A

B

C

D

Figure 4. Anti-HCV effects IFN in combination with GL. HCVcc-infected cells were treated with IFN alone, or IFN with GL for 72 hours. HCV production was assessed by measuring the HCV core antigen in culture medium (A) and cell (B). Specific nfectivity in culture medium (C) and cell (D) were measured. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. *P < 0.05, **P < 0.005 versus versus IFN mono-therapy.

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the efficiency of virus production and core protein accumulation on LDs was also observed that colocalization of HCV protein with LDs was low in cases of the chimera Jc1, supporting up to 1,000-fold higher infectivity titers compared with JFH1 [41], [29]. In this study, we demonstrated that GL did not affect the size of LDs in un-infected cells (Figure 2F right panel). On the other hand, the size of LDs increased in HCV-infected cells with GL-treatment (Figure 2F left panel), probably because accumulated-HCV enhanced the formation of LDs [29].

We demonstrated the importance of PLA2G1B in HCV release by PLA2G1B inhibitor and siRNA against PLA2G1B (Figure 3). The overexpression of PLA2G1B did not have any effect on HCV release (data not shown), probably because enough PLA2G1B existed in the cells. This result is generally observed in other host factors that involved in HCV lifecycle. For example, overexpression of the human homologue of the 33-kDa vesicle-associated membrane protein-associated protein (hVAP-33), which has a critical role in the formation of HCV replication complex, did not increase HCV replication [42]. PLA2 family proteins have been known as lipid-signaling molecules, inducing inflammation [43]. On the basis of the nucleotide sequence, the superfamily of PLA2 enzymes consists of 15 groups, comprising 4 main types: cytosolic PLA2 (cPLA2), calcium-independent PLA2, platelet activating factor acetyl hydrolase/oxidized lipid lipoprotein associated PLA2, and the secretory PLA2 (sPLA2) including PLA2G1B, 2A, and 4A [44]. In this study, we showed that GL, PLA2G1B inhibitor, and PLA2G1B siRNA inhibited HCV release and that GL and OPC had no additive effect when applied together, suggesting that suppression of HCV release by GL may be derived from its inhibitory effect on PLA2G1B. The role of PLA2G1B in the HCV lifecycle has not been reported. In this study, we also demonstrated that PLA2G2A inhibitor decreased entry, replication, and assembly of infectious HCV particles in cells (Figures 3A, 3B, 3C, and 3D). The role of PLA2G2A in the HCV lifecycle has not been reported. PLA2G2A is known to affect the secretion of VLDL (30). Therefore, PLA2G2A may contribute to HCV assembly. In the case of PLA2G4A, Menzel et al. showed that inhibition of PLA2G4A produces aberrant HCV particles [45]. These observations suggest that PLA2 has a role in several steps of the HCV lifecycle.

In this study, we showed that the EC_{50} of GL treatment for intracellular infectivity was 16.5 μM (Figure 1A). It has been reported that the maximum peripheral concentration of GL in normal patients is 145 μM [46]. The placebo-controlled phase I/II trial revealed no significant effect on viral titer [47]. In vivo, accumulated HCV in GL treated cells may cause lysis and apoptosis of the cells, leading to the release of infectious particles in the circulation. This may be a major limitation to use GL mono-therapy against HCV infection in patients. On the other hand, combination treatment with GL augmented the IFN-induced reduction in HCV core antigen levels (Figure 4A).

Although a number of natural compounds with anti-HCV activities were identified in recent years (Silymarin, EGCG, Ladanein, Naringenin, Quercetin, Luteolin, Honokiol, 3-hydroxy caruilignan C, and other things) [48], many aspects concerning their mechanisms of action remain unknown. In this study, GL is identified as a novel anti-HCV agent that targets the release

steps of infectious HCV particles. We found that the suppression of viral release by GL may be due to an inhibitory effect of PLA2G1B. These observations provide a basis for development of an improved IFN-based combination therapy against chronic hepatitis C.

