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Serum Cytokine Levels in Patients With Chronic Hepatitis B According to Lamivudine Therapy

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Background: Cytokines are known to play critical roles in the pathogenesis of chronic hepatitis B (CHB). However, the relationship between cytokines and treatment responses to drugs for CHB is not clearly defined yet. We measured the serum cytokine levels of interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, vascular endothelial growth factor, interferon-γ, tumor necrosis factor- (TNF-α), macrophage/monocyte chemotactic protein 1, and epidermal growth factor to elucidate the cytokine expression pattern according to the patients' responses to lamivudine. *Methods*: Fifty-eight specimens from 27 CHB patients and 98 specimens from healthy individuals were tested for 12 kinds of cytokines. The patients were grouped as: before treatment, ongoing treatment, during maintaining remission, and patients with viral breakthrough owing to resistance against lamivudine. The Evidence Investigator (Randox, Antrim, UK), a protein chip analyzer, was used to quantify serum cytokines. *Results*: Among 12 cytokines, IL-6, IL-8, IL-10, and TNF-α were significantly elevated in patients with resistance against lamivudine compared with patients maintaining response. IL-8, IL-10, and TNF-α levels also weak to moderate correlated with ALT and HBV-DNA concentrations. *Conclusions*: Serum cytokine levels would reflect the pathological differences of the individual treatment phases and may become useful indices in monitoring the treatment response of CHB. J. Clin. Lab. Anal. 25:414–421, 2011.

Key words: cytokine; chronic hepatitis B; hepatitis B virus (HBV); lamivudine; protein chip array

INTRODUCTION

The hepatitis B virus (HBV) is one of the most common etiologic agents of chronic liver infection, with varied clinical expressions ranging from asymptomatic conditions to liver cirrhosis and hepatocellular carcinoma. Because HBV is not a cytolytic virus, most of the infection-induced pathologies are known as the host's immunoreactions against the viral products (1–3). As agents of immunoreaction, cytokines are produced by various types of cells, including lymphocytes, macrophages, natural killer cells, and dendritic cells, and are known to play pivotal roles in the pathogenesis of chronic HBV infection (1,4–6). Inflammatory responses in the liver infected with the HBV are known to be caused by cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) (7). The same cytokines trigger elimination of the virus as well (1,8–12). Fibrosis of the liver is known to be induced and promoted by cytokines, such as transforming growth factor-β (TGF-β) (13,14). Also, it has been reported that genesis and expansion of hepatoma accompany anomalies of immunity involving various types of cytokines (15–19). In a recent study, interleukin-6 (IL-6) ensured early control of viral hepatitis, which limited the adaptive immune response activation and protected the HBV-infected hepatocyte from death (20). Therefore, cytokines are known to play critical roles in pathophysiology of the disease. Several studies for the association of the cytokine profiles and the sequence of the disease phases or clinical features have been reported (21–23). However, there has been no study on the relationship

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TABLE 1. Clinical Characteristics of Study Groups

Parameters	Control group ($n=20$)	Before lamivudine therapy ($n=10$)	During lamivudine therapy ($n=6$)	Remission ($n=22$)	Recurrence ($n=20$)
Age (years)	40±8	35±6	36±7	36±9	43±11
Gender (M/F)	12/8	10/0	4/2	17/5	16/4
ALT (IU/l)	21±8	185±145	45±21	23±11	183±121[a]
HBeAg/Anti-HBe[b]	–	10/0	5/1	0/15	17/3
HBV-DNA (pg/mL)	–	647±651	56±137	0±0	529±710[a]

All results are shown as mean±standard deviation. ALT, alanine aminotransferase (reference range: <46 IU/l).
[a]$P<0.05$ (ANOVA).
[b]Number of patients with HBeAg/anti-HBe positive.

between cytokine levels and the treatment responses to drugs for chronic hepatitis B (CHB).

