Comparative Study of C-Reactive Protein in Chronic Hepatitis B and Chronic Hepatitis C

MASAYOSHI SHIMA, KAZUHIKO NAKAO, YUJI KATO,
KEISUKE NAKATA*, NOBUKO ISHII* and SHIGENOBU
NAGATAKI

*The First Department of Internal Medicine, Nagasaki
University School of Medicine, Nagasaki 852, and *Health
Research Center, Nagasaki University, Nagasaki 852*

SHIMA, M., NAKAO, K., KATO, Y., NAKATA, K., ISHII, N. and NAGATAKI, S. *Comparative Study of C-Reactive Protein in Chronic Hepatitis B and Chronic Hepatitis C*. Tohoku J. Exp. Med., 1996, **178** (3), 287-297 — C-reactive protein (CRP) is a liver-specific acute-phase protein, and its expression in hepatocyte is regulated by cytokines such as interleukin-1, interleukin-6 and tumor necrosis factor- α . Although several alterations in cytokines have been found in patients with chronic viral hepatitis, it remains obscure how CRP expression is associated with progression of the disease in chronic viral infection. In the present study, CRP expression was evaluated in 45 patients with chronic hepatitis B and in 38 patients with chronic hepatitis C. By the immunohistochemical analysis, the intensity of CRP expression in hepatocyte was closely associated with the histology activity index (HAI) score in chronic hepatitis B. In contrast, the association was not found in chronic hepatitis C. When serial changes in serum levels of CRP were compared in long-term follow-up patients including 5 patients with chronic hepatitis B and 4 patients with chronic hepatitis C, serum levels of CRP fluctuated simultaneously with serum levels of alanine aminotransferase in chronic hepatitis B, whereas the correlation was not recognized in chronic hepatitis C. These results suggest that CRP expression correlates with progression of the disease in chronic hepatitis B, but not in chronic hepatitis C. It is also possible that cytokine-mediated response is more pronounced in chronic hepatitis B than in chronic hepatitis C. ——— chronic hepatitis B; chronic hepatitis C; C-reactive protein

The proteins synthesized by hepatocyte can be divided into three groups: (a) constitutively expressed proteins (e.g., albumin), (b) proteins subjected to developmental regulation (e.g., α -fetoprotein), and (c) acute-phase proteins including C-reactive protein (CRP). Among these proteins, synthesis of acute-phase protein is under a strong regulatory control of cytokines. In fact, CRP expression in human and rat hepatocytes is mainly regulated by interleukin-6

Received August 31, 1995; revision accepted for publication December 20, 1995.

Address for reprints: Shigenobu Nagataki M.D., the First Department of Internal Medicine, Nagasaki University School of Medicine, 1-7-1 Sakamoto, Nagasaki 852, Japan.

(IL-6), tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) (Baumann et al. 1984; Darlington et al. 1986; Goldman and Liu 1987; Castell et al. 1989).

The pathogenesis of viral hepatitis seems to be mediated by the immune response to the virus-infected hepatocytes (Dienstag and Bhan 1980; Mondelli et al. 1982; Anastassakos et al. 1988; Muto et al. 1988), where cytokines play an important role in eradicating the infected virus. Although CRP expression in hepatocyte is directly regulated by cytokines, little is known about the relationship between CRP expression and progression of the disease in chronic viral infection.

In the present study, CRP expression in hepatocyte and serial changes in serum CRP levels were analyzed in both patients with chronic hepatitis B and patients with chronic hepatitis C, to clarify how CRP expression was associated with progression of the disease.

MATERIALS AND METHODS

Patients

Paraffin-embedded liver biopsy specimens were obtained from 45 patients with chronic hepatitis B and 38 patients with chronic hepatitis C. The male to female ratio was not different between the two groups, but the mean age was relatively younger in patients with chronic hepatitis B than in patients with chronic hepatitis C ($p < 0.01$, Table 1). Sixteen of 38 patients with chronic hepatitis C had histories of receiving blood transfusion. The diagnosis of chronic hepatitis B was based on seropositivity of hepatitis B surface antigen (HBsAg) for more than 6 months with characteristic histological findings in liver tissue obtained at the time of peritoneoscopy. The diagnosis of chronic hepatitis C was made when patients with a liver biopsy-proven chronic hepatitis tested seropositive for antibody to hepatitis C virus (anti-HCV) with enzyme-immunoassay (Ortho Diagnostic Systems, Newark, NJ, USA) but tested seronegative for HBsAg. No patient was seropositive for both HBsAg and anti-HCV or had the history of drug or alcohol abuse.

