

Dose-response Trial of Lactoferrin in Patients with Chronic Hepatitis C

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Hepatitis C virus (HCV) is one of the most common causes of chronic hepatitis. Interferon is presently the only effective treatment for chronic hepatitis C (CH-C), though its effectiveness is limited. Lactoferrin (LF), which is an 80-kDa, iron-binding glycoprotein, has several biological activities including anti-viral activity, and it was recently reported to inhibit HCV infection in cultured human hepatocytes. The present trial was designed to assess the relationship between the dose of bovine LF (bLF) and the effect of bLF on serum alanine aminotransaminase (ALT) and HCV RNA levels in patients with CH-C. Forty-five patients entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day) received orally an 8-week course of bLF. There was no significant relation between the dose of bLF and the effect of bLF on serum ALT or HCV RNA levels. Biochemical (a 50% or greater decrease in the serum ALT level) and virological (a 50% or greater decrease in HCV RNA level) responses were observed in two and four patients, respectively, but all responders relapsed during the follow-up period after bLF treatment. The bLF treatment was generally well tolerated, and no patient had any serious adverse event. In conclusion, the excellent tolerance and potential anti-HCV activity of bLF shown in this trial suggest that further trials using a large number of patients are mandatory. We are currently conducting a double-blind randomized controlled trial comparing bLF with placebo to clarify the anti-HCV activity of bLF in patients with CH-C.

Key words: Lactoferrin — Chronic hepatitis C — Hepatitis C virus — Alanine aminotransaminase

Hepatitis C virus (HCV) is one of the most common causes of chronic hepatitis, and is also a major risk factor for hepatocellular carcinoma (HCC).¹⁾ Chronic hepatitis C (CH-C) is often asymptomatic and mild, but may slowly progress to liver cirrhosis and eventually to HCC.^{2,3)} Therefore, treatment of CH-C and prevention of its complications assume utmost importance. At present, interferon (IFN) is the only effective treatment for CH-C, though its effectiveness is limited; a sustained biochemical (normalization of the serum alanine aminotransaminase (ALT) level) and virological (loss of detectable serum HCV RNA) response with histological improvement occurs in only 15–30% of CH-C patients.^{4,5)} Moreover, adverse effects of IFN are sometimes severe enough to warrant cessation of treatment. To overcome these drawbacks of IFN, new formulations of IFN or combinations of IFN with ribavirin have been tested and have steadily improved sustained responses to treatment.^{6–10)} Nevertheless, further developments in the treatment of CH-C are still needed to improve the prognosis of CH-C. The new agents against HCV may offer options to patients unable to take IFN or unable to achieve a response to IFN.

Lactoferrin (LF) is an 80-kDa, iron-binding glycoprotein present mainly in external secretions, such as breast milk, and in the secondary granules of neutrophils.^{11,12)} Several biological activities of LF have been addressed, including regulation of iron homeostasis, primary nonspecific defense against pathogenic microorganisms, modulation of immunoreaction, and bactericidal and anti-viral effects. To date, it has been reported that LF has an anti-viral effect against human herpes simplex virus-1, human immunodeficiency virus-1, and human cytomegalovirus.^{13–16)} Moreover, LF was recently reported to markedly inhibit HCV infection in cultured human hepatocytes regardless of HCV strain.^{17–19)} In addition, a recent clinical pilot study reported that treatment with bovine LF (bLF) is associated with a marked decrease in serum HCV RNA and/or ALT levels in some patients with CH-C.²⁰⁾

Thus, bLF is one potential candidate for an anti-HCV reagent that may be effective for the treatment of patients with CH-C. bLF has the clinical advantage of a low risk of severe adverse effects, because it is a natural milk protein. The present dose-response trial was designed to assess the relationship between the dose of bLF and the effect of bLF on serum ALT and HCV RNA levels in patients with CH-C.