Therefore, in this study, we measured serum cytokine levels from the blood samples of CHB patients undergoing lamivudine therapy to examine the correlation between the cytokine levels and the progress of lamivudine therapy. We measured 12 serum cytokine levels of IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, vascular endothelial growth factor (VEGF), IFN-γ, TNF-α, macrophage/monocyte chemotactic protein 1 (MCP1), and epidermal growth factor (EGF) simultaneously from the specimens drawn at the time before admission, during response maintenance, and at the point of recurrence owing to resistance. Also, as the cytokine levels are known to be affected by the blood collection and storage condition (24), we studied the effect of storage conditions of serum samples on the levels of cytokines.

MATERIALS AND METHODS

Study Subjects

Fifty-eight blood samples were prospectively collected from 27 CHB patients undergoing lamivudine therapy. Venous blood samples were drawn from those patients with the consents at two to four times as follows: before lamivudine therapy, during lamivudine therapy (3 months and/or 6 months after initiation of the therapy), during remission maintenance, and during recurrence by drug resistance. Remission of CHB was defined as normalization of serum alanine aminotransferase (ALT) level, loss of serum HBV DNA, and HBeAg clearance, and patients with HBeAg seroconversion were maintained on treatment for 3 months after seroconversion was confirmed, according to the 2004 American Association for the Study of Liver Diseases guidelines. For all the subjects, two to four continuous specimens were taken from each patient. Diagnosis, remission, and recurrence of CHB were determined by hepatologists based on positive serum HBsAg for more than 6 months and positive HBeAg with HBV-DNA for more than 20,000 IU/ml or negative HBeAg with HBV-DNA for more than 2,000 IU/ml and persistent elevation of ALT. For the control group, 20 health screening examinees, who were deemed as normal by laboratory tests, particularly having negative serological markers for hepatitis viruses, including HBsAg, anti-HBc, and anti-HCV, were chosen considering age and gender matched the patients (Table 1).

Measurement of Cytokine Levels

Serum was separated within 2 hr of taking venous blood samples. If cytokine measurement was not possible on the same day, the specimen was immediately frozen. A protein chip analyzer, Evidence Investigator, and Cytokine Array I reagents (Randox, Antrim, UK) were used to quantify 12 cytokines, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, VEGF, IFN-γ, TNF-α, MCP1, and EGF in the sera. Tests were performed in accordance with the instructions of the manufacturer. The frozen serum specimen and the reagent carrier comprising nine protein chips was left at room temperature for about 15 min before the test so that there would be no moisture when unpacked. Monoclonal antibodies for the 12 kinds of cytokines were attached on each protein chip surface. Assay diluent of 200 µl was applied into each well of the chips, and then 100 µl of serum was added. The chips were placed in the thermo shaker at 37°C, 370 rpm for 1 hr for reaction. After washing the chips with washing fluid six times, 300 µl of conjugate fluid containing enzyme-labeled antibody was fed into each well. After another 1 hr reaction in the same conditions, the chips were washed again six times. Substrate reagent containing one part luminol and one part peroxidase was added into each well of the chip, and then after 2 min the carrier was inserted into the Evidence Investigator. The charge-coupled device camera detected the chip signals, saving images in the computer. The computer

program was then used to quantify cytokine levels and the results were read.

Testing Stability of Specimens

The following experiments were carried out to test stability of the specimens. Venous blood specimens were taken from each healthy control individual. Serum was separated as soon as possible, and cytokine levels were measured and compared between fresh serum specimens, refrigerated serum specimens, frozen serum specimens, and serum specimens which were frozen, refrigerated, and then frozen again.

The separated serum was divided into 200 μl, one of which was tested immediately as a fresh specimen, another was refrigerated for 5 days and then tested. The remaining specimens were immediately kept in the −80°C freezer. One of the latter specimens were taken out of the freezer and refrigerated at 4°C for 8 hr, frozen again, and tested on the 5th day.

Reference Values

Serum cytokine levels were measured in 98 healthy individuals. Analyse-it Method Evaluation Edition version 2.22 software (Analyse-it Software Ltd., City West Business Park, Leeds, UK) was used to set upper levels in the 95% confidence interval as reference values (Table 2).

