which results in oxidative stress [4]. A moderate decrease in leukocyte counts has also been reported in patients who receive combination therapy and those who received IFN monotherapy.

Increased oxidative stress has been shown to accompany chronic liver disease [5–8]. Various factors, including hepatocellular iron accumulation [9,10] and the direct effect of the HCV core protein [11,12], are currently considered to be the causes of oxidative stress, in addition to reactive oxygen species production that is related to activated phagocytes and inflammatory cytokines such as TNF-α and interleukin-1β during the immune response [13,14].

In a previous study [15], we observed a decreased level of plasma α-tocopherol and an increase in plasma thiobarbituric acid-reactive substances (TBARS) in patients who had HCV and cirrhosis and showed an abnormal fatty acid pattern, with low levels of polyunsaturated fatty acids (PUFAs) in phospholipids of mononuclear cells. We also reported that vitamin E supplementation induced a significant increase in ω 6 and ω 3 PUFAs in the phospholipids of erythrocyte membranes of HCV-infected patients, which was accompanied by an improvement in the alanine aminotransferase (ALT) level [16]. Previously, von Herbay et al. [17] observed that high vitamin E supplementation improves the aminotransferase status in patients who have chronic HCV. Vitamin E is one of the safest antioxidants [18], but the mechanisms of its physiologic functions are not thoroughly understood [19]. Vitamin C can regenerate α-tocopherol from the α-tocopherol radical and, hence, cooperates with α-tocopherol in inhibiting lipid peroxidation [20].

Peripheral blood mononuclear cells are involved in the antiviral immune response. Some reports have shown a change of immune state, such as the functional impairment of HCV-specific $CD4^+$ T cells, in patients with chronic hepatitis C [21]. Several human studies have suggested that PUFAs have immunomodulatory actions [22]. The PUFAs, especially arachidonic acid (AA) and eicosapentaenoic acid (EPA), in cell membrane phospholipids are precursors of the eicosanoids and are involved in lipid-mediated intracellular signaling.

In the present study, we investigated the effects of vitamin E and C supplementation on the fatty acid composition of mononuclear cell phospholipids and on the clinical observations in patients who had chronic hepatitis C and received IFN-α-2b and ribavirin combination therapy.

Materials and methods

Subjects

Thirty patients who had chronic hepatitis C (16 men and 14 women, average age 52.4 ± 2.0 y) were enrolled in this study. The criteria for enrollment were a persistently increased ALT level for longer than 6 mo before enrollment, positive results for HCV RNA in serum, and a high viral load ($>10^5$ IU/mL). Patients with decompensated cirrhosis, detectable hepatitis B virus surface antigen, other potential causes of chronic liver disease, hemoglobin (Hb) level lower than 12 g/dL, platelet count lower than 70 000 /mm^3, human immunodeficiency virus infection, poorly controlled diabetes mellitus, or cardiovascular disease were excluded.

The baseline characteristics of the patients in both groups were similar (Table 1). HCV genotypes were as follows: 1a/1b/2a/2b: 1/9/4/0 in the vitamin group and 0/10/5/1 in the non-vitamin group. No significant differences were observed in the distribution of the histologic diagnosis for grading inflammation (stages 1 to 3; not done: 3/4/2/5 in the vitamin group and 2/11/2/1 in the non-vitamin group) or for staging fibrosis (grades 1 to 3, not done: 4/4/1/5 in the vitamin group and 4/7/4/1 in the non-vitamin group) at baseline.

The control subjects were healthy volunteers (four men and four women, average age 54.3 ± 2.8 y).

Study design

We obtained informed consent from all patients and randomized them to receive daily 500 mg of vitamin E (Juvela, one tablet contains 50 mg of tocopherol acetate; Eisai, Tokyo, Japan) and 750 mg of vitamin C (HICEE, one packet contains 250 mg of ascorbic acid; Takeda Chemical Industries, Osaka, Japan) orally after all three meals (vitamin E: morning 150 mg, afternoon 150 mg, and evening 200 mg; vitamin C: morning 250 mg, afternoon 250 mg, and evening 250 mg; vitamin group: nine men and five women, average age 53.9 ± 2.6 y) or no supplementation (non-vitamin group: seven men and nine women, average age 51.1 ± 3.0 y), in addition to injections of 6 million units of IFN-α-2b (Intron A, Schering-Plough K.K., Osaka, Japan) six times weekly for 2 wk, followed by three times weekly for 24 additional wk, plus daily oral 600 mg (body weight ≤60 kg) or 800 mg (body weight >60 kg) of ribavirin (Revetol, Schering-Plough K.K.) for 24 wk. During treatment, the dose of ribavirin was decreased by 200 mg/d in patients whose Hb level decreased below 10 g/dL, and ribavirin was discontinued when the level decreased below 8.5 g/dL. Patients were required not to take iron or other antioxidant supplements for the treatment period.

Blood samples were obtained from each patient immediately before the initiation of therapy (0 wk) and at 2, 4, and 8 wk after the start of the therapy and were analyzed for the amount of α-tocopherol in red blood cells (RBCs) and plasma, ascorbic acid and TBARS in plasma, and the fatty acid composition in mononuclear cell phospholipids.

This experiment was designed in accordance with the principles of the Declaration of Helsinki of the World Medical Association and was approved by the ethics committee of Yamaguchi University Hospital.

Table 1
Effects of vitamin E and C supplementation on clinical data during combination therapy of IFN-α-2b and ribavirin*

	Controls (n = 8)	IFN-α-2b plus ribavirin treatment							
		Vitamin group (n = 14)				Non-vitamin group (n = 16)			
		0 wk	2 wk	4 wk	8 wk	0 wk	2 wk	4 wk	8 wk
Age (y)	54.3 ± 2.8	53.9 ± 2.6				51.1 ± 3.0			
Male/female	4/4	9/5				7/9			
ALT (U/L)	18 ± 1	87 ± 13‖	48 ± 9§	32 ± 8‡	29 ± 8‡	123 ± 15‖	48 ± 8†	30 ± 3†	25 ± 2†
RBC count (×10⁴/μL)	463 ± 7	434 ± 11	416 ± 11	375 ± 13‡	371 ± 15‡	431 ± 9†	401 ± 10‡	357 ± 11†	351 ± 15†
Hb (g/dL)	14.3 ± 0.3	14.0 ± 0.3	13.3 ± 0.3	11.9 ± 0.4‡	11.6 ± 0.4†	13.5 ± 0.4	12.5 ± 0.3§	11.6 ± 0.3‡	11.3 ± 0.4‡
WBC count (/μL)	5875 ± 495	5797 ± 660	4058 ± 33‡	3451 ± 294†	3541 ± 376‡	4813 ± 242	3669 ± 369†	3730 ± 452‡	3550 ± 339‡
Albumin (g/dL)	4.7 ± 0.1	4.3 ± 0.1¶	4.1 ± 0.1	4.2 ± 0.1	4.2 ± 0.1	4.3 ± 0.1#	4.1 ± 0.1‡	4.2 ± 0.1	4.2 ± 0.1
Transferrin (mg/dL)	258 ± 10	265 ± 11	238 ± 14‡	225 ± 9‡	238 ± 9‡	287 ± 13	241 ± 10†	233 ± 9†	244 ± 7†
Total cholesterol (mg/dL)	216 ± 14	165 ± 5#	152 ± 8	156 ± 6	159 ± 6	163 ± 8†	142 ± 6	143 ± 7	148 ± 7
Ferritin (ng/mL)	—	166 ± 38	—	—	305 ± 58†	106 ± 21	—	—	268 ± 43‡
TNF-α (pg/mL)	—	36.9 ± 21.0	—	—	33.4 ± 20.3	29.3 ± 8.8	—	—	10.1 ± 3.8§

—, no data; ALT, alanine aminotransferase; Hb, hemoglobin; HCV, hepatitis C virus; IFN-α-2b, interferon-α-2b; RBC, red blood cell; TNF-α, tumor necrosis factor-α; WBC, white blood cell.

* Mean ± standard error.

† $P < 0.001$ versus 0 wk (Wilcoxon's t test).

‡ $P < 0.01$ versus 0 wk (Wilcoxon's t test).

§ $P < 0.05$ versus 0 wk (Wilcoxon's t test).

‖ $P < 0.001$ versus control subjects at 0 wk (Mann-Whitney U test).

# $P < 0.01$ versus control subjects at 0 wk (Mann-Whitney U test).

¶ $P < 0.05$ versus control subjects at 0 wk (Mann-Whitney U test).

Analytical methods

Blood was drawn into tubes containing disodium ethylenediaminetetraacetic acid. Plasma was separated after centrifugation of blood samples at 1600*g* for 15 min at 4°C. The RBC layer was drawn off into another tube and washed three times with saline (4°C) for α-tocopherol determination. A sample of undiluted blood was layered onto Mono Poly Resolving Medium (Dainippon Pharmaceuticals, Osaka, Japan) and centrifuged at 400*g* for 20 min at 4°C. The mononuclear cell layer was drawn off into another tube and washed three times with saline (4°C) and stored at −80°C until assayed. Total lipid was extracted from mononuclear cells suspended in 0.5 mL of saline according to the method of Bligh and Dyer [23]. Phospholipids were separated by one-dimensional thin-layer chromatography using silica gel plates (Silica Gel 60; Merck, Darmstadt, Germany) using a solvent system of petroleum ether:ethyl ether:acetic acid (80:20:1, v/v). The spots corresponding to phospholipid were scraped from the plates and transmethylated for 2 h at 90°C using 2 mL of acetylchloride:methanol (5:50, v/v). A known amount of heptadecanoic acid (17:0) was used as an internal standard. Fatty acid methyl esters were analyzed by gas-liquid chromatography (Model GC-14A; Shimadzu, Kyoto, Japan) as described previously [24].

α-Tocopherol concentrations in plasma and RBCs were determined according to a modification of the method of Milne et al. [25]. Tocopherols were extracted from plasma and saponified RBC samples with ethanol and *n*-hexane or ethyl acetate and *n*-hexane (1:9, v/v), respectively. Extracts were evaporated under nitrogen and redissolved in ethanol. The α-tocopherol level was quantified by high-performance liquid chromatographic analysis in a TSK-gel ODS-80Ts column (25.0 cm × 4.6 mm; TOSOH, Tokyo, Japan). The mobile phase was methanol:1-butanol (80:20, v/v), including 10 mM of sodium acetate buffer (pH 3.6, 0.1%; v/v) at a flow rate of 1.0 mL/min (Model CCPM, TOSOH). The α-tocopherol was monitored at an excitation wavelength of 295 nm and an emission wavelength of 325 nm (Model 821-FP, JASCO, Tokyo, Japan). The α-tocopherol peak was identified and quantified against authentic D-α-tocopherol (vitamin E reference standards; Eisai) used as an external standard. The Hb concentration of each RBC sample was detected by using the method of Oshiro et al. [26]. The RBC α-tocopherol concentration was expressed as micrograms per gram of Hb.

Plasma ascorbic acid concentrations were determined according to a specific and sensitive method involving chemical derivatization and high-performance liquid chromatography described by Kishida et al. [27]. The extracted sample was analyzed on a μ-Bondasphere 5-μm C18-100A column (3.9 mm inner diameter × 150 mm; Waters), eluted with 50% acetonitrile containing 0.1% triethylamine at a flow rate of 1.0 mL/min (Waters 600E Multisolvent Delivery System, Waters). The absorbance at 505 nm was recorded (Waters 486 Tunable Absorbance Detector, Waters, Milford, CT, USA).

