smoking.

Results: After adjusting for the influence of other cytokines and laboratory tests, the serum level of IL-4 was associated with IL-5 and GM-CSF. In addition to IL-4, IL-5 was also associated with IL-10 and IFN- γ . IL-6 was associated with IL-10 and IFN- γ , and IL-10 and IFN- γ were associated with GM-CSF. CRP was significantly negatively associated with serum IL-4 level. IL-6 was weakly correlated with alcohol consumption level in this population. IL-5 among smokers tended to be lower than that among nonsmokers.

Conclusions: Close relations among serum cytokine levels were observed in a healthy population. It is interesting that the level of IL-4, an anti-inflammatory cytokine, was inversely related to CRP level, suggested to be a marker of inflammation.

Key words: cytokines, interleukin, interferon, serum, healthy population

Introduction

Cytokine is one term for a group of protein cell regulators, variously called lymphokines, monokines, interleukins and interferons, which are produced by a wide variety of cells in the body, and play an important role in many physiological responses (1). Serum cytokine levels are increasingly being used in population studies, yet there are few studies describing the epidemiology of cytokine levels in healthy subjects. This

information is important to set the stage for widespread use of cytokine levels in epidemiological investigations.

Among common laboratory tests, some have been reported to correlate with cytokine levels in serum or plasma. Log-normalized plasma concentration of interleukin-6 (IL-6) was correlated with C-reactive protein (CRP) or HDL-cholesterol among healthy males (2). The potential relationships between cytokines and CRP are interesting because IL-6 and CRP were associated with the risk of future cardiovascular events (3, 4). Pradhan et al. also reported that IL-6 was correlated with CRP among healthy middle-aged females, and IL-6 and CRP were associated with development of type 2 diabetes mellitus (5). In addition, Woodward et al. reported that plasma IL-6 correlated with white cell count (6).

Lifestyle factors such as alcohol consumption may influence cytokine levels. Tilg et al. demonstrated a higher mean

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Reprint requests to: Masashi TSUNODA

Department of Public Health, School of Medicine, Fukushima Medical University, Hikarigaoka 1, Fukushima, Fukushima, 960-1295, Japan

TEL: +81(24)548-2111 (ext. 2211), FAX: +81(24)548-4600

E-mail: mtsunoda@fmu.ac.jp

value of serum IL-6 among heavy drinkers compared with controls (7). Gonzalez-Quintela et al. reported higher mean values of IL-6 and IL-10 in alcoholic patients compared with healthy volunteers (8). Thus, we were interested in whether alcohol consumption affects serum cytokine levels in a healthy population.

We previously observed that cigarette smoking was associated with alterations in some, but not all serum cytokine levels in a population of American females by the method of a sensitive chemiluminescence ELISA (CL-ELISA) (9). However, Satoh et al. reported no significant difference between smokers and nonsmokers for any cytokines in a study using 38 Japanese and 10 American males (10). It is also interesting to clarify the relations between serum cytokine levels and smoking.

Using a sensitive chemiluminescence ELISA (CL-ELISA) (11), we measured a panel of cytokines (IL-4, IL-5, IL-6, IL-10, interferon- γ (IFN- γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF)) in a population of healthy Japanese males. We also examined the relations between cytokine levels and common laboratory tests, alcohol consumption and smoking.

Materials and Methods

Subjects

All subjects were healthy adult Japanese males who lived in an agricultural district in Iwate Prefecture, in northern Japan. Subjects were selected among males aged 40 to 60 years old with no history of asthma or severe diseases checked by a questionnaire, who visited a medical clinic for a routine medical check-up. Due to the possibility that allergic diseases such as asthma may be related to serum cytokine levels (12), subjects with a history of allergy or symptoms of allergy or acute infectious diseases were eliminated by questioning. Subjects with a history such as autoimmune disease, malignant tumor, diabetes mellitus, hypertension, or heart disease were also eliminated by a questionnaire.

The total number of participants in the study was 235. The mean value of age was 48.7 years of old. All participants agreed to join this study, completed a questionnaire, and gave blood samples. Subject names and other personal identifiers were deleted from the database after the cytokine and laboratory data were linked.

Serum

The blood specimens were drawn from fasting donors by routine venipuncture using sterile blood collection tubes which were not coated by reagents such as heparin to avoid the contamination of endotoxins (13). The blood samples were collected specifically for this study in addition to the routine blood taking. Some of the serum samples had too little volume for analysis of all cytokines. Therefore, the numbers of samples were: 217 for IL-4, IL-6, and GM-CSF, 235 for IL-5, IL-10, and IFN- γ . They were centrifuged at 3,000 r.p.m. for 5 minutes within two hours after drawing. Each serum sample was separated into aliquots (Nalgene Cryovials, Nalge Company, Rochester, NY), and stored at -80°C . Before CL-ELISA analysis, the samples were thawed at room temperature.