Statistical Analysis

Differences in clinical indices between the patient group and the control group were analyzed for the variance between the groups (ANOVA) and by χ^2 tests. Levels of cytokines according to storage conditions were compared using ANOVA and paired *t*-tests. Multiple comparison of cytokine levels between the patient subgroups and between the patient group and the control group was analyzed using Kruskal–Wallis test with Bonferroni compensation and/or Mann–Whitney *U* test. Correlation coefficient (*r*) between cytokine levels and clinical indices were calculated by Spearman's correlation tests. All statistical analyses were performed using Analyse-it Method Evaluation Edition version 2.22 software. A *P*-value less than 0.05 was determined as statistically significant.

TABLE 2. References Limits of Cytokines Established From 98 Healthy Subjects

Cytokines	Mean ± SD (pg/ml)	95% upper limit (pg/ml)
IL-1α	0.10 ± 0.41	0.91
IL-1β	1.42 ± 4.33	9.91
IL-2	1.83 ± 4.19	10.04
IL-4	0.69 ± 1.52	3.67
IL-6	3.15 ± 9.31	21.40
IL-8	160.45 ± 352.05	630.50
IL-10	0.11 ± 0.29	0.67
VEGF	198.75 ± 172.69	421.96
IFN-γ	0.45 ± 1.48	3.36
MCP1	296.68 ± 116.48	524.97
EGF	111.92 ± 87.06	282.56
TNF-α	5.05 ± 14.18	7.28

IL-, interleukin-; VEGF, vascular endothelial growth factor; IFN-γ, interferon-γ; MCP1, macrophage/monocyte chemotactic protein 1; EGF, epidermal growth factor; TNF-α, tumor necrosis factor-α.

RESULTS

Clinical Charactersteristics of the Patient and Control Groups

The patient group was divided into four subgroups: before lamivudine therapy, during lamivudine therapy, during remission maintenance, and recurrence by drug resistance. According to ANOVA between the subgroups, ALT and HBV-DNA showed significant differences ($P < 0.0001$, $P = 0.0012$, by ANOVA), but age did not show significant differences between the subgroups ($P > 0.05$, by ANOVA). The χ^2 test between the subgroups also showed that there were no statistically significant differences for gender and $HBeAg^+/HBeAb^+$ results (χ^2 test, $P > 0.05$; Table 1).

Stability by Storage Condition of Specimens

In order to determine whether storage conditions of each specimen had significant effect on cytokine measurements, we compared the cytokine levels from various storage conditions—fresh serum separated from blood immediately, refrigerated serum, frozen serum, and serum frozen, refrigerated, and then frozen again. The effects of storage conditions were estimated with the blood specimens of the same collections. ANOVA of cytokine levels within each specimen storage conditions showed no significant differences ($P > 0.05$, by ANOVA), and the paired *t*-test of cytokine levels in the fresh serum specimen and the other serum specimens showed statistically significant differences for the refrigerated specimen and the specimen frozen, refrigerated, and frozen again for VEFG and MCP1 (Table 3).

Serum Cytokine Levels of Patient and Control Groups

Levels of all cytokines, except TNF-α, IL-6, IL-8, and IL-10, were not different between the treatment phases and the healthy control group (median of the healthy control TNF-α: 2.29 pg/ml; IL-6: 1.76 pg/ml; IL-8: 98.23 pg/ml; IL-10: 0.00 pg/ml). As illustrated in Figure 1, median serum TNF-α, IL-6, IL-8, and IL-10