Among 83 patients, serum levels of alanine aminotransferase (ALT) clearly fluctuated in 5 patients with chronic hepatitis B and 4 patients with chronic hepatitis C for at least more than 2 years. To evaluate the correlation between

TABLE 1. *Sex and age distributions in patients with chronic hepatitis B and C*

	Sex (Male : Female)	Age (years, mean $\pm$ s.d.)
Chronic hepatitis B	37 : 8	31.9 $\pm$ 8.3
Chronic hepatitis C	30 : 8	51.8 $\pm$ 10.4

The mean age of patients with chronic hepatitis B was approximately 20 years younger than that of patients with chronic hepatitis C.

The statistical analysis was performed using Student's *t*-test ($p < 0.01$).

serum levels of CRP and ALT, the serial changes in serum levels of CRP were studied in these 9 patients. During the follow-up periods, the number of serum samples collected in each patient ranged from 25 to 60. Consequently, 373 serum samples were subjected to analysis of the correlation between serum CRP and ALT. Serum ALT was assayed using a sequential multiple autoanalyzer immediately after venipuncture. Serum samples were also stored at -40°C for later measurement of serum CRP.

Histology activity index score

The histological activity in each biopsy specimen was evaluated according to the method of Knodell et al. (1981). In brief, the categories include the following: (i) periportal/bridging necrosis; (ii) intralobular degeneration and focal necrosis; (iii) portal inflammation; and (iv) fibrosis. A numerical score ranges from 0 to 10 in category (i), and ranges from 0 to 4 in categories (ii), (iii) and (iv).

Expression of CRP in hepatocyte

The hepatic expression of CRP was evaluated in deparaffinized liver biopsy sections using the biotin-streptoavidin complex method (Bio Genex Laboratories, Dublin, CA, USA) with a rabbit antibody to human CRP (Sinotest, Tokyo) diluted with 1% bovine serum albumin in 0.1 M phosphate-buffered saline (working dilution; 1:500). The intensity of CRP expression was scored from 0 to 2 independently by three observers without the prior knowledge of the clinical data, and graded by the mean score as follows; $(-)/0-0.5$ (negative), $(1+)/0.5-1.5$ (weak), and $(2+)/\text{more than } 1.5$ (intense).

Measurement of serum CRP

The quantitative analysis of CRP was carried out using a solid phase enzyme immunoassay as described previously (Salonen 1982). Ten μl of serum and 90 μl of dilution buffer containing 1% bovine serum albumin were added to each microplate well coated with goat antibody to human CRP (kindly provided by Dr. Hidematsu Hirai, Tumor Laboratory, Tokyo). After 2 hr-incubation at room temperature, the wells were washed twice with 200 μl of 0.05% Tween-20 in 0.01 M phosphate-buffered saline, pH 7.4. One hundred μl of solution containing peroxidase-labeled goat antibody to human CRP was added to each well and reacted for 2 hr at room temperature. Then, the washing procedure as described above was repeated, and 200 μl of substrate containing 0.1 M Tris-HCl, pH 8.0, 0.02 M phenol, 0.0048% H_2O_2 and 0.001 M 4-amino-antipyrine was added to each well. Fifteen min later, the enzyme-substrate reaction was terminated by addition of 50 μl of 0.54% formalin. Absorbance was measured at 500 nm using a vertical-pathway spectrophotometer. All assays were carried out in duplicate. The normal serum CRP concentrations were considered less than 0.5 mg/ml based on the results from 1092 healthy volunteers.

Statistical analyses

Statistical analyses were carried out using the Student's *t*-test, Mann-Whitney U test or the chi-square test. Correlations were evaluated by linear regression and calculation of the correlation coefficient. *p* Values less than 0.05 were considered statistically significant.

RESULTS

Comparison between CRP expression in hepatocyte and total Histology Activity Index (HAI) score

CRP was stained in the cytoplasm of hepatocyte homogeneously throughout the lobule of the liver (Fig. 1). In some specimens, CRP was detected in both the cytoplasm and the nucleus. The localization of CRP in the nucleus does not seem to be artificial because of strong affinity of CRP for chromatin and nucleosome core particles as described previously (Robey et al. 1984; Shephard et al. 1986).

The intensity of CRP expression in chronic hepatitis B did not differ from that in chronic hepatitis C (Table 2). The distributions of the total HAI score

Fig. 1. Immunohistochemical localization of CRP in deparaffinized liver biopsy specimens.

A) Immunohistochemical staining of CRP in a liver biopsy specimen with weak staining in the cytoplasm ($\times 200$).