Supporting Information

Figure S1. Anti-HCV effect of GL. HCVcc-infected cells were treated with various concentrations of GL for 72 hours. HCV production was assessed by measuring the level of HCV core antigen in culture medium. Results are expressed as the mean ± SD of the percent of the control from four independent experiments. IFN (10 IU/ml) was used as a positive control. *P < 0.05, **P < 0.005 versus control (0 μM treatment).
(TIF)

Figure S2. Effect of GL on expression of PLA2G1B. A human PLA2G1B cDNA was inserted into the EcoRI site of pCAGGS, yielding pCAGPLA2G1B. Since there was no effective antibody to detect endogenous expression of PLA2G1B, 293T cells transfected with the pCAGPLA2G1B plasmid were treated with GL (500 μM) for 72 hours and lysed in lysis buffer, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies. OPC (10 μM) was used as a positive control to reduce PLA2G1B protein in the cells.
(TIF)

Figure S3. Effect of PLA2G1B siRNA on expression of PLA2G1B. HCVcc infected-Huh7 cells in a 24-well plate were transfected with siRNAs targeted to PLA2G1B and scramble negative control siRNA, followed by immunoblotting with anti-PLA2G1B and anti-actin antibodies.
(TIF)

Figure S4. Effect of GL on IFN induction. The pISRE-Luc vector contains the firefly luciferase reporter gene, downstream

of the IFN-Stimulated Response Element (ISRE) cis-acting enhancer element. The pRL-TK vector contains the renilla luciferase reporter downstream of the herpes simplex virus thymidine kinase (HSV-TK promoter), and was used as an internal control. Huh7 cells transfected with the pISRE-Luc vector and the pRL-TK vector were treated with various concentrations of GL for 72 hours, and luciferase activities were measured using the Dual-Luciferase Reporter Assay System. IFN (300 U/ml) was used as a positive control. Results are expressed as the mean ± SD percent of the controls (treatment with IFN).
(TIF)

Figure S5. Effect of GL on secretion of lipoprotein and the host proteins. Huh7 cells were treated or untreated with GL at 500 μM for 72 hours. ApoE and albumin in the culture supernatants were measured by immunoblotting and ELISA, respectively. Results are expressed as the mean ± SD of the percent of the control from four independent experiments.
(TIF)

Acknowledgements

Author Contributions

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BASIC AND TRANSLATIONAL—LIVER

Neutralizing Antibodies Induced by Cell Culture–Derived Hepatitis C Virus Protect Against Infection in Mice

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See editorial on page 285.

BACKGROUND & AIMS: Hepatitis C virus (HCV) infection is a major cause of liver cancer, so strategies to prevent infection are needed. A system for cell culture of infectious HCV particles (HCVcc) has recently been established; the inactivated HCVcc particles might be used as antigens in vaccine development. We aimed to confirm the potential of HCVcc as an HCV particle vaccine. **METHODS:** HCVcc derived from the J6/JFH-1 chimeric genome was purified from cultured cells by ultrafiltration and ultracentrifugation purification steps. Purified HCV particles were inactivated and injected into female BALB/c mice with adjuvant. Sera from immunized mice were collected and their ability to neutralize HCV was examined in naive Huh7.5.1 cells and urokinase-type plasminogen activator–severe combined immunodeficiency mice (uPA$^{+/+}$-SCID mice) given transplants of human hepatocytes (humanized livers). **RESULTS:** Antibodies against HCV envelope proteins were detected in the sera of immunized mice; these sera inhibited infection of cultured cells with HCV genotypes 1a, 1b, and 2a. Immunoglobulin G purified from the sera of HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection of cultured cells. Injection of IgG from the immunized mice into uPA$^{+/+}$-SCID mice with humanized livers prevented infection with the minimum infectious dose of HCV. **CONCLUSIONS:** Inactivated HCV particles derived from cultured cells protect chimeric liver uPA$^{+/+}$-SCID mice against HCV infection, and might be used in the development of a prophylactic vaccine.