PATIENTS AND METHODS

Patients Patients eligible for study entry had pathologically confirmed CH-C. Each patient was required to meet the following eligibility criteria: 20–74 years of age; liver biopsy performed within 6 months before entry; a serum ALT level \geq twice the upper normal limit for at least 6 months; positivity for anti-HCV antibody by enzyme-linked immunosorbent assay test; an HCV RNA level of 1–1000 Kcopies/ml; no evidence of HCC on the basis of ultrasonography and/or computed tomography (CT) performed within 3 months before entry; adequate renal function [normal serum creatinine and blood urea nitrogen levels], liver function [total bilirubin level \leq 2 mg/dl, serum albumin level \geq 3 g/dl, and serum transaminases (ALT) levels \leq 200 IU/liter], and bone marrow reserve [white blood cell count \geq 2000/mm³, platelet count \geq 50 000/mm³, and hemoglobin level \geq 9 g/dl]; written informed consent.

The exclusion criteria were as follows: positivity for hepatitis B surface antigen; IFN therapy within 6 months before entry; immunomodulatory or corticosteroid therapy within 3 months before entry; intravenous glycyrrhizin treatment within 1 month before entry; pleural or pericardial effusion which is difficult to control; gastrointestinal bleeding; pregnant or lactating females; females of child-bearing age unless using effective contraception; other serious medical conditions (e.g. active infection, severe cardiac or pulmonary disease, and psychiatric disorders).

Methods bLF tablets (450 mg/tablet), provided by Morinaga Milk Industry Co. (Tokyo), were administered orally twice or three times a day. Patients received an 8-week course of bLF on an outpatient basis and then were followed for the next 8 weeks. During the treatment, patients maintained a daily journal to record bLF intake and any adverse events experienced. Cohorts of 15 patients were entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day). The starting dose of bLF (1.8 g/day) was determined according to the results of the pilot study.²⁰ The first 15 were entered at the bLF dose of 1.8 g/day, and dose escalation to the next cohort was decided after evaluation by a safety review board of all the safety data collected on the first five patients who had completed the study period of 16 weeks. The safety review board consisted of two hepatologists (S. O. and K. T.) and one biostatistician (T. S.).

The protocol permitted dose modification (a 50% reduction in the assigned dose) for patients who had clinically significant adverse events or abnormalities in laboratory values. Patients were withdrawn from the study when they experienced adverse effects such as grade 4 hematological toxicity, total bilirubin level \geq 3 mg/dl, serum transaminases (ALT) levels \geq 300 IU/liter, serum creatinine \geq 1.5 times the upper normal limit, or other grade 3 non-hema-

tological toxicities. Patients were also removed from the study if it was difficult to continue bLF treatment because of the development of serious complications or if an investigator was concerned about their safety. Other reasons for withdrawal included a request to withdraw.

Pretreatment evaluation included a complete history and physical examination. In addition, a complete blood count, biochemistry tests, urinalysis, and serum tests were performed. All patients were assessed for 16 weeks in an outpatient setting for safety, tolerance, and efficacy of the 3 dose levels. Adverse events that were observed by the investigators or reported by the patients during the study period were recorded and graded for severity according to the Japan Society for Cancer Therapy criteria, which are fundamentally similar to the World Health Organization criteria and NCI Common Toxicity criteria.^{21,22} The analysis of safety included all the patients who received at least one dose of bLF.

Efficacy was assessed by measurements of serum HCV RNA and ALT levels. These levels were measured on day 1; during treatment at weeks 2, 4, and 8; and after treatment at weeks 4 and 8. Serum HCV RNA levels were measured by an Amplicor HCV Monitor (Nihon Roche, Tokyo) with a sensitivity of 1 Kcopy/ml. A biochemical response was defined as a 50% or greater decrease in the serum ALT level compared with the baseline when the post-treatment value was \leq twice the upper normal limit. A virological response was a 50% or greater decrease in the HCV RNA level compared with the baseline. Responses were measured at the end of treatment. The duration of both a biochemical response and a virological response was calculated from the first day of treatment until relapse to the pretreatment baseline.