TABLE 3. Cytokine Measurements by Specimen Storage Conditions

Cytokines	Fresh serum	Frozen	*P*-value[a]	Refrigerated	*P*-value[a]	Frozen, refrigerated, and frozen	*P*-value[a]	Total *P*-value[b]
IL-2	2.35±0.60	2.01±0.44	0.49	1.73±0.35	0.40	1.03±0.46	0.14	0.06
IL-4	2.01±1.03	2.25±2.25	0.93	0.37±0.37	0.20	0.29±0.29	0.17	0.64
IL-6	0.52±0.28	0.47±0.21	0.56	0.31±0.18	0.15	0.43±0.19	0.45	1.00
IL-8	4.07±0.82	4.02±0.76	0.90	3.13±0.51	0.06	3.35±0.63	0.08	0.89
IL-10	0.28±0.19	0.27±0.18	0.96	0.0±0.0	0.20	0.0±0.0	0.20	0.09
VEGF	234.35±72.40	214.75±68.42	0.05	192.32±64.02	0.01	222.85±64.26	0.50	1.00
IFN-γ	2.62±1.81	1.82±1.82	0.73	0.85±0.55	0.26	0.0±0.0	0.21	0.41
TNF-α	1.83±0.41	1.77±0.44	0.90	2.84±2.15	0.66	1.48±0.30	0.52	0.52
IL-1α	0.23±0.08	0.28±0.06	0.41	0.13±0.08	0.27	0.23±0.08	0.96	0.79
IL-1β	0.43±0.27	0.74±0.54	0.51	0.38±0.24	0.86	0.45±0.29	0.97	0.69
MCP1	225.69±16.78	210.97±11.69	0.09	195.10±13.51	0.00	197.17±15.18	0.00	0.57
EGF	40.09±11.70	39.09±11.04	0.54	38.53±9.99	0.52	37.72±10.24	0.44	1.00

All results are shown as mean±standard deviation.
[a]Paired *t*-test with fresh serum.
[b]Calculated by ANOVA.

levels in the recurrence by drug resistance group were higher than the remission maintenance group, and levels of these cytokines were not different between the healthy control and the remission groups, except IL-6 and IL-8, which were higher in the healthy control than the remission group ($P = 0.0218$ for IL-6; $P = 0.0016$ for IL-8). The TNF-α levels in the recurrence by drug resistance group were higher in comparison to the remission maintenance group (median of remission maintenance group, 0.00 pg/ml; median of recurrence by drug resistance group, 4.92 pg/ml; $P = 0.0025$). The IL-6 level also significantly increased in the group of recurrence by drug resistance in comparison to the remission maintenance group (median of remission maintenance group, 0.16 pg/ml; recurrence by drug resistance group, 0.79 pg/ml; $P = 0.0223$). The IL-8 level showed a significant increase in the group of recurrence by drug resistance (median 14.52 pg/ml) in comparison to the remission maintenance group (2.35 pg/ml) ($P = 0.0115$). In the case of IL-10, the pattern was similar to those of TNF-α (median of remission maintenance group: 0.00 pg/ml; recurrence by drug resistance group: 0.62 pg/ml; $P < 0.0001$).

$HBeAg^+$/Anti-HBe^+ Status and Serum Cytokine Levels

In order to analyze the serum cytokine levels and clinical indices for the $HBeAg^+$ and anti-HBe^+ status, all the involved subjects regardless of the treatment phases were divided into $HBeAg^+$ and anti-HBe^+ subgroups. There were no age differences between the subgroups. However, the $HBeAg^+$ subgroup had significantly high ALT and HBV-DNA results in comparison to the anti-HBe^+ subgroup ($P = 0.0016$ and $P = 0.0087$, respectively). The serum cytokine levels in either subgroup did not show any statistically significant differences (Table 4).

Correlations Between Clinical Indices and Cytokine Levels

In order to analyze the correlation of clinical indices and cytokine levels, ALT, HBV-DNA, and HBeAg test results were analyzed for correlations. The results for all patients regardless of treatment phases showed significant correlation of ALT with IL-4, IL-8, IL-10, and TNF-α (respectively, $r = 0.26$, $P = 0.0472$; $r = 0.43$, $P = 0.0006$; $r = 0.56$, $P < 0.0001$; $r = 0.39$, $P = 0.0018$; Table 5). A significant correlation was also observed between HBV-DNA and IL-1β, IL-6, IL-8, IL-10, and TNF-α (respectively, $r = 0.29$, $P = 0.0270$; $r = 0.27$, $P = 0.0388$; $r = 0.35$, $P = 0.0056$; $r = 0.60$, $P < 0.0001$; $r = 0.31$, $P = 0.0145$; Table 5). These results implied that the serum IL-8 and IL-10 levels are related to the degree of liver inflammation and the amount of virus.

