Changes of peripheral lymphocyte population in patients with chronic hepatitis C treated with herbal medicine (Maoto) and IFN-ß

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We recently reported that the herbal medicine (Maoto) might have immunomodulatory effects when used in conjunction with IFN-ß. In order to examine the relation between host immune reaction and virological effects upon treatment with Maoto and IFN-ß, we monitored the changes in lymphocyte populations of peripheral blood by flow-cytometry. Twenty-five patients with chronic hepatitis C were enrolled in this study. They received a daily dose of 6 million units of IFN-ß for 8 weeks. Maoto was given orally 4 times a day during the IFN-ß administration, and we monitored the changes in lymphocyte populations of peripheral blood by flow-cytometry. Six patients were sustained virological responders (SR), 10 were transient responders (TR), and 9 were nonresponders (NR). The percentage of $CD16^+CD56^+$ lymphocyte populations was decreased in all groups between pretreatment and 4 weeks, but it was significantly increased in SR compared with TR and NR between 4 and 8 weeks. The percentage of $HLADR^+CD8^+$ lymphocyte populations was significantly increased in SR and TR compared with NR between pretreatment and 8 weeks. Our results suggested that monitoring of changes in peripheral $CD16^+CD56^+$ and $HLADR^+CD8^+$lymphocyte populations could be useful to treat chronic hepatitis C with the combination therapy of Maoto and IFN-ß.

Key words herbal medicine, Maoto, chronic hepatitis C, $CD16^+CD56^+$lymphocyte, $HLADR^+CD8^+$lymphocyte.

Introduction

Interferon (IFN) is currently the most effective antiviral agent for the treatment of chronic hepatitis C.[1-3] Administration of IFN to patients with chronic hepatitis C may eliminate hepatitis C virus (HCV) and lead to long-term remission of the illness. Nevertheless, a high proportion of patients fails to eliminate HCV, eventually leading to serious consequences such as liver cirrhosis and hepatocellular carcinoma.[4,5] Recently, we reported that the herbal medicine (Maoto) reduces flu-like symptoms induced by IFN-β, and increases the biochemical response rate in chronic hepatitis C with a high viral load (≧1Meq/ml) of genotype 1b being treated with IFN-β.[6,7] Moreover, we found the increment of interleukin-1 receptor antagonist (IL-1ra) caused by Maoto to be one of the key factors involved in reducing the flu-like symptoms and improving the biochemical response rate.[8] Together with these findings, we considered that Maoto might possess immunomodulatory effects. Some investigators have reported that not only the factor of the virus but also that of the host immune response induced by interferon is responsible for the elimination of the hepatitis C virus.[9-11] It is believed that $CD8^+$ cytotoxic T cells (CTL) and NK cells are critical for viral clearance.[12,13] Therefore, the present study was designed to examine the relation between the host immune response and virological response in patients with chronic hepatitis C treated with Maoto and IFN-β, referring to the changes in peripheral lymphocyte populations monitored by flow-cytometry.

Materials and Methods

Patients. Twenty-five patients with chronic hepatitis C (7 males and 18 females; mean age, 55±10years) receiving combination therapy of Maoto and IFN-β were enrolled. Fifteen patients had HCV genotype 1b, 6 genotype 2a, and 4 genotype 2b. In all patients, diagnosis of chronic hepatitis C was made based on the following criteria: positivity for serum anti-HCV antibodies by second generation assay, presence of HCV-RNA in serum by PCR, elevated serum levels of alanine-aminotransferase (ALT) at least twice the upper limit of normal for more than 6 months before entry, and histological evidence of chronic hepatitis on liver biopsy. Patients with liver cirrhosis, lung fibrosis and emphysema were excluded from the study. All patients were seronegative for HBsAg and anti-HIV. None of them had been treated with IFN previously. This study was approved by the Human Subjects Committee of the Toyama Medical and Pharmaceutical University. All subjects gave their written informed consent in accordance with the ethical guidelines set out by the 1975 Declaration of Helsinki.