HCV serotypes were determined by a serotyping assay according to the method of Tsukiyama-Kohara *et al.* and Tanaka *et al.*^{23,24} In this analysis, HCV serotype 1 corresponds to genotypes 1a and 1b of Simmonds classification, and HCV serotype 2 corresponds to genotypes 2a and 2b.²⁵ A single pathologist who was unaware of the patient identification, dose level, or treatment response interpreted liver biopsy samples taken before treatment. The degree of hepatic fibrosis was scored using the METAVIR system.²⁶ The protocol did not require repetitive biopsies.

The primary endpoint of this trial was the changes in the serum ALT levels, and the secondary endpoints were the changes in the serum HCV RNA levels, virological and biochemical responses at the end of treatment, and safety. Analyses were conducted on all 45 patients for the changes in serum ALT levels and the biochemical responses. Since the pretreatment HCV RNA level decreased to an undetectable level in one patient (bLF of 3.6 g/day), the analyses of the change in serum HCV RNA levels and the virological responses were conducted on 44 patients.

Table I. Baseline Characteristics of Patients According to bLF Dose Group

	Dose of bLF		
	1.8 g/day	3.6 g/day	7.2 g/day
No. of patients	15	15	15
Age ^{a)} (years)	62 (54–73)	62 (38–71)	55 (37–74)
Gender (male/female)	6/9	9/6	11/4
History of IFN therapy	4 (27%)	6 (40%)	6 (40%)
ALT level ^{a)} (IU/liter)	82 (52–270)	107 (51–375)	99 (52–168)
HCV RNA level ^{a)} (Kcopies/ml)	341 (35–1492)	224 (<1–1354)	138 (3–1460)
HCV RNA serotype (1/2/ND ^{b)})	13/2/0	10/3/2	11/3/1
Hepatic fibrosis (F0,1/F2–4) ^{c)}	7/8	7/8	8/7

a) Median (range).

b) ND, not determined.

c) F, fibrosis (F0, no fibrosis, F1, portal fibrosis without septa, F2, portal fibrosis with rare septa, F3, numerous septa without cirrhosis, and F4, cirrhosis).

A linear dose-response was tested for the changes in the logarithm of serum ALT level and the changes in the logarithm of serum HCV RNA level using a linear regression model. The *P*-values calculated were one-sided, because only the decreasing trend of these two endpoints with an increase in bLF levels was of interest; the one-sided α level of significance was set at 0.05. A sample size of 15 patients per bLF dose was calculated to provide about 90% power (using a one-sided 0.05 level of significance) to detect a 50% reduction in the serum ALT level at the end of treatment in the bLF 7.2 g/day group. In addition to the above planned analyses, evaluations of the changes in serum HCV RNA levels at weeks 4 and 8 after the end of treatment were conducted. Since these were originally unplanned analyses, they were considered as exploratory. Analyses were performed by JMP 4.0 and PC SAS Release 6.02 (SAS Institute, Inc., Cary, NC).

The study was approved by the investigational review board of each participating institution according to the Declaration of Helsinki, and all the patients provided written informed consent.

RESULTS

Patients and treatment Forty-five patients were entered into this trial at three dose levels (15 patients at each level) from the two hospitals between March, 1999 and May, 2000. There were 26 males and 19 females with a median age of 62 years (range, 38–74). Characteristics of the patients at baseline are summarized in Table I. There were no significant differences between the three dose levels with respect to age, gender, history of IFN therapy, baseline ALT levels, baseline viral characteristics, or hepatic fibrosis. Of the 45 patients enrolled in the study, 16 (36%) had previously received IFN treatment, but had had no sustained virological response. Serum ALT levels on day 1 ranged from 52

Table II. Biochemical and Virological Responses at the End of Treatment According to bLF Dose Group

	Dose of bLF		
	1.8 g/day (%)	3.6 g/day (%)	7.2 g/day (%)
Biochemical response	1/15 (7)	1/15 (7)	0/15 (0)
Virological response	4/15 (27)	0/14 (0)	0/15 (0)

to 375 IU/liter (median, 99 IU/liter). Serum HCV RNA levels on day 1 ranged from ≤ 1 to 1492 Kcopy/ml (median, 227 Kcopy/ml), and the majority of the patients (76%) were infected with HCV serotype 1.