DISCUSSION

Liver inflammation by the HBV is immune mediated. Both acquired immunity and innate immunity are thought to play pivotal roles in the pathogenesis of HBV infection (1–3). In this study, we took simultaneous measurements of 12 kinds of cytokines in CHB patients, in order to assess the roles of cytokines according to the responses to a widely used therapeutic drug, lamivudine. We could measure the concentrations of IFN-γ, IL-4, IL-6, IL-8, IL-10, TNF-α, VEGF, MCP1, and EGF successfully in the serum, but IL-1α, IL-1β, and IL-2 could not be measured in most of the

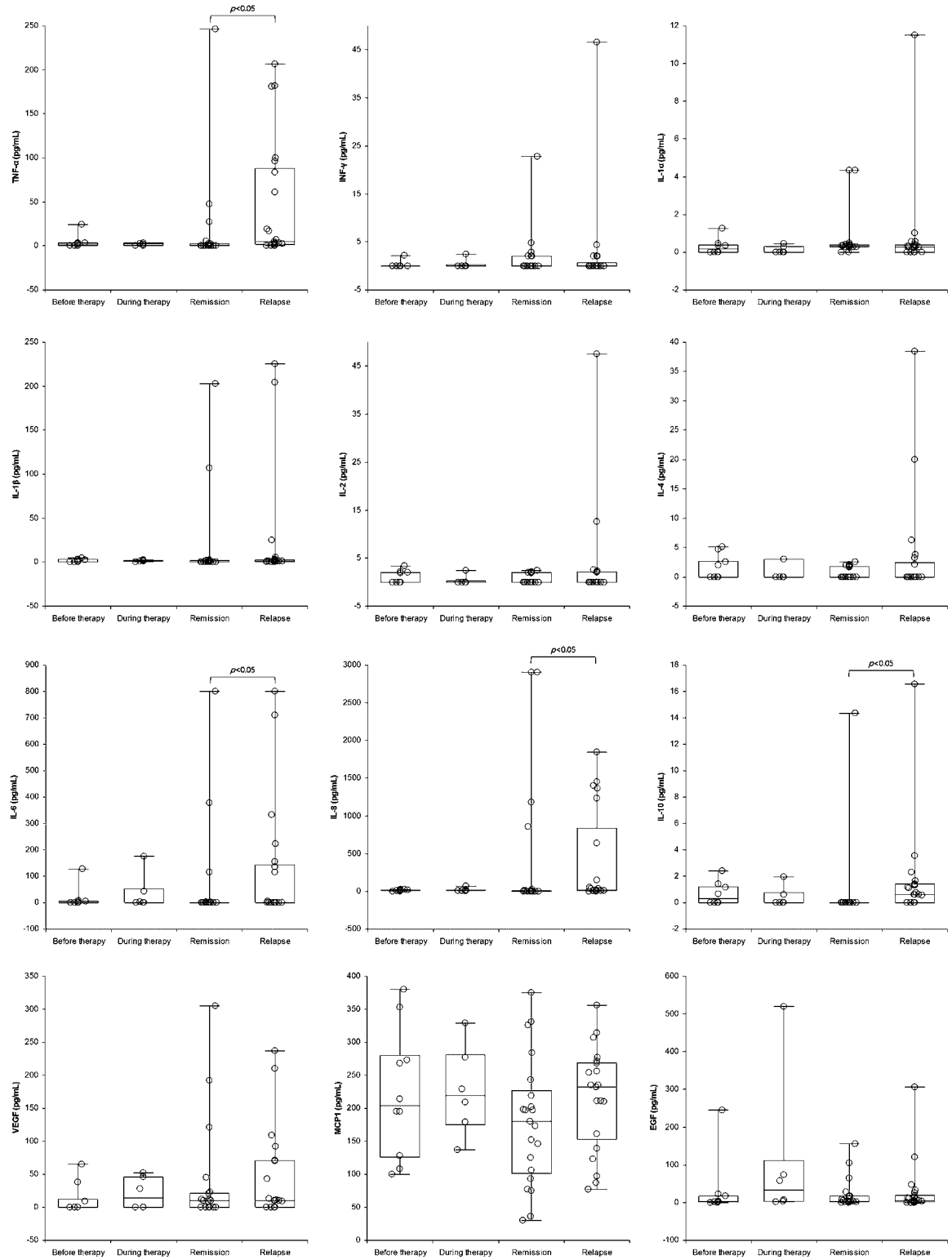

Fig. 1. Serum cytokine levels for chronic hepatitis B patients according to the phases of lamivudine therapy. TNF-α, IL-6, IL-8, and IL-10 levels of the recurrence group were higher than those of the remission group ($P < 0.05$), and there were no significant difference between the levels of other cytokines according to the treatment phases. The upper and lower ends of the boxes and box inner lines correspond to the upper and lower quartiles and median values, respectively. Whiskers denote extreme distribution values, and circles represent individual concentrations.

TABLE 4. Clinical Indices and Cytokine Levels for HBeAg$^+$ and Anti-HBe$^+$ Groups of Chronic Hepatitis B Patients Under All Treatment Phases

Parameters	HBeAg+ ($n = 32$)	Anti-Hbe+ ($n = 19$)	*P*-value