Plasma TBARS, primarily malondialdehyde, was measured according to the method described by Yagi [28].

Evaluation of plasma TNF-α was performed by using a commercially available enzyme-amplified sensitivity immunoassay (TNF-α enzyme immunoassay kit; Immunotech, Marseille, France). The minimum detectable concentration of TNF-α was 5 pg/mL.

Statistical analysis

Values are expressed as means ± standard errors of the mean. A non-parametric method, Wilcoxon's *t* test, or Mann-Whitney U test, was conducted to analyze the data, as appropriate. All *P* values are two-tailed and statistical significance was set at $P < 0.05$.

Results

Assignment of therapy and completion of assigned therapy

Treatment was discontinued 8 to 26 wk after its initiation because of side effects (general fatigue, anorexia, and/or depression) in three patients (21.4%) in the vitamin group and four patients (25.0%) in the non-vitamin group (not significant). Therefore, 30 patients (14 in the vitamin group and 16 in the non-vitamin group) were analyzed until 8 wk after initiation of therapy. The dose of ribavirin was decreased by 200 mg/d at some period (>4 wk or <20 wk) in two patients (14.3 %) in the vitamin group and six (37.5 %) in the non-vitamin group because of a decrease in the Hb level to lower than 10 mg/dL (not significant). IFN was not decreased in any patients in either group.

Concentration of α-tocopherol and ascorbic acid

At the start of treatment, plasma α-tocopherol (7.0 ± 0.3 μg/mL), RBC α-tocopherol (3.2 ± 0.2 μg/g of Hb), and plasma ascorbic acid (51.6 ± 3.2 nmol/mL) concentrations in patients were significantly lower than those in healthy control subjects (9.5 ± 0.7 μg/mL, $P < 0.01$; 4.6 ± 0.3 μg/g of Hb, $P < 0.01$; and 70.9 ± 4.5 nmol/mL, $P < 0.01$, respectively). There were no significant differences in α-tocopherol or ascorbic acid concentrations between the vitamin and non-vitamin groups before treatment. After vitamin supplementation, plasma α-tocopherol levels increased to 2.4-fold of the basal level at 2 wk ($P < 0.001$) and 2.9-fold at 4 wk ($P < 0.001$) in the vitamin group. RBC α-tocopherol levels of the vitamin group also increased 2.1-fold at 2 wk ($P < 0.001$) and were maintained at that level throughout supplementation. Plasma ascorbic acid levels in the vitamin group reached a plateau of 70.1 ± 3.4 nmol/mL, 1.6-fold of the basal level, at 2 wk ($P < 0.01$) and were maintained at

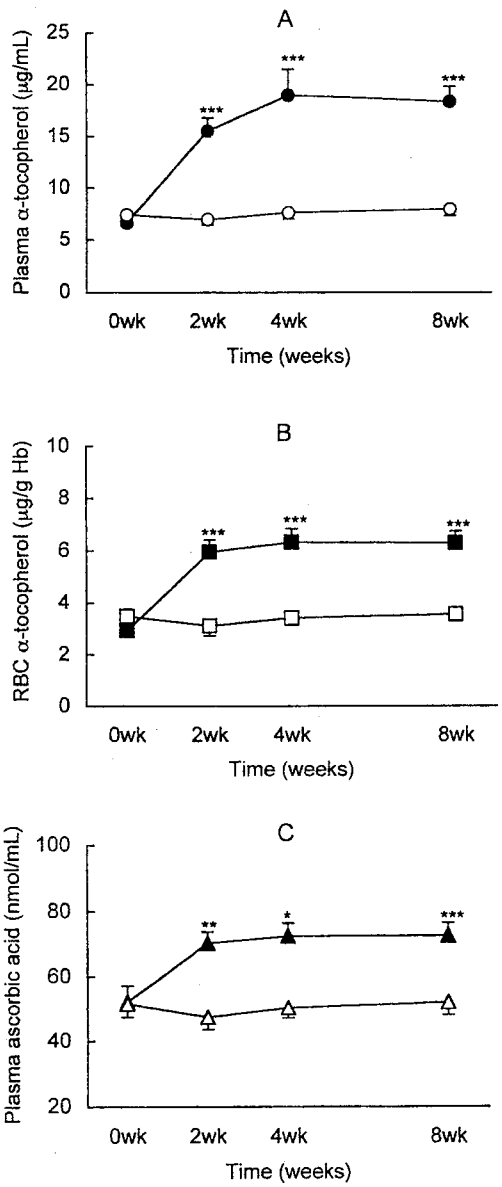

Fig. 1. Change in antioxidant vitamin levels during treatment in patients infected with hepatitis C virus. Values are means ± standard errors. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ versus 0 wk (Wilcoxon's t test). (A) Plasma α-tocopherol in the vitamin group (solid circles) and non-vitamin group (open circles). (B) RBC α-tocopherol in the vitamin group (solid squares) and non-vitamin group (open squares). (C) Plasma ascorbic acid in the vitamin group (solid triangles) and non-vitamin group (open triangles). RBC, red blood cell.

that level thereafter; this level was the same as that in control subjects. In the non-vitamin group, no significant change was seen in these antioxidant vitamin levels during treatment (Fig. 1).

Clinical data

Serum ALT levels decreased significantly after 2 wk of treatment in both groups (Table 1). In the vitamin group, RBC counts and Hb levels 2 wk after initiation of vitamin E and C supplementation were maintained at the same levels as observed at 0 wk. Serum ferritin levels increased at 8 wk, regardless of vitamin supplementation. Plasma TNF-α level significantly decreased in the non-vitamin group but did not change in the vitamin group after 8 wk of treatment.

Fatty acid compositions of mononuclear cell phospholipids

In the present patients, a lower level of EPA (1.00 ± 0.77 mol%) and a higher level of the molar ratio of AA to EPA (16.89 ± 1.46) were observed compared with those in the control subjects (1.71 ± 0.14 mol%, $P < 0.01$; 9.20 ± 0.67, $P < 0.001$, respectively) at the start (0 wk) of treatment; however, there were no significant differences in the fatty acid profile between the vitamin and non-vitamin groups (Table 2). The EPA level of mononuclear cells was maintained in the vitamin group during treatment, whereas a significant decrease was observed in the non-vitamin group at 4 and 8 wk after treatment. Linoleic acid had decreased at 4 and 8 wk after treatment in the non-vitamin group. AA level did not change throughout the therapy in either group. Tetracosanoic acid (24:1) decreased at 8 wk in both groups.

At the start of treatment, a significant correlation between the AA/EPA molar ratio in phospholipids of mononuclear cells and serum ALT level was seen in all patients who had chronic hepatitis ($r = 0.374$, $P < 0.05$; Fig. 2). The AA/EPA molar ratio did not change significantly during treatment in either group, although serum ALT levels clearly improved.

Level of TBARS

Plasma TBARS levels decreased significantly at 4 and 8 wk in the vitamin group and at 4 wk in the non-vitamin group after initiation of therapy compared with basal levels (Fig. 3).

Discussion

The present study showed that oral vitamin E and C supplementation (500 mg/d of tocopherol acetate and 750 mg/d of ascorbic acid) prevented the decrease of EPA in phospholipids of mononuclear cells during combination therapy of IFN-α-2b and ribavirin in patients with chronic hepatitis C. The lower level of EPA in mononuclear cells observed at the start of treatment may indicate enhanced lipid peroxidation [29] and/or a low dietary intake of EPA [30] in patients with chronic HCV. In the present study, we observed no dietary fatty acid intake in patients with chronic hepatitis C. Although dietary fatty acid intake differs according to dietary habits [30], a lower intake of total ω 3 fatty acids was observed in patients with chronic hepatitis C (2.6 ± 0.4 g/d) compared with healthy subjects (3.7 ± 0.5

Table 2
Effects of vitamin E and C supplementation on fatty acid composition of mononuclear cells phospholipids during combination therapy of IFN-α-2b and ribavirin (molar percentage)*

	Controls (n = 8)	IFN-α-2b plus ribavirin treatment							
		Vitamin group (n = 14)				Non-vitamin group (n = 16)			
		0 wk	2 wk	4 wk	8 wk	0 wk	2 wk	4 wk	8 wk
Myristic acid (14:0)	1.31 ± 0.18	1.63 ± 0.16	1.57 ± 0.27	1.53 ± 0.32	1.68 ± 0.30	1.43 ± 0.18	2.10 ± 0.41	2.15 ± 0.48	2.39 ± 0.50
Palmitic acid (16:0)	23.91 ± 0.67	23.48 ± 0.95	23.36 ± 0.80	23.62 ± 0.74	24.28 ± 1.12	22.91 ± 0.59	24.27 ± 1.34	23.94 ± 0.82	24.55 ± 0.76‡
Palmitoleic acid (16:1)	0.63 ± 0.16	0.92 ± 0.14	0.71 ± 0.07	0.68 ± 0.18	0.78 ± 0.14	0.67 ± 0.05	0.57 ± 0.07	0.80 ± 0.16	0.71 ± 0.15
Stearic acid (18:0)	19.19 ± 0.24	18.05 ± 0.37¶	18.37 ± 0.25	17.96 ± 0.58	17.72 ± 0.33	17.60 ± 0.36#	17.18 ± 0.44	17.97 ± 0.39	17.94 ± 0.28
Oleic acid (18:1)	15.79 ± 0.52	15.51 ± 0.43	16.54 ± 0.49‡	17.33 ± 0.52‡	16.41 ± 0.46	16.62 ± 0.57	16.70 ± 0.77	17.31 ± 0.62	15.61 ± 0.48
Linoleic acid (18:2 ω 6)	6.63 ± 0.21	6.14 ± 0.23	5.73 ± 0.27	5.37 ± 0.40‡	5.94 ± 0.42	6.58 ± 0.38	6.06 ± 0.46	5.33 ± 0.40‡	5.54 ± 0.30‡
Arachidic acid (20:0)	1.16 ± 0.08	1.16 ± 0.10	1.01 ± 0.08	1.08 ± 0.11	1.31 ± 0.22	1.03 ± 0.08	1.01 ± 0.11	0.89 ± 0.10	1.23 ± 0.09
Dihomo-γ-linolenic acid (20:3 ω 6)	1.06 ± 0.06	1.27 ± 0.08	1.33 ± 0.09	1.20 ± 0.11	1.26 ± 0.09	1.38 ± 0.06#	1.19 ± 0.06	1.19 ± 0.11	1.17 ± 0.08
Arachidonic acid (20:4 ω 6)	15.11 ± 0.40	13.91 ± 0.90	14.15 ± 0.90	13.75 ± 0.85	13.51 ± 0.66	14.70 ± 0.74	12.98 ± 1.06	14.07 ± 0.82	14.33 ± 0.86
Eicosapentaenoic acid (20:5 ω 3)	1.71 ± 0.14	1.02 ± 0.09‖	1.00 ± 0.10	1.02 ± 0.12	0.98 ± 0.16	0.98 ± 0.12#	0.89 ± 0.13	0.82 ± 0.11‡	0.76±0.07‡
Behenic acid (22:0)	1.87 ± 0.14	1.64 ± 0.11	1.87 ± 0.20	1.99 ± 0.16	1.91 ± 0.24	1.73 ± 0.11	1.61 ± 0.13	1.62 ± 0.11	1.77 ± 0.10
Docosapentaenoic acid (22:5 ω 3)	1.27 ± 0.20	1.50 ± 0.08§	1.29 ± 0.09	1.28 ± 0.09	1.19 ± 0.09†	1.35 ± 0.13	1.32 ± 0.16	1.20 ± 0.16	1.08 ± 0.07
Docosahexaenoic acid (22:6 ω 3)	2.74 ± 0.15	2.88 ± 0.19	2.93 ± 0.20	3.00 ± 0.22	2.54 ± 0.18	3.16 ± 0.25	3.01 ± 0.25	2.73 ± 0.21	2.67 ± 0.20
Lignoceric acid (24:0)	1.48 ± 0.12	1.44 ± 0.11	1.18 ± 0.08‡	1.39 ± 0.08	1.17 ± 0.12	1.32 ± 0.08	1.32 ± 0.09	1.26 ± 0.07	1.27 ± 0.07
Tetracosenoic acid (24:1)	1.76 ± 0.18	2.18 ± 0.18	2.02 ± 0.12	2.49 ± 0.25	1.68 ± 0.13‡	2.42 ± 0.22	2.34 ± 0.23	2.34 ± 0.24	1.87 ± 0.21‡
Total saturated fatty acid	49.93 ± 0.75	50.18 ± 1.16	49.57 ± 1.27	49.79 ± 1.21	51.10 ± 1.33	48.42 ± 0.93	50.49 ± 1.81	50.26 ± 1.25	52.23 ± 1.48‡
Total monounsaturated fatty acid	19.81 ± 0.55	20.23 ± 0.50	20.74 ± 0.45	21.94 ± 0.54‡	20.55 ± 0.48	20.97 ± 0.65	21.12 ± 0.85	21.81 ± 0.74	19.60 ± 0.63
Total polyunsaturated fatty acid	30.66 ± 0.42	29.59 ± 1.06	29.15 ± 1.10	28.27 ± 0.92	28.35 ± 1.28	30.61 ± 0.83	28.39 ± 1.58	27.93 ± 1.34	28.17 ± 1.04
PUFA/SFA ratio	0.62 ± 0.02	0.60 ± 0.03	0.60 ± 0.03	0.58 ± 0.03	0.57 ± 0.04	0.64 ± 0.03	0.59 ± 0.05	0.57 ± 0.04	0.55 ± 0.03
Total ω 6 PUFA	24.55 ± 0.41	23.87 ± 0.98	23.59 ± 0.97	22.73 ± 0.79	23.09 ± 1.04	24.90 ± 0.68	22.77 ± 1.23	22.91 ± 1.03	23.30 ± 0.94
Total ω 3 PUFA	6.11 ± 0.20	5.72 ± 0.31	5.57 ± 0.30	5.54 ± 0.39	5.26 ± 0.46	5.71 ± 0.45	5.62 ± 0.47	5.02 ± 0.39	4.87 ± 0.25
ω 6 PUFA/ω 3 PUFA ratio	4.05 ± 0.16	4.32 ± 0.31	4.35 ± 0.24	4.40 ± 0.37	4.77 ± 0.42	4.69 ± 0.32	4.26 ± 0.24	4.81 ± 0.25	4.93 ± 0.27
20:4 ω 6/20:5 ω 3 ratio	9.20 ± 0.67	14.95 ± 1.70#	16.06 ± 2.02	15.93 ± 1.93	18.89 ± 3.03	18.59 ± 2.25#	20.12 ± 2.82	20.23 ± 1.92	21.12 ± 2.10