Crude drugs. The composition of Maoto is listed in Table 1.

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Table 1 Constituent list of Maoto

Ephedrae Herba	8.0g
Armenicae Cortex	8.0g
Cinnamomi Cortex	5.4g
Glycyrrhizae Radix	2.7g

Maoto was decocted with 700 ml water in an earthen teapot on an electric heater at 600 W ('HP-634' Toshiba, Tokyo, Japan) for 40 minutes until the volume of extractant was reduced to about 400 ml.

Management protocol. Each patient received a dose of 6 million units (MU) of IFN-β (human fibroblast IFN, Feron, Toray Industries Inc., Tokyo, Japan) intravenously at 9 am once a day continuously for 8 weeks. Maoto was given orally 4 times a day during the IFN-β administration.

Virological tests. Serum anti-HCV antibody was measured by second-generation enzyme immunoassay (Dainabot, Tokyo, Japan). Serum HCV-RNA levels were tested by commercial PCR (Amplicor HCV v2.0, Roche Molecular Systems, Banchburg, NJ). The HCV genotype was determined by PCR amplification of the core region according to the method of Okamoto *et al.*[14]

Histology. The histological findings of the liver biopsy specimens were evaluated by two physicians in a blinded manner, using two scoring methods. For assessment, histological staging was divided into four categories (1: mild fibrosis, 2: moderate fibrosis, 3: severe fibrosis, 4: cirrhosis). For the assessment of histological grade, the total of the histological activity score was used.[15]

Definition of response. The long-term response to therapy was assessed virologically by repeated PCR assays of serum for HCV RNA and was categorized as follows: sustained responders (SR) in whom serum HCV RNA was not found at the end of therapy and for at least 6 months thereafter; transient responders (TR) in whom serum HCV RNA was not found at the end of therapy but was found within 6 months of follow-up; nonresponders (NR) in whom serum HCV RNA was found at the end of therapy.

Lymphocyte subpopulations. Sequential analyses of lymphocyte subpopulations were performed in patients as described elsewhere.[16] Briefly, mononuclear cells in peripheral blood were obtained by Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient centrifugation and incubated with saturating concentrations of directly labeled monoclonal antibodies to various cell surface determinants. Phenotypic markers on the surface of peripheral blood lymphocytes were characterized by automated two-dimensional flow-cytometric method in our laboratory (EPICS XL, Beckman Coulter, France). The following lymphocyte subpopulations were determined (antibodies from Immunotech, France): anti-CD3 (FITC) with anti-CD19, anti-CD4 and anti-CD8 (PE), anti-CD16 (FITC) with anti-CD56 (PE), and anti-HLADR (FITC) with anti-CD8 (PE). Lymphocyte subsets were identified by gating analysis and fluorescence profiles obtained for 100,000 cells of each sample. The total numbers of cells expressing each marker were calculated as the percentage of positive cells multiplied by the absolute cell count before treatment, at 4 weeks, and at the end of treatment (8 weeks).

Statistical analysis. Statistical differences in the data were determined by one-way ANOVA followed by Fisher's PSLD, with a P-value of <0.05 being considered significant. Differences in proportions were tested by Kruskal-Wallis test followed by Bonferroni correction, with a P-value of <0.0167 being considered significant. Statistical analysis of comparison in the change of lymphocyte subpopulations was performed by repeated measures ANOVA followed by Fisher's PSLD, with a P-value of <0.05 being considered significant.