B) Immunohistochemical staining of CRP in a liver biopsy specimen with intense staining in the cytoplasm ($\times 200$).

TABLE 2. CRP expression in chronic hepatitis B and C

	Intensity of CRP staining		
	—	+1	+2
Chronic hepatitis B	5	15	25
Chronic hepatitis C	2	27	9

Intensity of CRP was classified into 3 groups according to the method described in Materials and Methods.

The statistical analysis was performed using the chi-square test.

Fig. 2. The relationship between the intensity of CRP expression and the total HAI score. The statistical analysis was performed using Mann-Whitney U test.

were similar in patients with chronic hepatitis B and those with chronic hepatitis C (Fig. 2). The mean total HAI score was significantly higher in 2+ CRP group than in 1+ CRP group in patients with chronic hepatitis B. On the other hand, the total HAI score did not correlate with the intensity of CRP expression in patients with chronic hepatitis C. When the histological activities were stratified according to the two major categories, necroinflammatory reaction such as periportal/bridging necrosis, intralobular degeneration and focal necrosis and portal inflammation, and the extent of fibrosis, both scores clearly correlated with the intensity of CRP expression in patients with chronic hepatitis B. In contrast, any correlation was not found in patients with chronic hepatitis C (Table 3). Since the hepatic CRP synthesis increased together with natural aging process (Kindmark 1972), the age distribution was compared in both groups. However, the age distribution was similar irrespective of the intensity of CRP expression in

TABLE 3. *Relationship between CRP expression and score of each category*

CRP expression	Patients with HBV			Patients with HCV		
	— (n=4)	1+ (n=15)	2+ (n=25)	— (n=2)	1+ (n=27)	2+ (n=9)
Serum ALT ^a	49	72	89	69	73	84
Score of each category						
(I) Periportal/bridging necrosis	1.3 (0-3)	1.7 (0-6)	3.5** (0-6)	5 (5)	3.2 (0-6)	2.7 (0-6)
(II) Intralobular degeneration and focal necrosis	0 (0)	0.7 (0.3)	2.8** (0-4)	2.5 (1-4)	1.3 (0-4)	2.3 (0-4)
(III) Portal inflammation	1 (1)	1.8 (0-4)	3.0** (1-4)	3 (3)	2.4 (0-4)	2.6 (1-6)
(IV) Fibrosis	2.3 (1-3)	1.0 (1-3)	2.4** (1-3)	3 (3)	2.5 (1-3)	2.1 (1-3)

n, number.

^aSerum ALT represents the mean value of each group.

The score is expressed as mean (range). The statistical analysis was performed using Mann-Whitney U test.

***p* < 0.01 vs. 1+CRP group.

TABLE 4. *Relationship between CRP expression and age distribution in chronic hepatitis B and C*

	Intensity of CRP staining		
	—	+1	+2
Chronic hepatitis B	30.4 ± 7.8 (n=5)	30.4 ± 9.0 (n=15)	33.2 ± 8.1 (n=25)
Chronic hepatitis C	59.0 ± 5.7 (n=2)	52.1 ± 11.0 (n=27)	49.4 ± 9.3 (n=9)

Intensity of CRP was classified into 3 groups according to the method described in Materials and Methods.

Age is expressed as mean ± s.d.

The statistical analysis was performed using Student's *t*-test.

patients with chronic hepatitis B and in patients with chronic hepatitis C (Table 4).

Serial changes in serum levels of CRP

The clinical course of a patient with chronic hepatitis B (case 1) is shown in Fig. 3. This patient was admitted to our hospital because of general malaise, and received 30 mg prednisolone every day for 3 weeks. Serum levels of both ALT and CRP decreased immediately after initiation of steroid treatment. Thereafter, this patient was followed as an outpatient. Serum levels of ALT fluctuated

Fig. 3. Serological and biochemical profile in case 1.

A 39-year-old Japanese man with chronic active hepatitis B was followed for approximately 3 years. He took 30 mg prednisolone every day for 3 weeks immediately after enrollment.

C.S., corticosteroid treatment.

Fig. 4. Serological and biochemical profile in case 5.

A 35-year-old Japanese man with chronic active hepatitis B was followed for approximately 5 years.

simultaneously with serum levels of CRP during the follow-up period. Fig. 4 shows serial changes in serum levels of ALT and CRP in another patient with chronic hepatitis B (case 5). Although simultaneous elevation of serum levels of CRP and ALT was observed during the follow-up period, the serum level of CRP

Fig. 5. Serological and biochemical profile in case 6.