IFN-α-2b, interferon-α-2b; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid.

* Mean ± standard error.

† $P < 0.01$ versus 0 wk (Wilcoxon's t test).

‡ $P < 0.05$ versus 0 wk (Wilcoxon's t test).

§ $P < 0.05$ versus non-vitamin group at 0 wk (Mann-Whitney U test).

‖ $P < 0.001$ versus control subjects (Mann-Whitney U test).

# $P < 0.01$ versus control subjects (Mann-Whitney U test).

¶ $P < 0.05$ versus control subjects (Mann-Whitney U test).

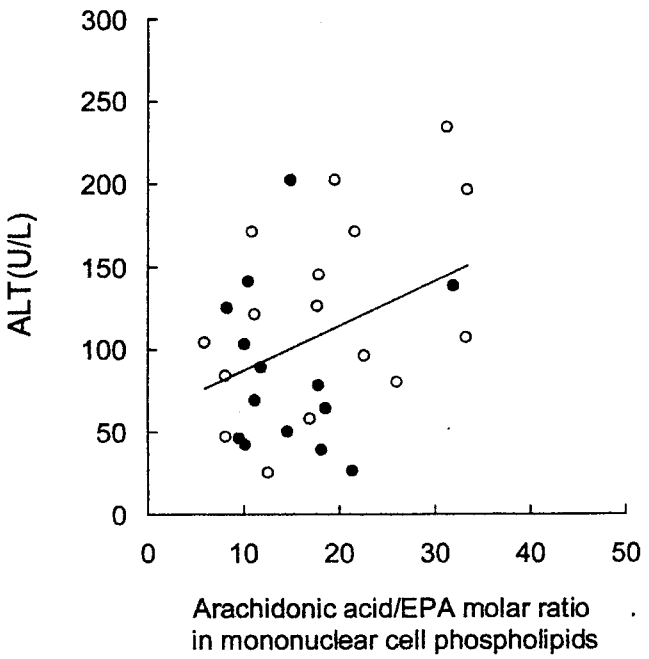

Fig. 2. Relation between serum ALT levels and molar ratios of arachidonic acid to EPA in mononuclear cell phospholipids before treatment in the vitamin group (solid circles) and non-vitamin group (open circles; $y = 2.7x + 60.5$, $r = 0.374$, $P < 0.05$). ALT, alanine aminotransferase; EPA, eicosapentaenoic acid.

g/d; unpublished data). α-Tocopherol and ascorbic acid deficiencies at baseline may also indicate oxidative stress in the present patients, as described in a previous report [5]. Mabile et al. [31] pointed out that a higher level of incorporation of ω 3 fatty acids into the erythrocyte membrane induced a higher level of α-tocopherol in the membrane. A deficiency of α-tocopherol, therefore, may induce a low level of EPA in mononuclear cells.

Side effects, including hemolytic anemia, may lead to dose reduction or discontinuation of IFN-α-2b and ribavirin therapy. Although no significant differences in hemolytic anemia or the continuance of treatment were recognized between the vitamin and non-vitamin groups, RBC counts and Hb levels 2 wk after initiation of vitamin E and C supplementation were maintained at the same levels as observed at 0 wk in the vitamin group. The long-term outcome is not yet known, but our findings are compatible with those of Saeian et al. [32] who reported that high-dose vitamin E monosupplementation had no effect on hemolytic anemia or sustained viral response in IFN and ribavirin therapy. Conversely, Morisco et al. [33] reported that an antioxidant-rich tomato-based functional food decreased the severity of hemolytic anemia during pegylated IFN and ribavirin therapy. Their findings suggest the importance of antioxidant-rich dietary management. Further studies are needed to clarify the efficacy of antioxidants on combination therapy.

Vitamin E and C supplementation prevented the decrease in EPA level in the present study. The ω 3 fatty acids in the erythrocyte membrane are expected to preserve membrane integrity, as reported by Mabile et al. [31]. A higher level of EPA in the erythrocyte membrane may be needed to prevent hemolysis. Ide et al. [34] observed that oral administration of ethyl ester of EPA (1800 mg/d) increased the Hb level in a chronic hepatitis C patient who had developed anemia during IFN-α-2b and ribavirin therapy. Because EPA is a highly unsaturated fatty acid and thus is thought to have a high susceptibility to oxidation, antioxidant supplementation may be needed for prevention of oxidative damage during EPA treatment.

The increase in serum ferritin level observed in the present study may have been due to hemolytic anemia caused by ribavirin therapy. Iron overload may generate oxygen-free radicals and cause peroxidative tissue injury [35]. Membrane oxidative damage also results from ribavirin accumulation in erythrocytes [4]. These results also indicate the necessity of antioxidant therapy during IFN-α-2b and ribavirin therapy.

Phospholipids in mononuclear cell membranes are characterized by having predominantly PUFAs such as AA, EPA, and docosahexaenoic acid that are esterified in position 2 of glycerol. Upon activation of phospholipases by physiologic or pathophysiologic stimuli, AA and EPA are released and oxidized by cyclooxygenase and lipoxygenase. This action leads immediately to the biosynthesis of various prostaglandins (PGs) and other eicosanoids. Accelerated synthesis of PGE_2 from ω 6 PUFAs, and especially from AA, suppresses the production of type 1 cytokines [36]. Therefore, it may be important to maintain a lower AA/EPA ratio for the combination therapy of patients with chronic hepatitis C. The significant correlation between the AA/EPA molar ratio and serum ALT level observed at the start of therapy supports this expectation. Vitamin E has also been reported to have non-antioxidant molecular mechanisms [19]. Wu et al. [37] demonstrated that in vitro sup-

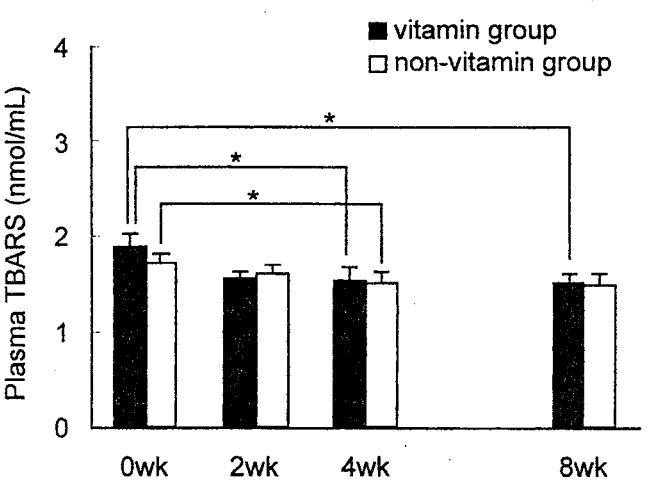

Fig. 3. Change in plasma TBARS levels during treatment in patients infected with hepatitis C virus. Values are means ± standard errors. *$P <$ 0.05 versus 0 wk (Wilcoxon's t test). TBARS, thiobarbituric acid-reactive substances.

plementation with α-tocopherol increased T-cell proliferation and inhibited cyclooxygenase and PGE_2 production by mouse peritoneal macrophages. Meydani et al. [38] also observed that vitamin E supplementation improved mitogen-stimulated lymphocyte proliferation and interleukin-2 production and suppressed PGE_2 synthesis by peripheral blood mononuclear cells in healthy elderly subjects. Although the leukocyte count decreased in both groups in the present study, plasma TNF-α level decreased only in the non-vitamin group. TNF-α is fluctuated by IFN-α [39,40] and a favorable clinical response to IFN-α therapy is associated with a decrease in serum type 2 cytokine levels. The maintenance of the level of serum TNF-α, a type 1 cytokine, observed in the vitamin group may have been favorable for the efficacy of therapy. From a number of reports, it is also supposed that ribavirin serves as an immunomodulator to enhance type 1 cytokine production [41]. Vitamin E supplementation, therefore, may be beneficial to support the immune response in combination therapy. We have data only about the fatty acid pattern in mononuclear cells. To clarify the effect of vitamin E on the immune response, parameters related to immunity, such as the T-helper 1/T-helper 2 ratio and lymphocyte blast transformation, should be examined.