Efficacy There was no significant relation between the dose of bLF and the effect of bLF on serum ALT ($P=0.30$) or HCV RNA levels ($P=0.20$) at the end of treatment; the patients at each level did not significantly differ with regard to the changes in serum ALT or HCV RNA levels.

Table II summarizes the biochemical and virological responses at the three dose levels at the end of treatment. Biochemical response was observed in two patients (bLF of 1.8 g/day and 3.6 g/day) at the completion of treatment, but no patient achieved ALT normalization. Virological response was observed in four patients (bLF of 1.8 g/day) at the end of treatment, although all had persistently detectable serum HCV RNA levels. All patients who responded to bLF relapsed during the follow-up period after bLF treatment; the durations of the biochemical responses were 5 and 6 months, and the durations of the virological responses ranged from 6 to 10 months. In some patients, serum HCV RNA levels were reduced even after the completion of treatment; the virological response was observed in seven patients at week 4 and eight patients at week 8 after the end of treatment.

In the virological responders, the reduction in HCV RNA levels, which was rapid after the initiation of treatment, was invariably associated with a decrease in serum ALT levels. The effects of bLF on serum HCV RNA and

ALT levels in one representative patient from the study are shown in Fig. 1. Although the decrease in serum HCV RNA levels preceded the decrease in serum ALT levels, the time course of serum HCV RNA levels paralleled serum ALT levels during and after bLF treatment.

The efficacy of bLF treatment was analyzed with respect to several baseline parameters. Table III shows the virological response rates at week 8 after the completion of treatment for these variables. Age was associated with the response to bLF treatment; in contrast to IFN therapy, the response rate was significantly higher among the patients of 60 years or older, although the reason for these inconsistent results remains unclear. However, the virological response was not significantly influenced by gender, a history of IFN therapy, granulocyte count, serum ALT levels, or hepatic fibrosis. The virological response was more common in patients with serum HCV RNA levels of less than 100 Kcopies/ml, compared with patients with levels equal to or greater than 100 Kcopies/ml, but there was no significant difference. Similarly, the response rate was somewhat higher in patients with HCV serotype 2, com-

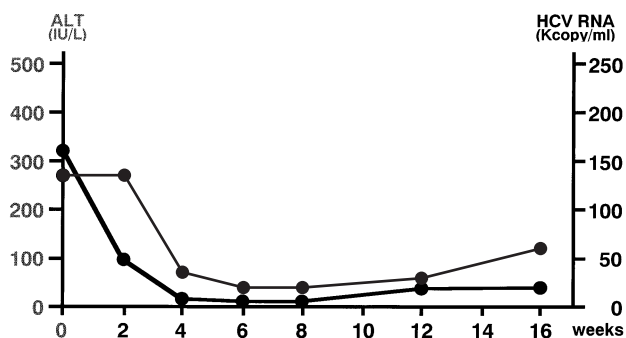


Fig. 1. Time course of changes in serum ALT and HCV RNA levels during and after bLF (1.8 g/day) treatment in a 71-year-old woman with chronic hepatitis C. — ALT, - - - HCV RNA.

Table III. Virological Response at Week 8 after the Completion of bLF Treatment with Respect to Baseline Characteristics of Patients