Keywords: HCV Particle; HCV Vaccine; Immunization; Virology.

Hepatitis C virus (HCV), an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family, is a human pathogen that is a major cause of chronic hepatitis, cirrhosis, and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated interferon and ribavirin. However, these agents are not very effective for patients with high titer HCV-RNA and genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV infection.

A cell culture–derived HCV (HCVcc) was developed using the genome of the genotype 2a JFH-1 strain. HCVcc was shown to be infectious in vitro and in vivo,[1-3] and its analysis has made it possible to understand the HCV lifecycle. Thus, HCVcc can be considered as a useful model for the screening of new anti-HCV drugs. However, the fact that only human beings and chimpanzees are susceptible to HCV has greatly slowed the in vivo study. As a tool for circumventing this problem, a human liver chimeric albumin enhancer/promoter urokinase plasminogen activator-severe combined immunodeficiency (uPA-SCID) mouse was established.[4] Thus, the HCV cell culture system and the human liver chimeric mouse are potent tools for pharmaceutical research concerning HCV.

Prevention of HCV infection is important for control of the pathogenesis of hepatitis C. However, there is no commercial vaccine for HCV at present, although vaccines are now being developed using HCV components, peptides, DNA, and viral vectors.[5] Because HCVcc is a structural mimic of the native HCV particle, it has the potential to function as an immunogen for the development of an anti-HCV vaccine. In this study, to use HCVcc as an HCV-particle vaccine, we purified HCVcc and determined the neutralizing effects of the HCV-particle vaccine against HCV infection in mice.

Materials and Methods

Cell Culture

Huh7 and Huh7.5.1 cells[2] (a generous gift from Dr Francis V. Chisari) were cultured in 5% CO_2 at 37°C in Dulbecco's modified

Eagle medium (DMEM) containing 10% fetal bovine serum (DMEM-10) as previously reported.[1]

Plasmids

pJ6/JFH1 was generated by replacing the JFH-1 structural region with that of the J6CF strain as previously reported.[6]

HCV Particle Purification

J6/JFH-1 chimeric HCVcc was purified by ultrafiltration and ultracentrifugation. The methods for HCVcc production and purification were as described in the Supplementary Materials and Methods section.

Infectivity Titration

Huh7.5.1 cells were used to determine the viral infectivity titer using end-point dilution and immunofluorescence methods as described previously.[7] The titer was expressed as focus-forming units per milliliter.

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[8] HCV pseudo-particle (HCVpp) harboring the envelope of the genotype 1a (H77[9]), 1b (TH[10]), or 2a (J6CF[11]) strain were generated as described in the Supplementary Materials and Methods section.

Preparation of Recombinant Proteins

HCV-E1 and HCV-E2 recombinant proteins were generated as described in the Supplementary Materials and Methods section.

Immunization of Mice With the HCV Vaccine

Mice were immunized as described in the Supplementary Materials and Methods section.

Enzyme Immunoassay

Anti-E1 and anti-E2 antibodies were detected by enzyme immunoassay for stabilized recombinant J6E1/FLAG and J6E2/FLAG protein, respectively, as described in the Supplementary Materials and Methods section.

HCV Inhibition Assay

Inhibition of HCV infection in cultured cells was assayed using HCVpp and HCVcc for infection as described in the Supplementary Materials and Methods section.

IgG Purification

Mouse IgG was purified using KAPTIV-GY resin (Technogen, Tehran, Iran) as described in the Supplementary Materials and Methods section.

Anti-HCV Envelope IgG Adsorption

Anti-HCV envelope antibodies were absorbed using recombinant FLAG-tagged HCV-E1 and HCV-E2 proteins. Briefly, 1 mg/mL IgG from HCV-particle-immunized mice (iHCV-IgG) was precleared with anti-FLAG M2 agarose, and then mixed with 50 μg each of J6E1/FLAG and J6E2/FLAG protein. The IgG recombinant protein mixture was incubated at 4°C overnight, and anti-FLAG M2 agarose then was added into the mixture, which

was centrifuged at 3000 × g for 2 minutes and the supernatant was collected as anti-envelope IgG-adsorbed IgG.