We observed decreased tetracosanoic acid in mononuclear cell phospholipids at 8 wk after treatment, regardless of vitamin supplementation. Large amounts of tetracosanoic acid are contained in sphingomyelin; however, other phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol, contain little tetracosanoic acid [42,43]. Sphingomyelin mainly exists outside the biomembrane lipid bilayer and is a constituent of lipid rafts. Lipid rafts are specialized membrane domains that contain high concentrations of cholesterol, sphingomyelin, and gangliosides and are thought to be involved in the regulation of signal transduction [44,45]. Although the reason for the decrease is uncertain, the decrease in tetracosanoic acid may have some implications for mononuclear cell function. A decreased level of linoleic acid was observed at 4 wk in the vitamin group and at 4 and 8 wk in the non-vitamin group after initiation of therapy. Linoleic acid is an essential fatty acid that originates from the diet; therefore, decreased consumption of vegetable oil or other dietary elements rich in linoleic acid because of appetite loss during IFN therapy may be a cause of the decrease. Linoleic acid is an oxidizable PUFA. Vitamin supplementation may control the oxidation or metabolism of linoleic acid. Changes in fatty acid profile, such as an increase in palmitic acid, a saturated fatty acid, and a decrease in linoleic acid and EPA may affect the membrane integrity and morphology of mononuclear cells in the same way as reported for erythrocytes [46]. We have found no published reports on changes in RBC ribavirin concentrations. The measurement of RBC ribavirin levels might be useful for clarifying the influence of RBC α-tocopherol and EPA on ribavirin accumulation in RBC.

In conclusion, antioxidant vitamin supplementation during combination therapy of IFN-α-2b and ribavirin prevented a decrease in EPA of mononuclear cell phospholipids. If a further decrease in the AA/EPA ratio could be achieved using oral EPA supplementation, it might be possible to enhance the efficacy of combination therapy of IFN-α-2b and ribavirin.

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Quantitative Analysis of Anti–Hepatitis C Virus Antibody–Secreting B Cells in Patients With Chronic Hepatitis C

Takeji Umemura,[1,2] Richard Y.-H. Wang,[1] Cathy Schechterly,[1] J. Wai-Kuo Shih,[1] Kendo Kiyosawa,[2] and Harvey J. Alter[1]

To investigate the quantitative characteristics of humoral immunity in patients with hepatitis C, we established an enzyme-linked immunosorbent spot (ELISpot) assay for detection of anti–hepatitis C virus (HCV)-secreting B cells. Receiver operating characteristic curve analysis demonstrated 100% specificity and 58% to 92% sensitivity for detecting B-cell responses to NS5b, NS3, E2, and core antigens. The median sum of anti-HCV–secreting B cells to all HCV antigens tested was significantly higher in 39 patients with chronic hepatitis C (47.3 spot forming cells [SFCs]/10^6 peripheral blood mononuclear cells [PBMCs]) than in 9 recovered subjects (15.3 SFCs/10^6 PBMCs; $P = .05$) or 11 uninfected controls (5.3 SFCs/10^6 PBMCs; $P < .001$); the significant difference ($P = .018$) in chronic versus recovered patients was in reactivity to nonstructural antigens NS3 and NS5b. Anti-HCV immunoglubulin M (IgM)–secreting B cells were also readily detected and persisted decades into HCV infection; there was no difference in IgM-positive cells between chronic and recovered patients. ELISpot reactivity to genotype 1–derived antigens was equivalent in patients of genotypes 1, 2, and 3. There was significant correlation between the numbers of anti-HCV IgG-secreting B cells and serum aminotransferase and to the level of circulating antibody. In conclusion, ELISpot assays can be adapted to study B-cell as well as T-cell responses to HCV. Measurement at the single-cell level suggests that humoral immunity plays a minor role in recovery from HCV infection and that B-cell immunity is strongest in those with persistent infection. (HEPATOLOGY 2006;43:91-99.)

Hepatitis C virus (HCV) infection is a major cause of chronic liver disease worldwide. More than half of patients with acute HCV infection develop chronic hepatitis, leading to cirrhosis and/or hepatocellular carcinoma in at least 20% of these patients.[1-3] Chronic HCV infection results in the induction of a strong humoral immune response, and measurement of anti-HCV antibodies in serum is widely used to screen for HCV infection. Although several studies have examined the features of the humoral immune response to HCV,[4-7] the quantitative characteristics of HCV-specific antibody production during infection remain undefined. In patients with acute hepatitis C, an early HCV-specific T-cell response is associated with viral clearance,[8-11] but the role of humoral immune responses in HCV clearance is unclear and appears to be subsidiary, because strong antibody responses are detected in all immunocompetent chronic HCV carriers. It is also unknown whether anti-HCV antibodies serve to control the level of viremia during chronic infection and whether they ameliorate horizontal or vertical transmission.

An enzyme-linked immunosorbent spot (ELISpot) assay for detecting individual B cells secreting specific antibodies has enabled investigators to study B-cell immunity at a cellular level in a variety of clinical applications.[12-15] The advantages of the ELISpot assay are that it can detect even a single cell out of 10^6 peripheral blood mononuclear cells (PBMCs), whose secretion level may not be sufficient for detection of circulating antibody, and distinguishes and quantifies only active immunoglobulin-secreting

Abbreviations: ELISpot, enzyme-linked immunosorbent spot; HCV, hepatitis C virus; SFC, spot-forming cell; PBMC, peripheral blood mononuclear cell; Ig, immunoglobulin; ALT, alanine aminotransferase; AST, aspartate aminotransferase; PBS, phosphate-buffered saline; ROC, receiver-operating characteristics; AUC, area under the curve; IQR, interquartile range.

From the [1]Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, MD; and [2]Department of Medicine, Shinshu University School of Medicine, Matsumoto, Japan.

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Address reprint requests to: Harvey J. Alter, M.D., M.A.C.P., Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, Building 10, Room 1C711, National Institutes of Health, Bethesda, MD 20892-1184. E-mail: halter@cc.nih.gov; fax: 301-402-2965.

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cells. This assay thus provides a useful tool for better understanding immunity to infectious diseases and improved analysis of the immune response to vaccination.[14] Although studies of antigen-specific antibody-secreting cells in various viral infections have been conducted,[15-19] there are no published data on detection and quantification of anti-HCV antibody–secreting B cells.

The objective of this study was to adapt the ELISpot assay for the detection of anti-HCV antibody–secreting B cells to (1) clarify the HCV-specific humoral immune responses in patients with chronic hepatitis C, (2) examine the correlation between the numbers of anti-HCV antibody–secreting B cells and clinical outcomes, and (3) examine humoral immune responses in patients with chronic hepatitis C compared with those who spontaneously clear HCV.

Patients and Methods

Subjects. Individuals who were identified by the Greater Chesapeake and Potomac Region of the American Red Cross as being positive for anti-HCV via enzyme immunoassay at the time of blood donation were referred to the Department of Transfusion Medicine at the National Institutes of Health for participation in a long-term study of the natural history of HCV infection[20,21]; 750 participants were enrolled from 1990 through September 2003. Of these, 48 subjects were selected randomly to assess humoral immune responses at the B-cell level. The chronic hepatitis C group included 39 subjects who were positive for anti-HCV antibodies (EIA-2 and RIBA-3) and positive for HCV RNA. The recovered group included 9 anti-HCV–positive subjects who were HCV RNA–negative via qualitative polymerase chain reaction on at least two consecutive visits. The patients' characteristics are summarized in Table 1. Eleven volunteer blood donors without a history of HCV infection served as controls. All subjects were negative for hepatitis B surface antigen and antibodies to the human immunodeficiency virus. The study protocols were reviewed and approved by the appropriate institutional review boards, and all subjects gave written informed consent to participate in the study.

Laboratory Testing. Antibodies to HCV were measured in serum samples via second-generation enzyme immunoassay (EIA-2; Abbott Laboratories, North Chicago, IL). EIA-2 reactive samples were subsequently tested via third-generation recombinant immunoblot assay (RIBA-3; Chiron Corp., Emeryville, CA). Reactivity to at least two of four HCV antigens (5-1-1/C100-3, C33, C22, and NS5) was considered a positive RIBA-3 result, no reactivity was considered a negative result, and reactivity to only one antigen was considered an indeterminate result. The serum levels of HCV RNA were determined using the qualitative and quantitative COBAS AMPLICOR assays (Roche Diagnostic Systems, Branchburg, NJ), which amplify HCV RNA via reverse-transcription polymerase chain reaction. HCV genotypes were determined using INNO-LiPA HCV II (Innogenetics, Gent, Belgium). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), and other relevant biochemical tests were performed using standard methods.

Table 1. Demographic and Clinical Characteristics of Patients With HCV Infection

Characteristics	All (N = 48)	Chronic (n = 39)	Recovered (n = 9)	*P* Value
Mean age, yrs (range)	51 (33-83)	52 (37-83)	49 (33-78)	.46
Male, n (%)	23 (48)	17 (44)	6 (67)	.28
Race, n (%)				
White	43 (90)	35 (90)	8 (89)	1.00
Black	5 (10)	4 (10)	1 (11)	
Source of infection, n (%)				
Transfusion	16 (33)	14 (36)	2 (22)	.30
Injection drug use	19 (40)	15 (38)	4 (44)	
Nasal cocaine use	4 (8)	2 (5)	2 (22)	
Occupational	6 (13)	6 (15)	0 (0)	
Unknown	3 (6)	2 (5)	1 (11)	
Genotype, n (%)				
1	25 (52)	24 (62)	1 (11)	.074
2	7 (15)	6 (15)	1 (11)	
3	2 (4)	1 (3)	1 (11)	
Unknown	14 (29)	8 (21)	6 (67)	
Mean values (range)				
ALT (IU/L)	52 (15-251)	58 (28-251)	25 (15-52)	.001
AST (IU/L)	43 (12-145)	48 (12-145)	24 (13-37)	.001
ALP (IU/L)	69 (32-171)	71 (35-171)	59 (32-74)	.20
Total bilirubin (mg/dL)	0.7 (0.3-1.5)	0.7 (0.3-1.5)	0.7 (0.4-1.4)	.72
Albumin (g/dL)	3.9 (3.3-4.5)	3.9 (3.3-4.5)	4.0 (3.6-4.3)	.53
GGTP (g/dL)	44 (8-286)	48 (8-286)	27 (8-102)	.025
HCV RNA level (10^5 IU/mL)	11.2 (<0.5-73)	14.1 (<0.5-73)	ND	<.001
Recombinant strip immunoblot assay				
C100	3.0 (0-4)	3.2 (0-4)	2.1 (0-4)	.042
C33	3.5 (1-4)	3.7 (1-4)	2.6 (1-4)	.011
C22	3.8 (0-4)	3.9 (1-4)	3.1 (0-4)	.068
NS5	2.1 (0-4)	2.3 (0-4)	1.3 (0-4)	.18

Abbreviations: ALP, alkaline phosphatase; GGTP, γ-glutamyltransferase; ND, below the limits of detection.

PBMCs. PBMCs were isolated from whole blood using cellular preparation tubes (Becton Dickinson, Franklin Lakes, NJ), washed one time in phosphate-buffered saline (PBS) and three times in medium (RPMI 1640 medium supplemented with 2 mmol/L L-glutamine, 5×10^{-5} mol/L 2 mercaptoethanol, 50 U/mL penicillin, 50 μg/mL streptomycin, and 10% fetal bovine serum), and were either studied immediately or cryopreserved in media containing 50% fetal bovine serum, 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO), and 10% RPMI 1640.

HCV Proteins. The recombinant full-length HCV core protein (amino acid residues 1-191), E2 protein

(amino acid residues 384-746), NS3 protein (amino acid residues 1027-1657), and NS5B protein (amino acid residues 2421-3011) were expressed and purified from *Escherichia coli* using the expression vector as previously described.[22,23] Control proteins were expressed as carboxy-terminal fusion proteins with human superoxide dismutase in *E. coli*.