	Dose of bLF			
	1.8 g/day (%)	3.6 g/day (%)	7.2 g/day (%)	All doses (%)
Age*				
<59 years	0/4 (0)	0/7 (0)	0/8 (0)	0/19 (0)
≥60 years	5/11 (45)	1/7 (14)	2/7 (29)	8/25 (32)
Gender				
Male	2/6 (33)	0/9 (0)	2/11 (18)	4/26 (15)
Female	3/9 (33)	1/5 (20)	0/4 (0)	4/18 (22)
History of IFN				
+	1/4 (25)	0/6 (0)	0/6 (0)	1/16 (6)
-	4/11 (36)	1/8 (13)	2/9 (22)	7/28 (25)
Granulocyte count				
<1.9×10 ³ /mm ³	2/3 (67)	1/4 (25)	0/5 (0)	3/12 (25)
≥2.0×10 ³ /mm ³	3/12 (25)	0/10 (0)	2/10 (20)	5/32 (17)
ALT level				
<99 IU/liter	3/8 (38)	0/6 (0)	1/8 (13)	4/22 (18)
≥100 IU/liter	2/7 (29)	1/8 (13)	1/7 (14)	4/22 (18)
HCV RNA level				
<99 Kcopies/ml	1/2 (50)	1/4 (25)	2/5 (40)	4/11 (36)
≥100 Kcopies/ml	4/13 (31)	0/10 (0)	0/10 (0)	4/33 (12)
HCV RNA serotype				
1	4/13 (31)	0/10 (0)	1/11 (9)	5/34 (15)
2	1/2 (50)	1/2 (50)	1/3 (33)	3/7 (43)
Hepatic fibrosis ^{a)}				
F0, 1	2/7 (29)	0/6 (0)	1/8 (13)	3/21 (14)
F2-4	3/8 (38)	1/8 (13)	1/7 (14)	5/23 (22)

a) F, fibrosis (F0, no fibrosis, F1, portal fibrosis without septa, F2, portal fibrosis with rare septa, F3, numerous septa without cirrhosis, and F4, cirrhosis).

* P<0.01.

Table IV. Adverse Effects of bLF Treatment for CH-C

Case	Dose of bLF	Adverse effect (grade)	Dose reduction
1	3.6 g/day	Diarrhea (grade 1)	(+)
2	7.2 g/day	Skin eruption (grade 2)	(+)
3	7.2 g/day	Anorexia (grade 1)	(-)
4	7.2 g/day	Fatigue (grade 1)	(-)
		Chills (grade 1)	
		Constipation (grade 1)	

pared with patients with HCV serotype 1, but the HCV serotype had no significant influence on the virological response to bLF treatment.

Toxicity All 45 patients received at least one dose of bLF and were therefore eligible for toxicity assessment. The bLF treatment was generally well tolerated, and no patient showed any serious adverse event. Minor, transient treatment-related symptoms occurred in four patients, and these adverse effects appeared to be dose-dependent; three of the four patients received bLF of 7.2 g/day, while no symptoms occurred in patients receiving bLF of 1.8 g/day (Table IV). Therapy was continued without any specific treatment in two of the four patients, but the doses were reduced in the remaining two. Moreover, there were no clinically significant abnormalities in laboratory values during or after treatment.

DISCUSSION

The incidence of new HCV infections has markedly declined in recent years because of the availability of HCV testing of donated blood.²⁷⁾ However, the number of patients infected with HCV remains high, and the number of CH-C patients likely to develop clinically significant liver cirrhosis has not yet peaked. CH-C may have a negligible clinical impact over the first 1 to 2 decades in the majority of patients, but without a highly effective treatment, patients with CH-C usually suffer from liver cirrhosis and eventually HCC.^{2,3)} The primary goal in the treatment of CH-C is to achieve a complete resolution of CH (at least a decrease in progression to cirrhosis) and an inhibition of HCC development. However, current therapies for CH-C, including IFN, are less than optimal even with new approaches such as pegylated IFN and IFN/ribavirin combination.⁶⁻¹⁰⁾ Furthermore, the adverse effects of IFN therapy may significantly impair the quality of life of patients during the course of treatment. Therefore, there remains a critical need for newer and more effective treatments without severe adverse effects for CH-C.

LF has several biological activities, which include anti-viral activity and immunomodulatory functions such as natural killer cell activation.^{11,12)} Anti-viral activity of

LF has been reported for some viruses, and LF is the first physiological substance other than IFN found to show anti-HCV activity.¹³⁻¹⁹⁾ With respect to mechanisms of anti-viral activity of LF, two distinct mechanisms were reported; the direct interaction of LF with the cells and the interaction of LF with the virus. In the case of HCV, the anti-viral activity *in vitro* is due to the direct binding of LF to HCV; the interaction between LF and HCV occurred immediately after mixing LF and serum containing HCV, resulting in the inhibition of adsorption and/or internalization of the HCV-LF complex into human hepatocytes.¹⁷⁻¹⁹⁾ In contrast, LF showed no anti-HCV activity after adsorption and/or internalization of HCV into human hepatocytes.