Age (years)	39±10	36±10	0.2041
ALT (IU/l)	160.6±122.9	51.5±93.5	0.0016
HBV-DNA (pg/ml)	504.2±666.9	65.5±265.2	0.0087
TNF-α (pg/ml)	28.8±58.5	17.9±56.6	0.5167
IL-6 (pg/ml)	84.6±193.6	68.5±198.3	0.7773
IL-8 (pg/ml)	259.7±543.1	420.9±930.4	0.4368
IL-10 (pg/ml)	0.75±0.9	0.85±3.3	0.8756

All results are shown as mean±standard deviation. ALT, alanine aminotransferase (reference range: <46 IU/l).

TABLE 5. Correlation of Clinical Indices and Cytokine Levels in the All Study Subjects Regardless of Treatment Phases

Parameter	Compared cytokine	Correlation coefficients (*r*)	*P*-value
ALT	IL-1α	−0.13	0.3230
	IL-1β	0.20	0.1265
	IL-2	0.09	0.5173
	IL-4	0.26	0.0472
	IL-6	0.23	0.0835
	IL-8	0.43	0.0006
	IL-10	0.56	<0.0001
	VEGF	0.19	0.1465
	IFN-γ	0.09	0.5144
	MCP1	0.17	0.1844
	EGF	0.22	0.0904
	TNF-α	0.39	0.0018
HBV DNA load	IL-1α	−0.15	0.2540
	IL-1β	0.29	0.0270
	IL-2	0.09	0.5177
	IL-4	0.24	0.0646
	IL-6	0.27	0.0388
	IL-8	0.35	0.0056
	IL-10	0.60	<0.0001
	VEGF	0.04	0.7348
	IFN-γ	−0.02	0.8693
	MCP1	0.17	0.1931
	EGF	0.09	0.4742
	TNF-α	0.31	0.0145

specimens, because their amount was probably below the detection limits. According to some previous reports, IL-2 could be measured, but IL-10 level was quite difficult to be determined (25). The differences between the previous study and our data are thought to be owing to varied sensitivity of the measurement methods, which demands further studies.