Results

Baseline characteristics of patients classified according to outcome of therapy (Table 2). Of the twenty-five

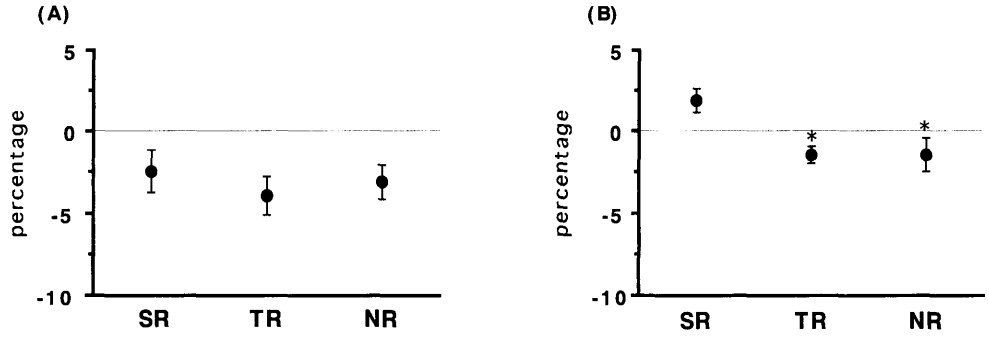
Table 2 Characteristization of 25 patients with chronic hepatitis C according to outcome of therapy

	Sustained responder (n=6)	Transient responder (n=10)	Nonresponder (n=9)
Sex			
male	1	5	1
female	5	5	8
Age (years)	49.0±13.2	56.5±9.6	59.3±8.2
ALT (IU/l)	139.1±77.9	98.8±61.4	131.1±56.8
HCV RNA (KIU/ml)	154.0±151.9*	599.8±443.7	778.9±396.8
HCV genotype#			
1b	1	5	9
2a	4	2	0
2b	1	3	0
Grade of inflammation			
mild	3	4	0
moderate	3	6	8
severe	0	0	1
Stage of fibrosis			
mild	4	3	2
moderate	0	7	3
severe	2	0	4

* one-way ANOVA followed by Fisher's PSLD
\# Kruskal-Wallis test followed by Bonferroni correction
Serum HCV RNA levels were significantly lower in SR than in TR and NR (P=0.03, 0.005)
The number of HCV genotype 1b in NR is significantly larger than SR (P=0.0014)

patients treated with combination therapy, 6 were SR, 10 were TR, and 9 were NR. The characteristics of the patients according to outcome of therapy are shown in Table 2. When we compared the SR group with the other two groups, serum HCV RNA levels were significantly lower than in TR and NR, and the number of HCV genotype 1b in NR is significantly larger than SR.

Change in percentage of peripheral lymphocyte subpopulations. The percentages of $CD3^+$, $CD19^+$, $CD3^+CD4^+$, and $CD3^+CD8^+$ lymphocyte subpopulations were not significantly different among the three groups (data not shown). The percentage of the $CD16^+CD56^+$lymphocyte population was decreased in all groups between pretreatment and 4 weeks (Fig.2A), but the percentage was significantly increased in SR compared with TR and NR between 4 and 8 weeks (Fig.2B). The percentage of the $CD16^+CD56^+$lymphocyte population was significantly decreased in TR and NR compared with SR between pretreatment and 8 weeks (Fig.2C). The percentage of the $HLADR^+CD8^+$lymphocyte population was not significantly different between pretreatment and 4 weeks (Fig.3A), 4 and 8 weeks (Fig.3B), but the percentage was significantly increased in SR and TR compared with NR between pretreatment and 8 weeks (Fig.3C).