In this dose-response trial, we examine the relation between the dose of bLF and the effect of bLF on serum HCV RNA and ALT levels in patients with CH-C. The patients who entered at each of the three dose levels (bLF of 1.8, 3.6, and 7.2 g/day) received an 8-week course of bLF and then were followed for the next 8 weeks. There was no significant relationship between the dose of bLF and the effect of bLF on serum ALT or HCV RNA levels. However, a biochemical (a 50% or greater decrease in serum ALT level) and/or virological (a 50% or greater decrease in HCV RNA level) response occurred in some CH-C patients; the durations of the biochemical responses were 5 and 6 months, and the durations of the virological responses ranged from 6 to 10 months. There was a major discrepancy between the durations of the responses in the present trial and the pilot study; in the pilot study, two of the three responders relapsed 2 weeks after cessation of the treatment.²⁰⁾ Although the reason for the conflicting results remains unclear, future trials using a large number of patients may resolve this issue.

With respect to toxicity, although mild, transient treatment-related symptoms, which appeared to be dose-dependent, occurred infrequently during bLF treatment, no clinically significant abnormalities were observed in laboratory values. Many patients with chronic liver disease, particularly cirrhosis, have leukocytopenia and thrombocytopenia, which limit the dose and duration of IFN therapy. Therefore, bLF treatment appears advantageous in comparison with IFN in terms of toxicity. Based on these results regarding the efficacy and toxicity, we suggest that the recommended dose for future trials using this schedule is 1.8 g/day.

Serum HCV RNA levels decreased even after cessation of bLF treatment in some patients; while the virological response was observed in only four patients at the end of treatment, eight patients achieved a virological response at week 8 after the end of treatment. These virological responses were highly correlated with a decrease in the serum ALT levels. Since progression to cirrhosis in patients with CH-C depends on the severity of hepatic

inflammation, bLF treatment, by reducing hepatic inflammation, may prevent progression of CH even in the presence of persistent viremia. Age was associated with the virological response to bLF treatment, but serum HCV RNA levels, HCV serotype, or hepatic fibrosis had no significant influence on the response to bLF treatment. Accordingly, the beneficial effect of bLF treatment may extend to subgroups of patients in whom IFN therapy has historically been unsuccessful, such as patients with high HCV RNA levels, HCV serotype 1, or advanced fibrosis.

LF is present in the secondary granules of neutrophils, and plasma LF is predominantly granulocyte-derived.²⁸⁾ Activated granulocytes release LF into circulation, and a rise in plasma LF concentrations is observed in some situations such as inflammation and pregnancy.^{29, 30)} Therefore, granulocytes may be associated with the response to bLF treatment. However, in the present trial, the virological response was not influenced by baseline granulocyte count, although plasma LF concentrations may or may not correlate with the granulocyte count, depending on the magnitude of degranulation and perhaps the contribution of other organs to the plasma content of LF.³⁰⁻³²⁾

Despite the potential anti-HCV activity of bLF shown in the current trial, several questions remain concerning bLF in the treatment of CH-C. It is unclear if bLF really has anti-HCV activity in patients with CH-C, or what the optimal treatment duration is for CH-C. It is also necessary to clarify the criteria for selecting patients to be treated with

bLF, and whether a response to bLF will alter the natural history of CH-C, reducing the likelihood of future development of cirrhosis, end-stage liver disease, HCC, or death. It must also be clarified if bLF has substantial anti-HCV activity in CH-C patients who showed no response to previous treatment with IFN, and in CH-C patients with relapse after IFN therapy. In addition, it needs to be clarified if the combination therapy of bLF and IFN would be a more effective means of clinical treatment. To resolve these questions and to define the exact role of bLF treatment in the treatment of CH-C, trials using large numbers of patients with a longer observation period are mandatory. We are currently conducting a double-blind randomized controlled trial comparing bLF with placebo to clarify the anti-HCV activity of bLF in patients with CH-C.

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