ELISpot Assay. Ninety-six–well plates containing high-protein binding membranes (MAIP S4510; Millipore Co., Bedford, MA) were coated with a 10-μg/mL purified recombinant HCV core, E2, NS3, NS5b, or control antigens in carbonate coating buffer (0.1 mol/L Na_2CO_3, 0.1 mol/L $NaHCO_3$; pH 9.6). After incubation at 4°C overnight, the plates were washed twice with PBS and blocked with 3% bovine serum albumin for more than 30 minutes at 37°C. Cryopreserved PBMCs were thawed and incubated for 44 hours at 37°C in a humidified atmosphere of 5% CO_2 at 1.25×10^5 or 2.5×10^5 cells/well in AIM V Media (Invitrogen, Carlsbad, CA). All determinations were run in triplicate. After incubation, the cells were removed by washing 6 times with PBS containing 0.05% NP-40, and the plates incubated with horseradish peroxidase-linked anti-human IgG or IgM antibodies (1:1,000; KPL, Gaithersburg, MD) at 37°C for 2 hours. After the plates were washed twice with PBS and 6 times with PBS containing 0.05% NP-40, an optimal 4CN peroxidase substrate (Bio-Rad, Hercules, CA) was added and incubated for 20 to 30 minutes at room temperature to develop the spots. The reaction was stopped by washing with distilled water. The plates were dried overnight, and the spots were counted automatically by an ELISPOT reader (Carl Zeiss Vision, Hallbergmoos, Germany). The frequencies of anti-HCV antibody–secreting B cells were calculated by subtracting the mean number of spots in the control wells from the HCV antigen-coated wells, and expressed as the mean of triplicates of spot-forming cells (SFCs) per 10^6 PBMCs. Assays with a high background (>5 spots/well in the negative control) were excluded.

Assay of Anti-HCV/NS3 Antibodies. Anti-HCV/NS3 IgG was assayed via ELISA as described previously.[23] Briefly, MaxiSorp Nunc-Immuno plates were coated with recombinant HCV NS3 protein at 6 μg/mL in coating buffer (20 mmol/L sodium bicarbonate buffer [pH 9.6], 0.15 mol/L NaCl) and overcoated with 0.1% bovine serum albumin in PBS buffer (pH 7.4). The sera were tested via two-fold serial dilution in 0.3% IGEPAL CA-630 (Sigma), 5% milk diluent (Kirkegaard & Perry Laboratories, Gaithersburg, MD), and PBS [pH 7.4], with initial dilution at 1:250. Biotinylated anti-human IgGγ (Kirkegaard & Perry Laboratories) and strepavidin–horseradish peroxidase (Kirkegaard & Perry Laboratories) were added sequentially. One hundred microliters per well ABTS microwell peroxidase substrate was used to develop the color and 100 μL per well peroxidase stop solution (Kirkegaard & Perry Laboratories) was added to stop the reaction. Absorbance was read at 405 nm. The IgG titer was determined via end point dilution.

Statistical Analysis. The Mann-Whitney *U* test or Student *t* test was used to analyze continuous variables as appropriate. Spearman's rank order correlations were used to evaluate the frequencies of anti-HCV antibody–secreting B cells to each antigen and to the clinical features. A *P* value of .05 or less was considered significant. Although SFCs/10^6 PBMCs were expressed in this study, the statistics were significant whether this was used or the raw counts were used. Statistical analyses were performed using SigmaStat (version 2.03; SPSS, Chicago, IL). Receiver-operating characteristic (ROC) curve analysis was performed using MedCalc 7.0 software (http://www.medcalc.be). The best cutoff values of the ELISpot assays were chosen automatically by MedCalc 7.0 as the SFCs with the highest diagnostic accuracy (*i.e.*, the sum of the false-negative and false-positive rates was minimized). The respective overall diagnostic values were expressed using the area under the curve (AUC).

Results

Optimal Cutoff Values for ELISPOT Assay. To determine the optimal cutoff values for the B-cell ELISpot assay in differentiating patients with HCV infection from HCV seronegative blood donors, ROC curve analysis was performed. The ROC curves for the ELISpot assay detecting anti-HCV IgG-specific B cells were obtained via calculations made using the values obtained from 48 patients with HCV infection and the 11 HCV-negative volunteer blood donors. The selection of the optimal cutoff point value was based on the level at which the accuracy was maximum (see Patients and Methods). The optimal cutoff values, sensitivity, specificity, positive predictive values, negative predictive values, and calculated AUCs to all HCV antigens are listed in Table 2. In our ELISpot assay, the values of sensitivity ranged from 58% to 92%, and the values of specificity were 100%. The AUC results were constantly high in the ELISpot assays for all antigens, and AUC values were between 0.71 (NS5B antigen) and 0.94 (core and E2 antigens).

After we defined the optimal cutoff value for each antigen, we determined the frequencies of anti-HCV IgG-secreting B cells in 48 patients with HCV infection. The prevalence of anti-HCV IgG-secreting B cells during HCV infection specific for the various antigens were:

Table 2. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgG-Secreting B Cells in ELISpot Assay in 48 Patients With Chronic Hepatitis C and 11 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	13.4	92 (80-98)	100 (71-100)	0.94 (0.84-0.98)	100	73
E2	10.7	92 (80-98)	100 (71-100)	0.94 (0.85-0.99)	100	73
NS3	5.4	77 (63-88)	100 (71-100)	0.83 (0.71-0.92)	100	50
NS5B	5.4	58 (43-72)	100 (71-100)	0.71 (0.58-0.82)	100	36

NOTE. All AUC values were significantly higher than a 0.50 nonpredictive value ($P < .001$ for all comparisons).
Cutoff values were determined by making ROC curves and are expressed as SFCs/10^6 PBMCs.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

core, 92%; E2, 92%; NS3, 77%; and NS5B, 58% (Table 2).

We further assessed the optimal cutoff values for the ELISPOT assay detecting anti-HCV IgM-secreting B cells using ROC curve analysis in 43 patients with HCV infection and in 6 HCV-negative blood donors (Table 3). The AUC values ranged from 0.73 (NS5B antigen) to 0.94 (core antigen). The prevalence of anti-HCV IgM-secreting B cells ranged from 54% (NS5B antigen) to 84% (core antigen) (Table 3).

Detection and Quantitation of Anti-HCV Antibody–Secreting B Cells. Forty-eight PBMC samples obtained from patients with HCV infection and 11 samples from healthy volunteer blood donors were examined for detection of anti-HCV IgG-secreting B cells. The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (38.3 SFCs/10^6 PBMCs; interquartile range [IQR], 10.7-149.3) compared with control anti-HCV negative donors (5.3 SFCs/10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). Figure 1A shows box plots for the numbers of anti-HCV IgG-secreting B cells to all 4 HCV antigens in patients with HCV infection and in the controls. Among 48 patients with HCV infection, the median numbers of anti-HCV IgG-secreting B cells ranged from 10.7 SFCs/10^6 PBMCs (NS5B antigen) to 119.0 SFCs/10^6 PBMCs (E2 antigen). The median numbers of anti-HCV IgG-secreting B cells in patients with HCV infection were significantly higher than those in controls for each HCV antigen (Fig. 1A).

Subsequently, we developed an ELISpot assay for detecting anti-HCV IgM-secreting B cells. Detection of the anti-HCV IgM-secreting B cells was performed in 43 patients with HCV infection and in 6 anti-HCV negative blood donors (Fig. 1B). The median numbers of the sum of anti-HCV IgM-secreting B cells to all HCV antigens were significantly higher in patients with HCV infection (21.3 SFCs/10^6 PBMCs; IQR, 9.2-48.0) compared with the controls (8.0 SFCs/10^6 PBMCs; IQR, 0.0-10.7; $P < .001$). The median numbers of anti-HCV IgM-secreting B SFC to the core (31.1 vs. 4.0 SFCs/10^6 PBMCs; $P < .001$) and E2 (32.0 vs. 8.0 SFCs/10^6 PBMCs; $P = .005$) antigens in patients with HCV infection were significantly higher than those in controls. (Fig. 1B).

Relationship Between Anti-HCV Antibody–Specific B Cells and HCV Genotypes. Because the antigens used were derived from HCV genotype 1a, the numbers of anti-HCV IgG-secreting B cells were compared between 25 patients with HCV genotype 1 infection (10 with 1a, 11 with 1b, and 4 not subtyped) and 9 infected with another single genotype (1 with 2a, 4 with 2b, 2 with 2 unsubtyped, and 2 with 3a). The median value of the anti-HCV IgG-secreting B cells to each antigen was not statistically different between the genotype 1 group and the other genotype groups (Fig. 2). In addition, there were no statistically significant differences in detecting anti-HCV IgM-secreting B cells to all HCV antigens in those with genotype 1 versus non-1 infections (data not shown).

Table 3. Optimal Cutoff Values, Sensitivity, Specificity, AUC, and Predictive Values of Anti-HCV IgM-Secreting B Cells in ELISpot Assay in 43 Patients With Chronic Hepatitis C and 6 Volunteer Blood Donors

Antigen	Cutoff Value	Sensitivity, % (95% CI)	Specificity, % (95% CI)	AUC (95% CI)	PPV, %	NPV, %
Core	12.1	84 (69-93)	100 (54-100)	0.94 (0.84-0.99)	100	46
E2	17.4	72 (56-85)	100 (54-100)	0.86 (0.73-0.94)	100	33
NS3	10.7	70 (54-83)	100 (54-100)	0.74 (0.60-0.86)	100	32
NS5B	8.1	54 (38-69)	100 (54-100)	0.73 (0.58-0.85)	100	23

NOTE. All AUC values were significantly higher than a 0.500 nonpredictive value ($P < .001$ for all comparisons). Cutoff values were determined by making ROC curves and are expressed as SFCs/10^6 PBMCs.
Abbreviations: PPV, positive predictive value; NPV, negative predictive value.

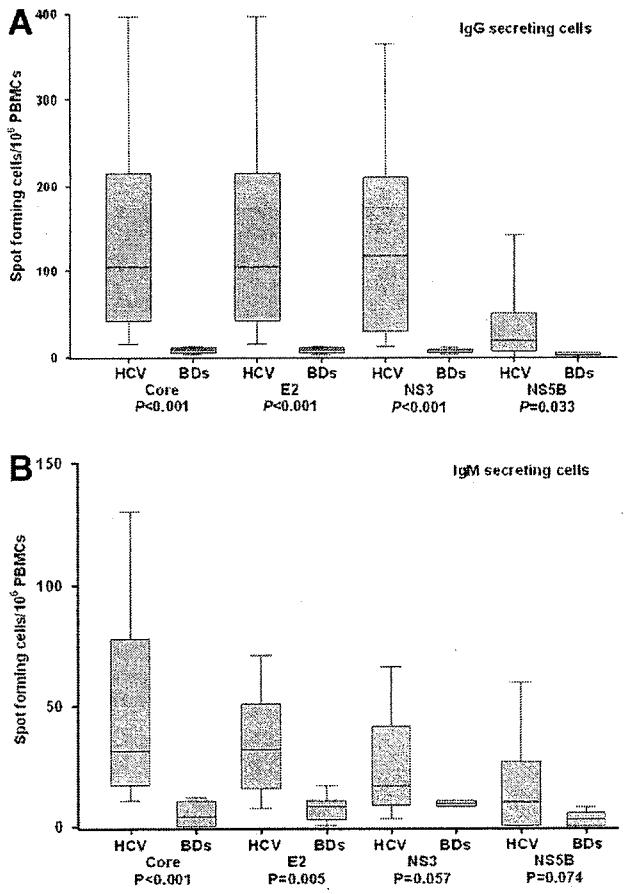

Fig. 1. Detection of anti-HCV antibody-secreting B cells in patients with HCV infection and volunteer blood donors. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. (A) The frequencies of anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 48 patients with HCV infection and in 11 volunteer blood donors. (B) The frequencies of anti-HCV IgM-secreting B cells were detected in 43 patients with HCV infection and in 6 volunteer blood donors. PBMCs, peripheral blood mononuclear cells; IgG, immunoglobulin G; HCV, hepatitis C virus; BDs, blood donors; IgM, immunoblobulin M.