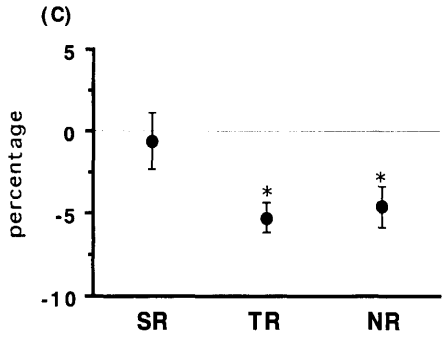
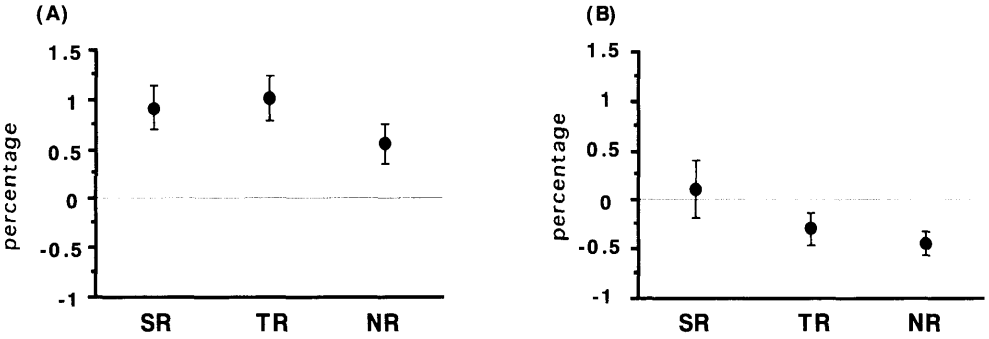


Fig. 1 Change in percentage of $CD16^+CD56^+$lymphocyte populations between pretreatment and 4 weeks (A), 4 and 8 weeks (B), pretreatment and 8 weeks (C).
The percentage of $CD16^+CD56^+$lymphocyte population decreased in all groups between pretreatment and 4 weeks (A), but the population was significantly increased in SR compared with TR and NR between 4 and 8 weeks (B). The percentage of $CD16^+CD56^+$lymphocyte population was significantly decreased in TR and NR compared with SR (C). Data are mean ± S.E.M. $^*P<0.05$ compared with SR
SR: sustained responder, TR: transient responder, NR: nonresponder



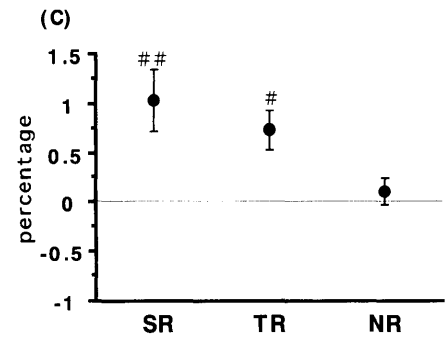


Fig. 2 Change in percentage of $HLADR^+CD8^+$lymphocyte populations between pretreatment and 4 weeks (A), 4 and 8 weeks (B), pretreatment and 8 weeks (C).
The percentage of the $HLADR^+CD8^+$lymphocyte population was significantly increased in SR and TR compared with NR between pretreatment and 8 weeks (C). Data are mean ± S.E.M. $^{\#}P<0.05$, $^{\#\#}P<0.01$ compared with NR
SR: sustained responder, TR: transient responder, NR: nonresponder

Discussion

Recently, we found that the concentrations of plasma IL-6 and IL-1receptor antagonist induced by IFN-β were significantly increased by Maoto,[8] leading us to consider that Maoto has immunomodulatory effects. Furthermore, it has been reported that NK cells and $CD8^+$cytotoxic T cells are critical for viral clearance.[12,13] Therefore, we measured the changes of lymphocyte subpopulations in this study. Our data demonstrated that the percentage of peripheral $CD16^+$ $CD56^+$ lymphocyte populations was decreased between pretreatment and 4 weeks in all groups, but then significantly increased in SR compared with TR and NR from week 4 to week 8. Furthermore, the population was significantly decreased in TR and NR compared with SR between pretreatment and 8 weeks. Several investigators have reported that NK cells are recruited to the liver after the administration of IFN and other biologic response modifiers,[17,18] and our data would support these observations. We considered that CD 16^+CD56^+ lymphocytes were recruited from peripheral blood to the liver, from where they returned to peripheral blood again in SR, whereas they remained in the liver in TR and NR in this study.