Alcohol consumption and smoking

All participants completed a questionnaire that included questions about alcohol consumption and smoking. We asked about alcohol consumption and information about the frequency of drinking, and the quantity of drinking per day for each alcoholic beverage (Japanese sake, beer, Japanese "shochu", and whiskey). Each alcoholic beverage was converted into the equivalent volume of ethanol, and the consumption of ethanol per day was calculated for each subject. The total number of drinkers was 206 (101 who drank greater than 58 ml ethanol per day), and the number of nondrinkers was 28. In Japan, the unit for Japanese sake, "gou" (equivalent to 29 ml of alcohol) is a widely recognized measure of alcohol consumption. Drinking less than 2 gou, 58 ml per day is generally considered to represent a low level of alcohol consumption. One participant did not answer the questions on alcohol consumption.

Smoking status (current smokers, ex-smokers and never smokers) was checked by a questionnaire. For the present analysis, never smokers and ex-smokers were combined as "nonsmokers". The total number of current smokers was 115, and the total number of nonsmokers was 120 (the total number of ex-smokers was 61, and that of nonsmokers was 59).

Laboratory data

We obtained the following laboratory data for this population: glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), γ -glutamyl transpeptidase (γ -GTP), alkaline phosphatase, total cholesterol, triglyceride, C-reactive protein (CRP), the total white blood cell count, the red blood cell count, hemoglobin, and hematocrit.

CL-ELISA

The concentration of cytokines in serum were determined by chemiluminescence enzyme-linked immunosorbent assay (CL-ELISA) as previously described (14). Briefly, purified cytokine capture (coating) monoclonal antibodies (MAB) [anti-IL-4, anti-IL-5 and anti-IL-10 MAB (Pharmingen, San Diego, CA); anti-IL-6, anti-IFN- γ and anti-GM-CSF MAB (Endogen, Boston, MA)] for each cytokine were diluted in coating buffer (0.1 M NaHCO_3 , pH 8.2). Approximately, 100 μl of the appropriate MAB was added to each well of a 96 well flat bottom opaque microtiter immunoassay plate (Microlite 1, Dynatech Laboratories, Inc., Chantilly, VA). After overnight incubation, each well was blocked with 200 μl /well of 2.5% (weight/volume) BSA/PBS-Tween. One hundred μl /well of serum diluted 1:4 with PBS-Tween or recombinant human cytokine standard [IL-4, IL-5 and IL-10 (Pharmingen); IL-6 and GM-CSF (Genzyme); and IFN- γ (Endogen)] diluted with 1.0% BSA/PBS-Tween was added in triplicate wells. After incubation, each plate was washed with PBS-Tween, and biotinylated anticytokine detecting MAB in 0.5% BSA/PBS-Tween was added to each well [anti-IL-4 and IL-5 MAB (Pharmingen); anti-IL-6, anti-IL-10, anti-IFN- γ and anti-GM-CSF MAB (Endogen)]. Extravidin Alkaline phosphatase (Sigma), diluted 1:10,000 with 0.5% BSA/PBS-Tween, was then added at 100 μl /well, followed by 100 μl /well LumiPhos 530 (Lumigen, Southfield, MI). Light emission from each plate was measured using a microtiter plate luminometer (ML 1000; Dynatech Laboratories, Inc.). A stan-

standard curve for each plate was computed, and results for the test samples were calculated from the standard curve by extrapolation using BioCalc Data Analysis Software (BioCalc J. V., Herdon, VA). The detection limits were also determined at that time: 0.15 pg/ml for IL-4, IL-5 and IL-6; and 0.30 pg/ml for IL-10; and 0.60 pg/ml for IFN- γ ; and 2.50 pg/ml for GM-CSF. Samples with nondetectable cytokine levels were assigned the concentration of the detection limit.

These CL-ELISA assays have been validated for accuracy and sensitivity using recombinant human cytokines, including WHO standard recombinant cytokines where available. WHO standards included: Recombinant IFN- γ (Catalogue No. Gg23-901-530) from the National Institute of Allergy and Infectious Disease, NIH, Bethesda, MD; Recombinant Human IL-4 (88/656) from the National Institute for Biological Standards and Control, Hertfordshire, England; and Recombinant Human IL-5 (90/586) from the National Institute for Biological Standards and Control, Hertfordshire, England. The assays were extensively tested using media and human serum samples spiked with mixtures of known quantities of recombinant cytokines. Each assay is highly specific for the cytokine of interest, with no detectable cross-reactivity with other cytokines. These studies confirmed the quality assurance data provided by the manufacturers of the monoclonal antibody pairs.