Correlation Between Anti-HCV IgG-Secreting B Cells and Clinical Features in Patients With HCV Infection. Several demographic (age and sex) and clinical (viral load, genotype, ALT, AST, alkaline phosphatase, total bilirubin, albumin, γ-glutamyltransferase, intensity of RIBA assay, and anti-HCV antibodies) findings were examined for their correlation with anti-HCV IgG-secreting B-cell frequency in patients with HCV infection. The circulating anti-HCV IgG-secreting B-cell frequency to the core antigen (Fig. 3A) was significantly correlated with the value of ALT ($P = .048$, $r = 0.29$) and inversely correlated with serum albumin ($P = .048$, $r = -0.33$). Similarly, the number of anti-HCV IgG-secreting B cells to the E2 antigen was significantly correlated with the value of ALT ($P = .037$, $r = 0.30$) (Fig. 3B) and AST ($P = .033$, $r = 0.31$) (Fig. 3C) and was inversely correlated with serum albumin ($P = .029$, $r = -0.36$). Furthermore, the number of SFCs to the NS3 antigen was significantly correlated with the circulating antibody level to the NS3 antigen in 38 patients with available serum samples ($P = .008$, $r = 0.43$) (Fig. 3D). There was no significant correlation between the numbers of anti-HCV IgG-secreting B cells to NS3 or NS5b antigens and any of the biochemical, demographic, or clinical parameters specified above.

Comparison of the Number of Anti-HCV Antibody-Secreting B Cells Between Patients With Chronic Hepatitis C and Patients Who Recovered. As shown in Table 1, patients with chronic hepatitis C had significantly higher mean serum levels of ALT (58 vs. 25 IU/L; $P = .001$), AST (48 vs. 24 IU/L; $P = .001$), and γglutamyltransferase (48 vs. 27 IU/L; $P = .025$) compared with the recovered patients. The mean HCV RNA level in the chronic group was 14.1×10^5 IU/mL. There were significant differences in the mean intensity of the RIBA assay against the C33 and C100 proteins in chronic vs. recovered subjects (C33, 3.2 vs. 2.1, $P = .042$; C100, 3.7 vs. 2.6, $P = .011$). We found no significant difference between patients with chronic hepatitis C and patients who had recovered when their age, sex, race, source of infection, HCV genotypes, total bilirubin, or albumin were compared.

The median numbers of the sum of anti-HCV IgG-secreting B cells to all HCV antigens were significantly higher in patients with chronic hepatitis C (47.3 SFCs/

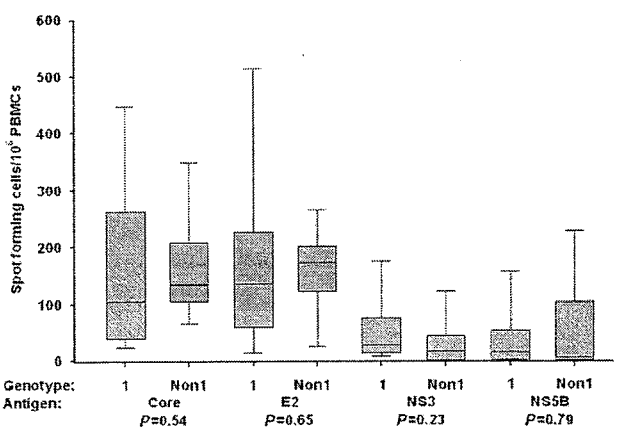

Fig. 2. Detection of anti-HCV IgG-secreting B cells in patients infected with HCV of genotype 1 and nongenotype 1. Boxes represent the IQR of the data. The lines across the boxes indicate the median values. The hash marks above and below the boxes indicate the 90th and 10th percentiles for each group, respectively. The frequencies of anti-HCV-secreting B cells were detected in patients infected with genotype 1 (n = 25) and in those with other genotypes (n = 9). PBMCs, peripheral blood mononuclear cells; Non1, nongenotype 1.

10^6 PBMCs; IQR, 13.3-149.7) than in recovered patients (15.3 SFCs/10^6 PBMCs; IQR, 3.3-142.7; $P = .05$) and normal controls (5.3 SFCs/10^6 PBMCs; IQR, 2.7-8.0; $P < .001$). The median numbers of the sum of anti-IgG–secreting B cells to structural antigens were not significantly higher in patients with chronic hepatitis C (108.3 SFCs/10^6 PBMCs) than in those who recovered (97.4 SFCs/10^6 PBMCs) (Fig. 4A). In contrast, the median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C (19.0 SFCs/10^6 PBMCs) than in patients who recovered (4.9 SFCs/10^6 PBMCs; $P = .018$), particularly for NS3 antigen (26.7 vs. 5.3 SFCs/10^6 PBMCs; $P = .032$) (Fig. 4B). Furthermore, patients with chronic hepatitis C had a significantly higher frequency of anti-HCV IgG-secreting B cells to the NS3 antigen than those who recovered (85% vs. 44%; $P = .02$) (Fig. 4C).

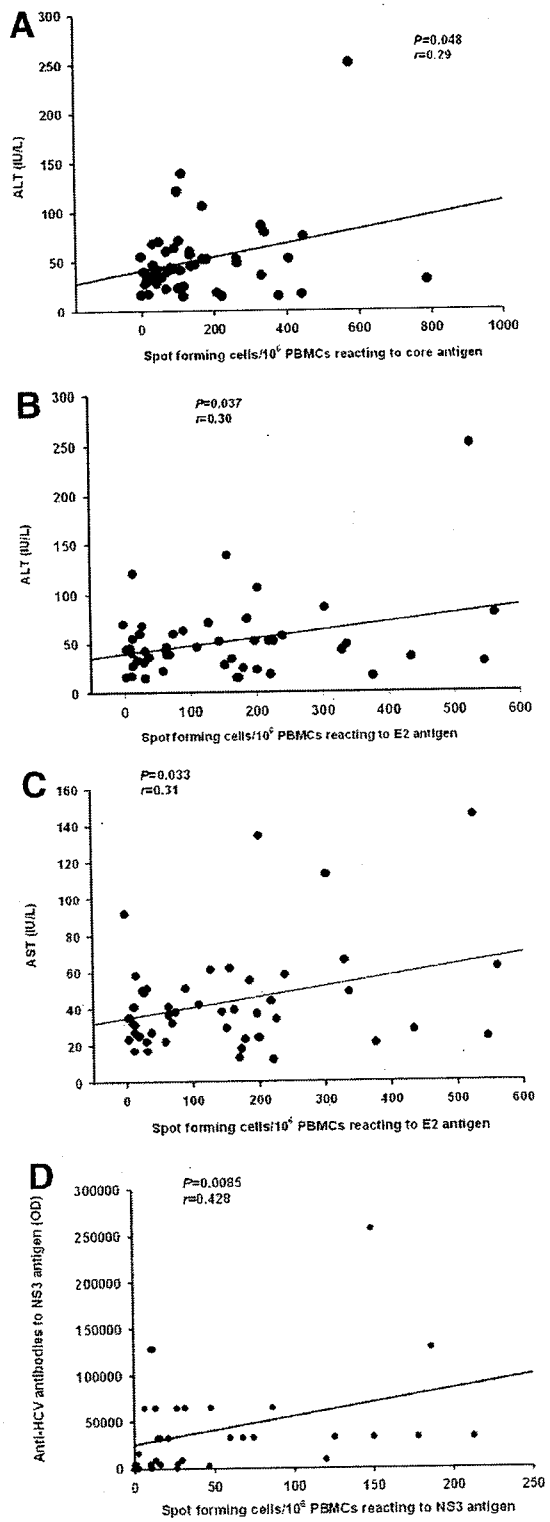

Fig. 3. Correlation of the number of anti-HCV IgG-secreting B cells and clinical characteristics in 48 patients with HCV infection. (A) Frequency of circulating anti-HCV IgG-secreting B cells to core antigen was significantly correlated with the value of ALT ($r = 0.29$, $P = .048$). (B-C) Frequency of circulating anti-HCV IgG-secreting to E2 antigen was correlated with the value of (B) ALT ($r = 0.30$, $P = .037$) and (C) AST ($r = 0.31$, $P = .033$), respectively. (D) Frequency of circulating anti-IgG–secreting B cells to NS3 antigen was correlated with the value of anti-HCV antibodies to NS3 antigen ($r = 0.43$, $P = .0085$). ALT, alanine aminotransferase; PBMCs, peripheral blood mononuclear cells; AST, aspartate aminotransferase; HCV, hepatitis C virus.

Anti-HCV IgM-secreting B cells to all HCV antigens were similar in patients with chronic hepatitis C (22.0 SFCs/10^6 PBMCs; IQR, 8.2-49.3) and recovered patients (20.7 SFCs/10^6 PBMCs; IQR, 12.2-36.7) and were significantly higher than in the controls (8.0 SFCs/10^6 PBMCs; IQR, 0.0-10.7; $P < .001$) (Fig. 4A). When the responses were analyzed for structural and nonstructural antigens, the median numbers of the sum of anti-HCV IgM-secreting B cells were not significantly different in patients with chronic hepatitis C and recovered subjects for either structural antigens (30.7 vs. 31.6 SFCs/10^6 PBMCs) or nonstructural antigens (20.7 vs. 12.7 SFCs/10^6 PBMCs) (Fig. 4A).