Another important finding in the present study was that the percentage of the $HLADR^+CD8^+$lymphocyte population was significantly increased in SR and TR than NR at 8 weeks. Although there has been no report regarding the increased population of $HLADR^+CD8^+$ lymphocytes after IFN-β administration, we considered that an increment in activated $CD8^+$lymphocytes might be needed to eliminate HCV. Further studies including the analysis of HCV antigen-specific activated $CD8^+$lymphocytes will be required to prove this hypothesis.

A lower level of viremia, and infection by HCV genotypes other than 1b are reported to be predictors of sustained response.[19-21] Our results are consistent with those findings. Furthermore, changes in serum levels of HCV as monitored by real-time quantitative PCR, changes of intracellular analysis of peripheral $CD4^+$ T cells, and serum IL-18 levels are predictive factors for response during the treatment,[9,22] but there has been no report about deciding the end-point of treatment. In this study, our results might cast a light on how to set the optimum end-point, by depending on the monitoring of changes in peripheral $CD16^+CD56^+$and $HLADR^+CD8^+$lymphocyte populations. Although 6 of 25 patients (24%) were SR in this study, and our clinical study showed results similar to those of other reports,[23-25] we considered that sustained response was further increased when the treatment period was prolonged, based on the change in lymphocyte subpopulations. In practice, we have already experienced that a patient with a high viral load of genotype 1b became SR when the treatment period was extended from 8 weeks to 12 weeks, referring to the change in lymphocyte populations.

Finally, because there have been no reports about the change of lymphocyte populations after IFN-β administration, and we could not conduct a controlled study, whether the recruitment of NK cells and the activation of $CD8^+$ lymphocytes could be attributed to the effect of Maoto is not clear. However, our results suggested that monitoring of changes in peripheral $CD16^+CD56^+$and $HLADR^+CD8^+$lymphocyte populations could be useful to treat chronic hepatitis C with the combination therapy of Maoto and IFN-β.

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References

1) Davis, GL., Balart, LA., Schiff, ER., Lindsay, K., Bodenheimer, HC., Perrillo, RP., Carey, W., Jacobson, IM., Payne, J., Dienstag, JL., Van Thiel, DH., Tamburro, C., Lefkowitch, J., Albrecht, J., Meschievitz, C., Ortego, TJ., and Gibas, A.: Treatment of chronic hepatitis C with recombinant interferon alpha. A multicenter randomized, controlled trial. *N. Engl. J. Med.,* **321**, 1501-1506, 1989.

2) Dibisceglie, AM., Martin, P., Kassianides, C., Lisker-Melman, M., Murray, L., Waggoner, J., Goodman, Z., Banks, SM., and Hoofnagle, JH.:Recombinant interferon alpha therapy for chronic hepatitis C. *N. Engl. J. Med.,* **321**, 1506-1510, 1989.

3) Hoofnagle, JH., Mullen, KD., Jones, DB., Rustgi, V., Di Bisceglie, A., Peters, M., Waggoner, JG., Park, Y., and Jones, EA.:Treatment of chronic non-A, non-B Hepatitis with recombinant human alpha interferon. *N. Engl. J. Med.,* **315**, 1575-1578, 1986.

4) Seeff, LB., Buskell-Bales, Z., Wright, EC., Durako, SJ., Alter, HJ., Iber, FL., Hollinger, FB., Gitnick, G., Knodell, RG., Perrillo, RP., Stevens, CE., and Hollingsworth, CG.: Long-term mortality after transfusion-associated non-A,non-B hepatitis. *N. Engl. J. Med.,* **327**, 1906-1911, 1992.

5) Tong, MJ., el-Farra, NS., Reikes, AR., and Co, RL.:Clinical outcome after transfusion-associated hepatitis C. *N. Engl. J. Med.,* **332**, 1463-1466, 1995.