Statistical analyses

Serum cytokine levels were approximately log-normally distributed, therefore all cytokine levels were logarithmically transformed prior to statistical analysis. Additional data available for analysis included information from a questionnaire and laboratory data. The population was divided into three groups based on their alcohol consumption; nondrinkers, drinkers who consumed less than 58 ml ethanol per day, and those who consumed 58 ml and over ethanol per day. In addition, the population was divided using the CRP value whether it exceeded 0.34 mg/dl or not. The CRP value of 0.34 mg/dl was used as the cut-off point whether it was normal or not following the criteria of the institute. The population was also divided into smokers and nonsmokers. For categorical comparisons, the difference between log-transformed mean values of each cytokine among the categories was analyzed by t test or ANOVA.

The relations between cytokine levels and laboratory data were initially analyzed using linear correlation analysis. Those variables that were correlated significantly or tended to be correlated with serum cytokine levels were then tested as covariates in multiple regression analyses. In addition, the categorical variable that demonstrated a significant or borderline difference among groups was tested as a covariate in multiple regres-

sion analyses. The multiple regression analysis was applied to clarify whether the relations observed in the correlation analysis were independent associations.

All statistics were computed using StatView version 5.0 (SAS, Berkeley, CA) for Macintosh.

Results

The number of samples with cytokines under detection limits were as follows; IL-4, 21/217 (9.7%), IL-5, 28/235 (11.9%), IL-6, 5/217 (2.3%), IL-10, 19/235 (8.1%), IFN- γ , 80/235 (34.0%), GM-CSF, 3/217 (1.4%). The geometric mean values of cytokines are summarized in Table 1. The maximum value of each cytokine was as follows; IL-4, 386.3 pg/ml, IL-5, 1013.0 pg/ml, IL-6, 1451.6 pg/ml, IL-10, 482.9 pg/ml, IFN-g, 295.7 pg/ml and GM-CSF, 28847.5 pg/ml. There were no correlations between cytokine levels and age.

We first evaluated individual laboratory test results for correlations with serum cytokine levels. Significant, or borderline significant correlations were observed for the following relations: IL-6 with GOT ($r=-0.192$, $p=0.005$), GPT ($r=-0.161$, $p=0.018$), γ -GTP ($r=-0.164$, $p=0.016$), triglycerides ($r=-0.147$, $p=0.030$), and white blood cell count ($r=0.117$, $p=0.084$); IL-10 with red blood cell count ($r=0.179$, $p=0.006$), and IFN- γ with triglycerides ($r=0.117$, $p=0.074$). For categorical variables, IL-4 tended to be lower among those that showed CRP values greater than 0.34 mg/dl compared with the others (Fig. 1). For smoking, only IL-5 tended to be lower among smokers compared with nonsmokers (geometric mean $\pm$ standard error, 5.93 ± 1.10 pg/ml for nonsmokers, 3.78 ± 0.67 pg/ml for smokers, $p=0.059$). These laboratory tests and smoking status were included in the multiple regression analyses.

The results of the multiple regression analyses are summarized in Table 2 for IL-4, IL-5 and IL-6 and Table 3 for IL-10, IFN- γ and GM-CSF. Serum level of IL-4 was associated with IL-5 and GM-CSF. In addition to IL-4, IL-5 was also associated with IL-10 and IFN- γ . IL-6 was associated with IL-10 and IFN- γ , IL-10 was associated with GM-CSF, and IFN- γ was associated with GM-CSF. CRP was significantly associated with serum levels of IL-4. Among other laboratory findings, GOT and

Table 1 Serum cytokine levels among healthy Japanese males

Cytokine	Geometric mean $\pm$ standard error (pg/ml)
IL-4	7.45 $\pm$ 1.08
IL-5	4.75 $\pm$ 0.61
IL-6	12.97 $\pm$ 1.32
IL-10	8.05 $\pm$ 0.78
IFN- γ	1.17 $\pm$ 0.11
GM-CSF	469.89 $\pm$ 50.11

Fig. 1 Serum IL-4 levels among CRP(+) and CRP(-) groups. CRP(+) was CRP >0.34 mg/dl. Each bar represents geometric mean, error bars represent standard errors. $p=0.058$.