Discussion

We developed an ELISpot assay for sensitive quantitative assessment of anti-HCV antibody-secreting B cells in PBMCs from patients with HCV infection and used this technique to analyze the induction of humoral immune responses at the single-cell level. IgG and IgM anti-HCV antibody secreting B cells to core, E2, NS3, and NS5 were detected and quantified in patients with chronic HCV infection and compared with recovered patients and uninfected controls. The key findings were: (1) anti-HCV secreting B-cell responses were greater in chronically infected patients than in recovered patients, suggesting that antibody does not play a major role in recovery from acute

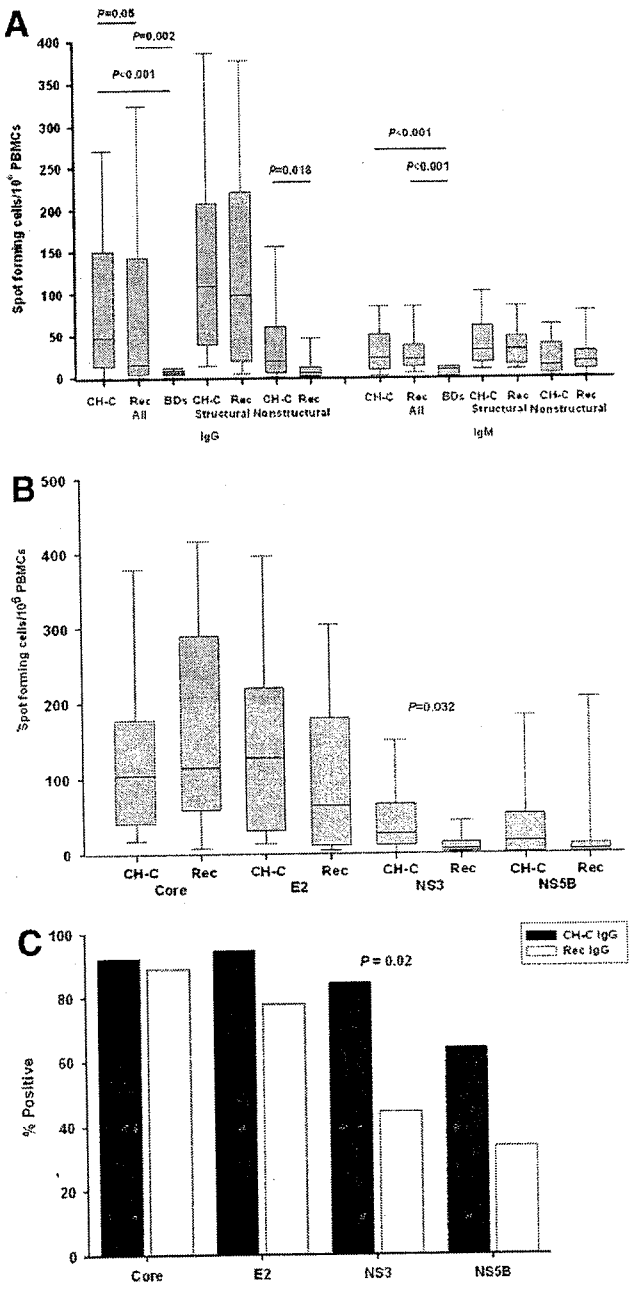

Fig. 4. Detection of anti-HCV antibody-secreting B cells in patients with chronic hepatitis C and in patients who had recovered from HCV. (A) Circulating anti-HCV IgG-secreting B cells were detected in 39 patients with chronic hepatitis C, 9 patients who had recovered from HCV infection, and 11 volunteer blood donors. Circulating anti-HCV IgM-secreting B cells were detected in 34 patients with chronic hepatitis C, 9 patinets who had recovered from HCV infection, and 6 volunteer blood donors. (B) Frequency of circulating anti-HCV IgG-secreting B cells to 4 HCV antigens were detected in 39 patients with chronic hepatitis C and in 9 recovered patients. (C) The prevalence of anti-HCV IgG-secreting B cells in 39 patients with chronic hepatitis C and 9 recovered patients. PBMCs, peripheral blood mononuclear cells; CH-C, chronic hepatitis C; BDs, blood donors; Rec, recovered; IgG, immunoglobulin G; IgM, immunoblobulin M.

HCV infection, as also indicated by recently developed pseudotype assays for HCV-neutralizing antibodies[24,25]; (2) the primary difference between chronically infected and recovered subjects was in the greater reactivity of the former to nonstructural antigens; (3) in chronic infection, HCV antibodies were cross-reactive against genotypes, again consistent with recent findings by neutralizing antibody assays[26,27]; (4) the ELISpot assay can measure IgM as well as IgG responses at the single-cell level, providing a new means to measure the more elusive IgM response; (5) IgM responses were surprisingly well maintained during chronic infection; and (6) IgG responses correlated positively with serum transaminase levels.

In this study, the B-cell ELISpot assay showed high specificity (91% to 100%) and sensitivity (58% to 92%) to all HCV antigens through analysis of the ROC curves and thus achieved high diagnostic accuracy. Although there was a general problem that raw numbers of SFCs were low, statistical analysis and prior publications[28,29] suggest that these small differences are consistent and relevant. Of note, individuals infected with nongenotype 1 variants were strongly positive in this assay, which used only genotype 1–derived antigens. This suggests that genotype 1 contains conserved epitopes that will allow the ELISpot assay to assess humoral immune responses to HCV irrespective of genotype (Fig. 2), with the caveat that we did not assess genotypes 4, 5, and 6, all of which are rare in the United States.

ELISpot assay has been used as a sensitive and specific tool to measure B-cell responses in autoimmune diseases[28,29] and viral infections such as cytomegalovirus,[15] rotavirus,[16] measles virus,[17] and hepatitis B virus,[18,19] as well as to evaluate responses to bacterial[30] and viral vaccines.[18,19] Other reports demonstrate that ELISpot is able to detect and numerate antigen-specific memory B cells in PBMCs after *in vitro* stimulation in both autoimmune diseases and viral infection.[31,32] Thus, the B-cell ELISPOT assay might be a useful tool to detect anti-HCV-specific memory B cells, and to monitor the efficacy of future HCV vaccines.

Interestingly, this study showed a strong correlation between the numbers of anti-HCV IgG-secreting B cells to the core and E2 antigens and the values of serum transaminases. The clinical significance of these observations is unknown, but raises the possibility that antibodies can contribute to liver cell injury. In addition, Ni et al.[33] recently reported that 10 of 36 hepatitis C patient samples showed increased B-cell frequencies that correlated with the degree of hepatic fibrosis. There are insufficient histological data in our study to assess whether the numbers of anti-HCV antibody–secreting B cells correlate with the degree of fibrosis as well as biochemical evidence of inflammation.

The median numbers of the sum of anti-HCV IgG-secreting B cells to nonstructural antigens were significantly higher in patients with chronic hepatitis C than in recovered patients. Similarly, an HCV-specific B-cell response was more frequently detected in patients with chronic hepatitis C than in recovered subjects (92% vs. 56%; $P = .017$) and was directed against a broader range of HCV antigens, particularly to NS3. In contrast, CD4 T-cell responses to NS3 epitopes are greatest in patients who recover from HCV infection.[34,35]

We have also developed and evaluated the ELISpot assay for detecting anti-HCV IgM-secreting B cells. It has been reported that IgM anti-HCV in serum might be predictive of viral clearance in acute hepatitis C or response to interferon therapy.[36-40] However, these results have been controversial and other studies have shown a significant correlation between IgM anti-HCV levels in serum and the recurrence of hepatitis C after liver transplantation.[41,42] In this study, we found that IgM-secreting B cells persisted during chronic infection so that the usefulness of IgM detection for assessing acute versus chronic HCV infection would have to depend on quantitative differences in IgM level rather than the simple presence or absence of IgM antibody. The fact that there are no standardized assays for measuring IgM anti-HCV in serum and the ready detection of IgM-secreting B cells in this study suggests that the ELISpot assay could be used to better define the clinical relevance of IgM antibody in acute and chronic HCV infection.

Overall, this study, as do studies of HCV-specific neutralizing antibodies,[26,27] suggest that the humoral arm of the HCV immune response is not a critical element of spontaneous viral clearance. However, because of the difficulty in obtaining serial acute-phase PBMC collections from recovering subjects, our study does not exclude a role for antibody-mediated viral clearance early in HCV infection. Sequential acute phase ELISpot IgM testing of PBMCs is planned in forthcoming chimpanzee infectivity studies. Nonetheless, studies of neutralizing and antienvelope antibodies that measured serial acute phase serum samples from recovering subjects[26,27] did not show that such antibodies correlated with viral clearance. Rather, it appears in those studies and the current study that antibodies to HCV increase in strength and broadness of reactivity during the course of chronic infection, presumably because of persistent antigenic stimulation. This is in contrast to cell-mediated immunity that is markedly diminished in chronically infected compared with recovered subjects. This dichotomy between the humoral and cellular immune response to HCV is intriguing and suggests T-cell tolerance in the absence of B- cell tolerance.

It is interesting to speculate on the role that antibodies might play in HCV infection. First, it seems reasonable that such antibodies complexed to virus would reduce the level of free virus and diminish transmission to others. This reduction in free virus in addition to lowered viral load might explain the relative rarity of sexual and perinatal transmission during chronic HCV infection. More intriguing is whether such antibodies establish the set point for viral load during chronic infection. It is known that viral loads are highest early in HCV infection prior to the appearance of antibody[43] and that chronically infected patients establish a lower and relatively constant level of viremia.[44] It appears that production and elimination of virus achieve a steady state. This steady state is probably multifactorial in origin, but antibody may play a key role. When patients in a steady state are immunosuppressed at the time of transplantation[45] or when coinfected with human immunodeficiency virus,[46] the viral load increases, supporting an immunological role for viral containment even in the absence of clearance. A deleterious function of anti-HCV is that it serves to drive quasispecies evolution making it increasingly hard for the immune system to achieve viral clearance. Farci et al.[47] have shown in both humans and chimpanzees that the appearance of antibody coincides with increasing viral diversity and complexity and predicts progression to chronic infection.

In conclusion, there is much to explore regarding the function and relevance of IgG and IgM antibodies in HCV infection, and we believe the ELISpot assay, by measuring antibody production at the single-cell level, provides a new and useful tool for understanding the complex interplay between HCV and the host immune response.

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Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy

Akinori Rokuhara[1], Akihiro Matsumoto[1], Eiji Tanaka[1], Takeji Umemura[1], Kaname Yoshizawa[1], Tatsuji Kimura[2], Noboru Maki[2], and Kendo Kiyosawa[1]

[1] Department of Medicine, Shinshu University School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan
[2] Advanced Life Science Institute Inc., Wako, Japan

Background. Changes in the serum hepatitis B virus (HBV) RNA level during lamivudine therapy were compared to those in the serum HBV DNA and HBV core-related antigen (HBVcrAg) levels in 24 patients with chronic hepatitis B. ***Methods.*** For measurement of HBV RNA, total nucleic acid was extracted from serum samples and treated with RNase-free DNase I. After cDNA synthesis from extracted RNA, HBV RNA was measured by real-time detection polymerase chain reaction. ***Results.*** The peak fraction of HBV RNA in serum samples was consistent with peak fractions of HBV DNA and HBV core protein in a sucrose gradient analysis, indicating that HBV RNA was incorporated into virus particles. All levels of HBV DNA, HBV RNA, and HBVcrAg decreased gradually during lamivudine therapy ($P < 0.001$ for all). The amount of decrease from the start of lamivudine therapy was significantly higher for HBV DNA than for HBV RNA or HBVcrAg during 6 months of lamivudine therapy ($P < 0.001$ for all). However, a similar difference was not seen between HBV RNA and HBVcrAg levels during that period. The HBV RNA level was significantly correlated ($P < 0.001$ for all) with levels of HBV DNA and HBVcrAg both at the beginning and 2 months after the start of lamivudine therapy. ***Conclusions.*** HBV RNA is detectable in serum in a form indicating incorporation into virus particles, and its serum level might serve as a new viral marker with a significance different from that of HBV DNA in lamivudine therapy.

Key words: chronic hepatitis B, viral load, viral replication, cccDNA, sucrose gradient analysis

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Reprint requests to: E. Tanaka

Introduction

Approximately 350 million people are chronic carriers of hepatitis B virus (HBV) worldwide.[1] In some countries, hepatocellular carcinoma and cirrhosis account for more than 50% of all deaths among HBV carriers.[2] Treatment of patients with hepatitis B has improved remarkably with the advent of oral nucleoside analogs such as lamivudine.[3,4] Lamivudine administration has been shown to cause a rapid decrease in the serum HBV DNA level followed by a decrease in the alanine aminotransferase level and improvement of the liver histology.[5–8] Therefore, measurement of serum HBV DNA is widely used in the clinical setting to monitor the effect of lamivudine.