6) Kainuma, M., Hayashi, J., Sakai, S., Imai, K., Mantani, N., Kohta, K., Mitsuma, T., Shimada, Y., Kashiwagi, S., and Terasawa, K.: The efficacy of herbal medicine (Kampo) in reducing the adverse effects of IFN-β in chronic hepatitis C. *Am. J. Chin. Med.,* **30**, 355-367, 2002.

7) Kainuma, M., Ogata, N., Kogure, T., Kohta, K., Mitsuma, T., and Terasawa, K.: The efficacy of a herbal medicine (Maoto) in combination with intravenous natural interferon-β for patients with chronic hepatitis C, genotype 1b and high viral load:a pilot study. *Phytomedicine.,* **9**, 365-372, 2002.

8) Kainuma, M., Sakai, S., Sekiya, N., Mantani, N., Ogata, N., Shimada, Y., and Terasawa, K.: The effect of a herbal medicine (Maoto) in patients with chronic hepatitis C after injection of IFN β. *Phytomedicine.,* **11**, 5-10, 2004.

9) De Maeyer,E., and De, Maeyer-Guignard, J.: Immunomodulating properties of interferons. *Philos. Trans. R. Soc. Lond. [Biol.].,* **299**, 77-89, 1982.

10) Enomoto, M., Nishiguchi, S., Tanaka, M., Fukuda, K., Ueda, T., Tamori, A., Habu, D., Takeda, T., Shiomi, S., Yano, Y., and Otani, S.: Dynamics of hepatitis C virus monitored by real-time quantitative polymerase chain reaction during first 2 weeks of IFN-β treatment are predictive of long-term therapeutic response. *J. Interferon. Cytokine. Res.,* **22**, 389-395, 2002.

11) Lohr, HF., Gerken, G., Roth, M., Weyer, S., Schlaak, JF., and Meyer zum Buschenfelde, KH.: The cellular immune response induced in the follow-up of interferon-α treated patients with chronic hepatitis C may determine the therapy outcome. *J. Hepatol.,* **29**, 524-532, 1998.

12) Guidotti, LG., and Chisari, FV.: Noncytolytic control of viral infections by the innate and adaptive immune response. *Annu. Rev. Immunol.,* **19**, 65-91, 2001.