A 47-year-old Japanese man with chronic active hepatitis C was followed for approximately 7.5 years.

TABLE 5. Correlation coefficient between serum levels of CRP and ALT

	Case	Age (years)	Sex	Diagnosis	Blood transfusion	Observation period (months)	Number of collected samples	Correlation coefficient (r-value)
Type B	1	39	M	CAH	—	36	38	0.855***
	2	33	M	CAH	—	39	39	0.690***
	3	33	M	CAH	—	63	58	0.703***
	4	37	M	CAH	—	45	33	0.433*
	5	35	M	CAH	—	55	46	0.103 n.s.
Type C	6	47	M	CAH	—	43	37	0.029 n.s.
	7	37	M	CAH	+	52	37	−0.153 n.s.
	8	52	M	CAH	—	26	25	0.333 n.s.
	9	48	M	CPH	—	96	60	0.117 n.s.

CAH, chronic active hepatitis; CPH, chronic persistent hepatitis.

Correlation was evaluated by linear regression and calculation of the correlation coefficient.

* $p < 0.05$; *** $p < 0.001$.

n.s., not significant.

was also elevated without the elevation of ALT at the times when the patient suffered from upper respiratory tract infections. Thus, the correlation between serum levels of CRP and ALT was not statistically significant in this case. Fig. 5 shows serial changes in serum levels of ALT and CRP in a patient with chronic hepatitis C (case 6), in whom serum levels of CRP fluctuated independently of serum levels of ALT during follow-up as well as in other patients with chronic

hepatitis C. These results are summarized in Table 5. Serum levels of CRP closely correlated with serum levels of ALT in 4 of 5 patients with chronic hepatitis B, whereas the correlation was not observed in any of 4 patients with chronic hepatitis C.

DISCUSSION

The underlying abnormalities in chronic viral hepatitis result from the enhanced T-cell-mediated immune response, which causes a necrosis of the virus-infected hepatocyte but cannot eradicate the infected virus completely (Dudley et al. 1972). Although some investigators have documented that the serum levels of IL-1 and TNF- α were elevated in patients with chronic viral hepatitis (Anastassakos et al. 1988; Muto et al. 1988), the association of the cytokine-inducible CRP expression with progression of the disease is not yet understood. Each cytokine does not have a direct cytotoxic effect on hepatocyte, but rather network of varying cytokines mediates liver inflammation through activating the cytotoxic T cell response in viral hepatitis. Since CRP expression is regulated by many cytokines, its expression in hepatocyte is applicable to evaluating the alterations in network of cytokines during chronic hepatitis viral infection. We, therefore, studied the changes in CRP expression in patients with chronic viral hepatitis.

The level of CRP mRNA might reflect its expression in hepatocyte more correctly than the immunohistochemical staining, because CRP expression is regulated at the transcriptional level. However, only a paraffin-embedded liver specimen where RNAs may be degraded by formalin treatment was obtained from each patient. Accordingly, CRP expression in hepatocyte was evaluated by the immunohistochemical method. In this study, the hepatic expression of CRP correlated with HAI score in chronic hepatitis B. Serum levels of CRP also correlated with serum levels of ALT in patients with chronic hepatitis B. In contrast, these correlations were not observed in patients with chronic hepatitis C. These results suggest that CRP expression is associated with progression of the disease in chronic hepatitis B, but not in chronic hepatitis C. Since CRP expression is mainly regulated by cytokines, our result indicates that progression of the disease is more closely associated with cytokine intervention in chronic hepatitis B than in chronic hepatitis C. In addition, our result also suggests that the cytokine network is differently modulated in chronic hepatitis B and chronic hepatitis C. The intensities of CRP expression in hepatocyte in patients with chronic hepatitis B and patients with chronic hepatitis C were similar. However, it was, in part, due to the difference in the age distribution between the two groups. In fact, the mean age of patients with chronic hepatitis B was approximately 20 years younger than that of patients with chronic hepatitis C in this study. Alternatively, it is possible that the hepatic CRP expression is relatively higher in chronic hepatitis B than in chronic hepatitis C, because CRP expression increases together with aging (Kindmark 1972).

In acute viral hepatitis, Atono et al. (1989) showed that serum levels of CRP markedly increased during acute phase of illness and rapidly decreased during convalescent phase in acute hepatitis A or acute hepatitis B, whereas the serum level of CRP was relatively lower in patients with acute non-A, non-B hepatitis compared with patients with acute hepatitis A or acute hepatitis B. These observations coincide with our result that the cytokine-mediated immune response to the virus-infected hepatocyte or a mechanism by which the infected virus is eradicated may be different between hepatitis B virus and hepatitis C virus infection.