It has been postulated that measurement of the HBV covalently closed circular (ccc) DNA level in hepatocytes is valuable in a different way than serum HBV DNA for monitoring the effects of antiviral therapy, because cccDNA is a key molecule in HBV replication.[9–12] In practice, the intrahepatic HBV cccDNA level has been reported to be superior to serum HBV DNA for predicting a sustained virologic response to antiviral therapy, including lamivudine.[13] However, the measurement of cccDNA seems ill-suited for clinical use because it requires a liver biopsy. Thus, serum markers that reflect the cccDNA level in the liver are desired.

Recently, an HBV core-related antigen (HBVcrAg) assay developed by our laboratory has been shown to possibly correlate with the cccDNA level, especially during lamivudine therapy.[14–16] This possibility is based on the fact that transcription of messenger RNA from cccDNA and subsequent translation of viral proteins are not inhibited by nucleoside analogs such as lamivudine. The same has been said for synthesis of pregenomic RNA.[7] Therefore, in the present study, we measured serum HBV RNA and analyzed its virologic characteristics. In addition, changes in the serum HBV

RNA level during lamivudine therapy were compared with those of serum HBV DNA and HBVcrAg levels to clarify whether HBV RNA measurement in serum has any clinical significance.

Patients and methods

Patients

A total of 24 patients with chronic hepatitis B consented to participate in the present study. They were selected from a pool of 32 consecutive patients who underwent lamivudine therapy at Shinshu University Hospital between July 2002 and June 2003. The patients comprised 18 men and 6 women, and their median age was 55 years (range, 39–79 years). Chronic hepatitis B was defined as positive HBV surface antigen for more than 6 months with liver histological findings consistent with chronic hepatitis. All patients had had elevated levels of serum alanine aminotransferase and HBV DNA for at least 6 months. Immediately prior to lamivudine administration, 16 patients were positive for HBV e antigen and 8 were positive for HBV e antibody but negative for HBV e antigen. The HBV genotype was C in all patients. Patients received 100-mg doses of lamivudine daily for at least 6 months. No patient was treated with other antiviral agents, such as interferon, before or during the present study, and all patients were negative for hepatitis C virus and human immunodeficiency virus antibodies. This study was approved by the ethics committee of our institution. Written informed consent was obtained from each patient.

Serum samples were collected at the start of lamivudine therapy, and at 2 and 6 months after commencement. Samples were stored frozen at −20°C or below until assayed.

Routine laboratory tests

HBV surface antigen, HBV e antigen, HBV e antibody, hepatitis C virus antibody, and human immunodeficiency virus antibody were measured by commercially available enzyme-linked immunosorbent assay kits (Abbott Japan, Tokyo, Japan). HBV genotypes were determined by the method reported by Mizokami et al.[17] and classified into six major genotypes, A to F.

The serum level of HBV DNA was determined using an Amplicor HBV Monitor kit (Roche Diagnostics, Tokyo, Japan), which has a quantitative range from 2.6 to 7.6 log copies/ml. Sera containing over 7.0 log copies/ml HBV DNA were diluted 10- or 100-fold with normal human serum and retested to obtain the end titer.

HBV core and core-related antigen assay

HBV core antigen (HBVcAg) and HBVcrAg in serum were measured using a chemiluminescence enzyme immunoassay as reported previously.[14,15,18] In brief, 100 µl serum was mixed with a pretreatment solution containing 15% sodium dodecyl sulfate. After incubation at 70°C for 30 min, 50 µl of pretreated serum was added to wells coated with monoclonal antibodies against denatured HBV core and e antigens (HB44, HB61, and HB114) and filled with 100 µl of assay buffer. The mixture was then incubated for 2 h at room temperature. After washing with buffer, either alkaline phosphatase-labeled HB50 monoclonal antibody (specific for denatured HBV core antigen) or a mixture of HB91 and HB110 monoclonal antibodies (against denatured HBV core and e antigens) were added to wells and incubated for 1 h at room temperature. After another washing, CDP-Star with Emerald II (Applied Biosystems, Bedford, MA, USA) was added and plates were incubated for 20 min at room temperature. The relative chemiluminescence intensity was measured, and HBVcAg and HBVcrAg concentrations were read by comparison to a standard curve generated using recombinant pro-hepatitis B e antigen (amino acids −10 to 183 of the precore/core gene product). The concentrations of HBVcAg and HBVcrAg were expressed as units/ml, and the immunoreactivity of recombinant pro-hepatitis B e antigen at 10 fg/ml was defined as 1 unit/ml. The cutoff value of both assays was set at 3 log units/ml.[11] Sera containing over 7 log units/ml of antigen were diluted 10- or 100-fold in normal human serum and measured again to obtain the end titer.

HBV RNA assay

A High Pure Viral Nucleic Acid kit (Roche Diagnostics) was used for isolation of HBV RNA from serum. Briefly, 200 µl of serum was added to 250 µl of freshly prepared working solution (6 M guanidine-HCl; 10 mM urea; 10 mM Tris-HCl, pH 4.4; and 20% vol/vol Triton X-100) supplemented with 20 µg of poly(A) carrier RNA and 900 µg proteinase K. After incubation for 10 min at 72°C, 100 µl of isopropanol was added and the mixture was transferred into a High Pure filter tube combined with a collection tube. The filter tube was centrifuged for 1 min at 3500 g in a standard tabletop centrifuge at room temperature and combined with a new collection tube. The inhibitor removal buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6, in ethanol) was added to the upper reservoir and centrifuged for 1 min at 3500 g. After washing with 250 µl of wash buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5, in ethanol), 80 µl of RNase-free DNase I solution (QIAGEN, Hilden, Germany) was added and incubated to digest HBV DNA for 15 min at room temperature. A volume of

200 µl of wash buffer was added to the filter tube, which was then centrifuged for 15 s at 5000 *g*. After being washed with 450 µl of buffer, the filter was placed in a new collection tube and 50 µl of RNase- and DNase-free water was added to elute the RNA. After centrifugation for 1 min at 3500 *g*, the eluted RNA was stored at −80°C.

Synthesis of cDNA was performed at 42°C for 30 min in a 20-µl reaction mixture containing 10 µl of the extracted RNA; 50 mM Tris-HCl, pH 8.3; 75 mM KCl; 3 mM $MgCl_2$; 1 mM dNTP (1 mM each dATP, dGTP, dCTP, and dTTP); 1 mM dithiothreitol; 100 nM reverse primer for the HBV surface gene (5′-GGTTGG TGAGTGATTGGAGGTT-3′; nt 345 to 324); 40 units of RNasin (TaKaRa, Kyoto, Japan); and 200 units of SuperScript II RNase H—reverse transcriptase (Invitrogen, Carlsbad, CA, USA). The reaction mixture was inactivated by heating to 70°C for 15 min and then cooled to −80°C until the real-time detection polymerase chain reaction (RTD-PCR) assay. A 4-µl aliquot of cDNA solution was used for RTD-PCR, which was performed with a Light Cycler system (Roche Diagnostics) as reported previously.[14] The two primers and TaqMan probe used were designed from a region of the HBV surface gene: forward primer, 5′-ACAACAT CAGGATTCCTAGGAC-3′ (nt 166 to 187); reverse primer as stated above (nt 345 to 324); and TaqMan probe, 5′-FAM-CAGAGTCTAGACTCGTGGTGGA CTTC-TAMRA-3′ (nt 244 to 269). An HBV genome (nt 20 to 1805) that had been subcloned into a pUC vector was used as an internal standard. The lower detection limit for the HBV RNA assay was set at 2.6 log copies/ml. HBV DNA was tested on extracted HBV RNA samples not having undergone the preceding process by RTD-PCR and was confirmed to be negative in all samples.

Sucrose density gradient ultracentrifugation

Serum (0.1 ml) was layered on a linear 10%–60% (wt/wt) sucrose gradient, then centrifuged at 200000 *g* (45000 rpm) for 15 h at 4°C with a Beckman SW50.1 rotor (Beckman Coulter, Fullerton, CA, USA). In total, 24 fractions of 200 µl were collected by micropipette. Each fraction was diluted fivefold and tested for HBV DNA, HBV RNA, and HBVcAg.

Statistical analyses

Statistical analyses with the Mann-Whitney's U test, Friedman's test, and the Spearman rank correlation test were performed using the SPSS 10.0J statistical software package (SPSS, Chicago, IL, USA). A P value of less than 0.05 was considered statistically significant.

Results

Serum levels of HBV DNA ($P < 0.001$), HBV RNA ($P < 0.001$), and HBVcrAg ($P < 0.001$) all decreased significantly throughout the course of lamivudine therapy (Fig. 1). The amount of decrease at 2 months following commencement of lamivudine therapy was significantly higher for HBV DNA than for HBV RNA or HBVcrAg (median, 2.45; 25%–75% range, 1.90–3.00 log copies/ml vs median, 0.40, 25%–75% range, 0.00–0.85 log copies/ml, $P < 0.001$, and median, 0.30, 25%–75% range, 0.10–0.65 log units/ml, $P < 0.001$, respectively). Similarly, the amount of decrease after 6 months of treatment was significantly higher for HBV DNA than for HBV RNA or HBVcrAg (median, 3.20; 25%–75% range, 2.00–4.55 log copies/ml vs median, 0.90; 25%–75% range, 0.45–1.90 log copies/ml, $P < 0.001$, and median, 0.90; 25%–75% range, 0.20–1.55 log units/ml, $P < 0.001$, respectively). The amount of decrease did not differ between HBV RNA and HBVcrAg at either 2 ($P > 0.2$) or 6 ($P > 0.2$) months after commencement.

As shown in Fig. 2, the serum level of HBV RNA was significantly correlated with HBV DNA both at the start of lamivudine therapy ($r = 0.801$, $P < 0.001$) and 2 months afterward ($r = 0.837$, $P < 0.001$). Serum HBV

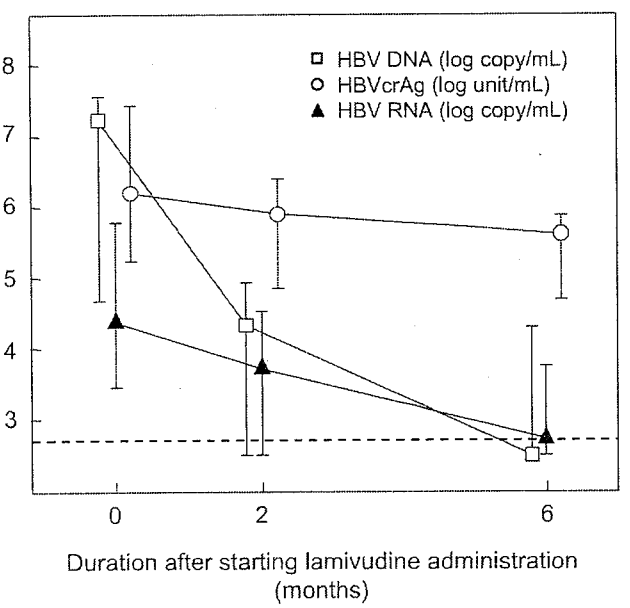

Fig. 1. Changes in serum levels of HBV DNA, HBV RNA, and HBVcrAg during lamivudine therapy in 24 patients with chronic hepatitis B. *Open squares* indicate HBV DNA, *open circles* indicate HBVcrAg, and *closed triangles* indicate HBV RNA. Data are expressed as medians and 25th and 75th percentiles. *HBV*, hepatitis B virus; *HBVcrAg*, HBV core-related antigen