Even after drawn specimens, cytokines continue to be produced by various cells and undergoes receptor binding or degradation. As a means to minimize such effects, it is recommended that the cytokine levels should be analyzed at least within 5 hr after drawing specimens. However, owing to practical difficulties, the cytokine levels are often measured in batches after some period of storage. When storing, it is recommended that the blood need to be collected in uncontaminated containers, centrifuged as soon as possible, frozen immediately, and stored at −80°C. Repeated freezing and thawing should be avoided in all instances (24). In these respects, we carried out experiments to examine stability of cytokines in various storage conditions of the specimen. Most cytokines, except VEGF, MCP1, and EGF, in healthy control subjects were below minimum measurable analytical levels, and thus analyses were difficult. Also, although ANOVA did not show any significant differences, the paired *t*-tests of VEGF and MCP1 in refrigerated specimens and refrigerated and then frozen specimens in comparison with fresh serum specimens showed significant decreases in cytokine levels. This implies that, if immediate testing of the specimen is difficult, the serum should not be left at room temperature or kept refrigerated, but frozen and stored at −80°C as soon as possible.

In our results, the serum levels of some cytokines changed in CHB patients according to their progress in lamivudine therapy. This implies that the cytokine levels of each subgroup in lamivudine therapy may reflect the changes in individual patients. It was also found that cytokine levels were affected by various factors, including inflammations, virus load, and virus antigen load (Tables 4 and 5). IL-6, which showed elevation in the group with recurrence by drug resistance, is an endogenous pyrogen which causes fever in case of inflammatory response in the body. IL-8 plays the role of promoting activation of neutrophils. TNF-α promotes creation of other inflammation-related cytokines, including IL-6, and activates inflammation-related leucocytes (24). All cytokines with significant elevations in our results were ones which are known to be related to inflammations. Thus, in cases of the recurrence by drug resistance, increment of cytokine production was thought to be owing to inflammations caused by viral replication. At the same time, IL-8, IL-10, and TNF-α showed weak-to-moderate correlation with ALT and HBV-DNA load in all patients, whereas IL-4 and IFN-γ showed correlation only with ALT. Although there have been a number of reports that IL-10 is related to liver inflammation and HBV-DNA load (26–29), it is currently difficult to provide practical explanations for such correlations. The cytokines may only be the inflammatory markers and not the direct markers for the viral replication, and this might be an explanation of the weak correlation between the levels of some cytokines and ALT and/or HBV-DNA load. Nevertheless, analyses of all changing factors and

changes in cytokine expression pattern by progress of disease and progress of pharmaceutical therapy are thought to prove useful in understanding pathogenesis and predicting development of resistance. Such studies, for example, can be used for predicting development into liver cirrhosis or hepatocellular carcinoma in CHB or recurrence of hepatitis owing to resistance after some time of maintaining remission following treatment with drugs, including lamivudine. Also, when the same patient has been maintaining remission and then begins to exhibit resistance, the HBV-DNA level increased, but the ALT level did not show any significant increase. It was learned that cytokine expression exhibited anomaly before ALT, which is a biochemical index. Therefore, changes in cytokine expression pattern could be used to predict development of resistance. In this study, levels of some cytokines were found to elevate in the recurrence by drug resistance group than in the remission maintenance by lamivudine therapy group. In particular, there were significant increases in TNF-α, IL-6, IL-8, and IL-10.

There were limited subjects in this study, and unfortunately we could not collect all samples from all treatment phases per patient. Therefore, our data imply a trend of increased cytokine levels according to the treatment phases. Further follow-up studies with more subjects are expected to prove useful for cytokine levels as a predictive factor of resistance against lamivudine.

From our data, serum cytokine levels in CHB patients reflected various clinical indices, including viral markers, inflammatory response of the body, viral load, and state of lamivudine treatment. Therefore, serum cytokine profile analysis would not only help understand the etiology of disease but also play a useful role in predicting efficacy of and resistance against therapeutic drugs, including lamivudine.

In this study, we performed simultaneous measurements of 12 kinds of cytokines in the serum of CHB patients to examine expression patterns of cytokines according to the treatment phases. IL-6, IL-8, IL-10, and TNF-α levels were higher in patients with recurring hepatitis owing to drug resistance than in patients with maintaining remission. Serum cytokine values may reflect the pathological differences of the individual treatment phases, and might become useful indices in monitoring the treatment response of CHB.

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