In summary, our result indicates that CRP expression correlates with progression of the disease in chronic hepatitis B, whereas the correlation is not observed in chronic hepatitis C. Our result also indicates that cytokine intervention more directly leads to progression of the disease in chronic hepatitis B than in chronic hepatitis C.

Acknowledgment

We would like to express sincere thanks to Miss Masako Matsuo for her excellent secretarial assistance.

References

- 1) Anastassakos, C.H., Alexander, G.J.M., Wolstencroft, R.A., Avery, J.A., Portmann, B.C., Panayi, G.S., Dumonde, D.C., Eddleston, A.L.W.F. & Williams, R. (1988) Interleukin-1 and interleukin-2 activity in chronic hepatitis B virus infection. *Gastroenterology*, **94**, 999-1005.
- 2) Atono, Y., Sata, M. & Tanikawa, K. (1989) Kinetics of C-reactive protein in acute viral hepatitis. *Gastroenterol. Jpn.*, **24**, 655-662.
- 3) Baumann, H., Jahreis, G.P., Sauder, D.N. & Koj, A. (1984) Human keratinocytes and monocytes release factors which regulate the synthesis of major acute phase plasma proteins in hepatic cells from man, rat, and mouse. *J. Biol. Chem.*, **259**, 7331-7342.
- 4) Castell, J.V., Gomez-Lechon, M.J., David, M., Andus, T., Geiger, T., Trullenque, R., Fabra, R. & Heinrich, P.C. (1989) Interleukin-6 is the major regulator of acute phase protein synthesis in adult human hepatocytes. *FEBS Lett.*, **242**, 237-239.
- 5) Darlington, G.J., Wilson, D.R. & Lachman, L.B. (1986) Monocyte-conditioned medium, interleukin-1, and tumor necrosis factor stimulate the acute phase response in human hepatoma cells in vitro. *J. Cell. Biol.*, **103**, 787-793.
- 6) Dienstag, J.L. & Bhan, A.K. (1980) Enhanced in vitro cell-mediated cytotoxicity in chronic hepatitis B virus infection: Absence of specificity for virus-expressed antigen on target cell membranes. *J. Immunol.*, **125**, 2269-2276.
- 7) Dudley, F.J., Tox, R.A. & Sherlock, S. (1972) Cellular immunity and hepatitis associated (Australia) antigen liver disease. *Lancet*, **1**, 723-726.
- 8) Goldman, N.D. & Liu, T.Y. (1987) Biosynthesis of human C-reactive protein in cultured hepatoma cells is induced by a monocyte factor(s) other than interleukin-1. *J. Biol. Chem.*, **262**, 2363-2368.
- 9) Kindmark, C.O. (1972) The concentration of C-reactive protein in sera from healthy individuals. *Scand. J. Clin. Lab. Invest.*, **29**, 407-411.
- 10) Knodell, R.G., Ishak, K.G., Black, W.C., Chen, T.S., Craig, R., Kaplowitz, N., Kiernan,

- T.W. & Wollman, J. (1981) Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology*, **1**, 431-435.
- 11) Mondelli, M., Vergani, G.M., Alberti, A., Vergani, D., Portmann, B., Eddleston, A.L.W.F. & Williams, R. (1982) Specificity of T lymphocyte cytotoxicity to autologous hepatocytes in chronic hepatitis B virus infection: Evidence that T cells are directed against HBV core antigen expressed on hepatocytes. *J. Immunol.*, **129**, 2773-2278.
 - 12) Muto, Y., Nouri-Aria, K.T., Meager, A., Alexander, G.J.M., Eddleston, A.L.W.F. & Williams, R. (1988) Enhanced tumor necrosis factor and interleukin-1 in fulminant hepatic failure. *Lancet*, **2**, 72-74.
 - 13) Robey, F.A., Jones, K.D., Tanaka, T. & Liu, T.Y. (1984) Binding of C-reactive protein to chromatin and nucleosome core particles. *J. Biol. Chem.*, **259**, 7311-7316.
 - 14) Salonen, E.M. (1982) A rapid and sensitive solid-phase enzyme immunoassay for C-reactive protein. *J. Immunol. Methods*, **48**, 45-50.
 - 15) Shephard, E.G., Van Helden, P.D., Strauss, M. Bohm, L. & De Beer, F.C. (1986) Functional effects of CRP binding to nuclei. *Immunology*, **58**, 489-494.
-