13) Nelson, DR., Marousis, CG., Ohno, T., and Lau, JY.: Intrahepatic hepatitis C virus-specific cytotoxic T lymphocyte activity and response to interferon alpha therapy in chronic hepatitis C. *Hepatology.*, **28**, 225-230, 1998.
14) Okamoto, H., Sugiyama,Y., Okada, S., Kurai, K., Akahane, Y., Sugai, Y., Tanaka, T., Sato, K., Tsuda, F., Miyakawa,Y., and Mayumi, M.: Typing hepatitis C virus by polymerase with type-specific primers: application to clinical surveys and tracing infectious sources. *J. Gen. Virol.*, **73**, 673-679, 1992.
15) Knodell, RG., Ishak, KG., and Black, WC.: Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology.*, **1**, 431-435, 1981.
16) Wachoitz, MC., Patel, SS., and Lipsky, PE. : Leukocyte function-associated antigen 1 is an activation molecule for human T cells. *J. Exp. Med.*, **170**, 431-448, 1989.
17) Yoo, YK., Gavaler, JB., Chen, K, Whiteside, TL., and Van Thiel, DH.: The effect of recombinant interferon-alpha on lymphocyte subpopulation and HLA-DR expression on liver tissue of HBV-positive individuals. *Clin. Exp. Immunol.*, **82**, 338-343, 1990.
18) Wiltrout, RH., Pilaro, AM., Gruys, ME., Talmadge, JE., Longo, DL., Ortaldo, JR., and Reynolds, CW.: Augmentation of mouse liver-associated natural killer activity by biologic response modifiers occurs largely via rapid recruitment of large granular lymphocytes from the bone marrow. *J. Immunol.*, **143**, 372-378, 1989.
19) Garson, JA., Brillanti, S., Whitby, K., Foli, M., Deaville, R., Masci, C., Miglioli, M., and Barbara, L.: Analysis of clinical and virological factors associated with response to alpha interferon therapy in chronic hepatitis C. *J. Med.Virol.*, **45**, 348-353, 1995.
20) Martinot-Peignoux, M., Marcellin, P., Pouteau, M., Pouteau, M., Castelnau, C., Boyer, N., Poliquin, M., Degott, C., Descombes, I., Le Breton, V., Milotova, V., Benhamou, JP., and Erlinger, S.: Pretreatment serum hepatitis C virus RNA levels and hepatitis C virus genotype are the main and independent prognostic factors of sustained response to interferon alpha therapy in chronic hepatitis C. *Hepatology.*, **22**, 1050-1056, 1995.
21) Soma, M., Sasaki, M., Sakamaki, M., Tanaka, S., Shimizu, H., and Kunihiro, Y.: Post marketing surveillance of natural IFN-β preparation for chronic hepatitis C. *Jpn. J. Pharmacoepidemiol.*, **6**, 5-28, 2001.
22) Ishii, K., Takamura, N., Shinohara, E., Shin, HY., Ikehara, T., Hata, S., Kawafune, T., Sumino, Y., and Ohmoto, Y.: Intracellular cytokine analysis of CD4-positive T cells predictive of sustained response to interferon therapy for patients with chronic hepatitis C. *Dig. Di. Sci.*, **47**, 778-783, 2002.
23) Fukutomi, T., Fukutomi, M., Iwao, M., Watanabe, H., Tanabe, Y., Hiroshige, K., Kinukawa, N., Nakamuta, M., and Nawata, H.: Predictors of the efficacy of intravenous natural interferon-beta treatment in chronic hepatitis C. *Med. Sci. Monit.*, **6**, 692-698, 2000.
24) Kobayashi, Y., Watanabe, S., Konishi, M., Yokoi, M., Kakehashi, R., Kaito, M., Kondo, M., Hayashi, Y., Jomori, T., and Suzuki, S.: Quantitation and typing of serum hepatitis C virus RNA in patients with chronic hepatitis C treated with IFNβ. *Hepatology.*, **18**, 1319-1325, 1993.
25) Kurosaki, M., Enomoto, N., Murakami, T., Sakuma, I., Asahina, Y., Yamamoto, C., Ikeda, T., Tozuka, S., Izumi, N., Marumo, F., and Sato, C.: Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-β therapy. *Hepatology.*, **25**, 750-753, 1997.

Japanese abstract

我々は最近，麻黄湯にはIFNβと併用療法時において免疫修飾作用があるのではないかと報告してきた。そこで今回我々は麻黄湯とIFNβ併用療法時における宿主の免疫反応とウイルス学的効果の関連性について検討する為に，フローサイトメトリー解析を用いて，併用療法前後での末梢血リンパ球サブセットの変化について検討した。25名のC型慢性肝炎患者を対象として8週間の麻黄湯とIFNβの併用療法を行った。また麻黄湯はIFN投与中1日4回服用とした。ウイルス学的検討では，6名が著効群（SR），10名が一過性有効群（TR），9名が無効群（NR）だった。$CD16^+CD56^+$陽性リンパ球数の比率は，治療前と比較して，4週後には3群ともその比率が低下したが，4週から8週後では，SR群でTR群とNR群と比較して有意な上昇が認められた。また$HLADR^+CD8^+$リンパ球数の比率は治療前後においてSR群とTR群でNR群と比較して有意な上昇が認められた。我々の結果より，C型慢性肝炎に対する麻黄湯とIFNβ併用療法時において，$CD16^+CD56^+$，$HLADR^+CD8^+$リンパ球数の変化をモニタリングすることが有用である可能性が示唆された。

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