Table 2 The multiple regression analyses for IL-4, IL-5 and IL-6 as dependent variables, and cytokines and laboratory tests as independent variables

Dependent variable	Independent variable	Regression coefficient	Standard error	p value
IL-4 ($R^2=0.487$, $p<0.0001$)				
	IL-5	0.399	0.072	<0.0001
	IL-6	0.034	0.090	0.7091
	IL-10	0.065	0.102	0.5254
	IFN- γ	-0.077	0.097	0.4299
	GM-CSF	0.537	0.092	<0.0001
	GOT	0.003	0.004	0.5587
	triglyceride	0.0004	0.001	0.5076
	CRP	-1.268	0.510	0.0137
	WBC count	0.002	0.003	0.4695
	smoking	0.026	0.097	0.7856
IL-5 ($R^2=0.505$, $p<0.0001$)				
	IL-4	0.324	0.059	<0.0001
	IL-6	-0.028	0.082	0.7302
	IL-10	0.293	0.089	0.0012
	IFN- γ	0.337	0.084	<0.0001
	GM-CSF	0.109	0.089	0.2244
	GOT	0.002	0.004	0.6860
	triglyceride	0.00006	0.001	0.9077
	CRP	0.119	0.466	0.7983
	WBC count	-0.005	0.003	0.0713
	smoking	0.083	0.087	0.3418
IL-6 ($R^2=0.390$, $p<0.0001$)				
	IL-4	0.020	0.054	0.7091
	IL-5	-0.021	0.059	0.7302
	IL-10	0.360	0.074	<0.0001
	IFN- γ	0.401	0.069	<0.0001
	GM-CSF	-0.101	0.076	0.1864
	GOT	-0.013	0.003	0.0002
	triglyceride	-0.002	0.0005	0.0009
	CRP	0.161	0.398	0.6870
	WBC count	0.005	0.003	0.0720
	smoking	0.055	0.074	0.4648

triglycerides were significantly associated with serum IL-6 levels, and triglycerides were significantly associated with IFN- γ .

In the analysis of alcohol consumption, there were no significant differences in cytokine levels between drinking categories. Only IL-6 tended to be correlated with the logarithm of alcohol consumption with a low correlation coefficient ($r=-0.125$, $p=0.065$).

Discussion

In the current study, for the comparison of the previous study (Kuniak et al. 1995), we measured the same panel of cytokines, IL-4, IL-5, IL-6, IL-10, IFN- γ and GM-CSF. The panel includes the two major classifications of cytokine, that is, Th1/Th2 classification (15) and the classification of proinflammatory/anti-inflammatory cytokines (16). According to the Th1/Th2, IFN- γ is included in Th1 cytokines. IL-4, IL-6, and IL-10 are included in Th2 cytokines. According to the classification of proinflammatory and anti-inflammatory cytokines, proinflammatory cytokines include IL-6 and IFN- γ , and anti-inflammatory cytokines include IL-4 and IL-10. GM-CSF, which is produced

by T cells, fibroblasts, vascular epithelial cells (17) also has an important role in the immune system. GM-CSF induces maturation (18, 19) and proliferation of macrophages (20, 21).

For serum cytokine levels, there are differences among studies (10). The differences among studies may be partly due to ethnic or gender differences in serum cytokine levels. The geometric mean of IL-6 in the current study was 12.97 pg/ml. This was higher than the values among American middle-aged females (4, 5). However, it was close to the value among healthy subjects who were of Caucasian origin in Spain (22).

In this study, we selected healthy adult Japanese males who lived in the northern part of Japan and visited a clinic for a routine annual medical check-up. Given the narrow age range of the study sample, it is not surprising that we found no correlation between ages and cytokine levels. Previous studies reported that serum IL-6 (23) or plasma IL-6 (24) was correlated with age.

Serum levels of several of the cytokines were strongly correlated, including both T-Helper 1 (TH1) and TH2 cytokines (15). The close correlations among cytokines were also observed in previous studies (9, 10).

Table 3 The multiple regression analyses for IL-10, IFN- γ and GM-CSF as dependent variables, and cytokines and laboratory tests as independent variables

Dependent variable	Independent variable	Regression coefficient	Standard error	p value
IL-10 ($R^2=0.519$, $p<0.0001$)				
	IL-4	0.030	0.048	0.5254
	IL-5	0.169	0.052	0.0012
	IL-6	0.285	0.059	<0.0001
	IFN- γ	-0.023	0.066	0.7252
	GM-CSF	0.379	0.063	<0.0001
	GOT	0.003	0.003	0.2713
	triglyceride	0.0003	0.0004	0.4819
	CRP	0.163	0.354	0.6461
	WBC count	0.0002	0.002	0.1454
	smoking	0.0066	0.066	0.2572
IFN- γ ($R^2=0.409$, $p<0.0001$)				
	IL-4	-0.040	0.050	0.4299
	IL-5	0.213	0.053	<0.0001
	IL-6	0.349	0.060	<0.0001
	IL-10	-0.026	0.073	0.7252
	GM-CSF	0.204	0.070	0.0040
	GOT	0.004	0.003	0.1712
	triglyceride	0.001	0.0004	0.0086
	CRP	0.297	0.371	0.4239
	WBC count	-0.00008	0.002	0.9718
	smoking	-0.056	0.069	0.4166
GM-CSF ($R^2=0.550$, $p<0.0001$)				
	IL-4	0.263	0.045	<0.0001
	IL-5	0.066	0.054	0.2244
	IL-6	-0.084	0.063	0.1864
	IL-10	0.396	0.066	<0.0001
	IFN- γ	0.194	0.067	0.0040
	GOT	-0.001	0.003	0.6995
	triglyceride	-0.0004	0.0004	0.3091
	CRP	0.424	0.363	0.2408
	WBC count	-0.003	0.002	0.2549
	smoking	-0.121	0.067	0.0744

The mean value of IL-4 among those who had CRP levels above 0.34 mg/dl tended to be lower than that of subjects with lower CRP levels. This relation was confirmed in the multiple regression analysis. The clinical significance of this observation is uncertain. However, it is interesting that IL-4 is classified by many as an “anti-inflammatory” cytokine (16), and CRP is a protein that reflects inflammatory status. Thus, the level of IL-4, an anti-inflammatory cytokine, was inversely related to CRP level, suggested to be a marker of inflammation.

It is reported that plasma concentration of IL-6 was correlated with CRP among healthy males (3) and healthy females (5). A significant correlation has also been reported between serum IL-6 and CRP bioactivity in patients with rheumatoid arthritis (25). In the current study, the mean value of IL-6 was not significantly related to CRP values, in agreement with a report by Hager et al. (23).

IL-6 tended to be negatively correlated with the logarithmic value of ethanol consumption per day. In previous studies, Tilg et al. demonstrated a high mean value of serum IL-6 among heavy drinkers (7). It was also reported that the mean value of serum IL-6 determined by a bioassay was significantly

higher among alcoholic hepatitis patients than that among a healthy group who consumed less than one drink per day (26). For alcoholic liver cirrhosis, the mean value of serum IL-6 bioactivity among the patients was high, and that among controls was under the detection limit (27). The association between IL-6 and alcohol consumption in the current study did not confirm the relationships among healthy subjects.

Smoking was associated with alterations in some, but not all serum cytokine levels in a population of American females (9), however, no significant difference between smokers and nonsmokers for any cytokines was observed in a study using 38 Japanese and 10 American males (10). In the current study, only IL-5 tended to be lower among smokers compared with nonsmokers. Ethnic or gender differences may affect the discrepancies among studies.

Triglyceride was a significant variable for IL-6, but the regression coefficient was negative. Fernandez-Real et al. reported that serum IL-6 levels correlated positively with fasting triglyceride among 32 healthy subjects (17 females) who were of Caucasian origin (22). In an experimental study, intravenous IL-6 administration increased the level of triglyceride in serum

in a dose-dependent manner in rats (28). These results were not in agreement with the relation between serum IL-6 and triglyceride levels in the current study.

For IFN- γ , triglyceride level was also a significant variable. In a previous study, patients with chronic psoriasis who were treated with intramuscular administration of human recombinant IFN- γ showed a mild elevation in serum triglyceride level (29).

In summary, we measured serum levels of six cytokines (IL-4, IL-5, IL-6, IL-10, IFN- γ , and GM-CSF) in a healthy Japanese male population using CL-ELISA. Serum level of IL-4 was associated with IL-5 and GM-CSF. In addition to IL-4, IL-5 was also associated with IL-10 and IFN- γ . IL-6 was asso-

ciated with IL-10 and IFN- γ , and IL-10 and IFN- γ were associated with GM-CSF. After adjusting for the influence of other cytokines and laboratory tests, CRP was significantly negatively associated with serum IL-4 level. Triglyceride was a significant variable for IL-6 (the regression coefficient was negative) and for IFN- γ . Finally, IL-6 was weakly correlated with alcohol consumption level in this population ($r=0.118$, $p=0.078$). Although some of these relations among cytokines and common laboratory tests were not in agreement with previous findings, it is possible that cytokine levels and common laboratory tests affect each other. Further studies are required.

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