Inhibition of HCV Infection in Chimeric Mice in Which the Liver Was Repopulated With Human Hepatocytes

SCID mice that were transgenic for the urokinase-type plasminogen activator gene and in which the liver was repopulated with human hepatocytes (chimeric mice) were purchased from Phoenix Bio Co, Ltd (Hiroshima, Japan). Only mice showing levels of human albumin greater than 7 mg/mL were used in the experiments. The mice were injected intraperitoneally with 100 μg of control (Cont)-IgG or iHCV-IgG at 1 week and at day -1 before virus challenge. J6/JFH-1 HCVcc particles (10^3, 10^4, and 10^5 RNA copies/mouse) were mixed with 50 μg/mL of Cont-IgG or iHCV-IgG, and used to challenge the mice. Blood samples were collected within 1 week, and HCV RNA was determined using real-time detection reverse-transcription polymerase chain reaction (RTD-PCR).

Western Blot Analysis

Purified HCV particle samples were resolved on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Western blot was performed using an anti-E1 or an anti-E2 antibody as described in the Supplementary Materials and Methods section.

Statistical Analysis

The Dunnett test was used to compare the statistical significance of differences between groups of data. A P value less than .05 was considered significant. Data are reported as means ± SEM as indicated.

Results

HCV Particle Purification

HCV particles for mouse immunization were obtained using the cell culture system described in the Materials and Methods section. J6/JFH-1 chimeric HCV was secreted into culture medium supplemented with 2% fetal bovine serum, which was collected and centrifuged; the supernatant was concentrated by ultrafiltration using a 500-kilodalton cut-off membrane. The concentrated culture fluid was diafiltered using phosphate-buffered saline. These purification procedures removed approximately 90% of the contaminating proteins in the solution of virus particles. Sucrose cushion or gradient centrifugation then was performed to purify the virus particles further. After each procedure, the fractionated viruses were concentrated by ultrafiltration and the amount of HCV core and RNA, the concentration of total protein, and viral infectivity were determined (Supplementary Table 1). As shown in Figure 1, HCV particles, which had 2 distinct characteristics, were obtained in different fractions after sucrose gradient fractionation. One fraction (Fraction [Frac] 6) had low infectivity and a relatively high level of HCV core and RNA, and the other fraction (Frac 8) had high infectivity and a lower level of HCV core and RNA. Thus, the yield of HCV particles was highest in sucrose gradient Frac 6 (Supplementary Table 1), whereas Frac 8 displayed
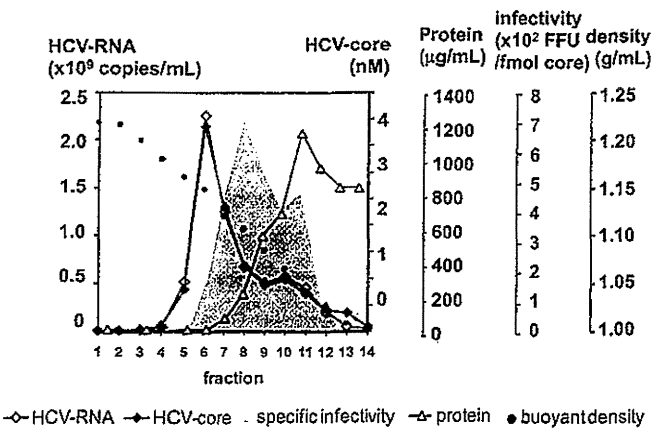
Figure 1. HCV-particle purification from cell cultures. Huh7 cells were inoculated with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2) and infected cells were passaged into CellSTACK (Corning, Corning, NY). The cell supernatant was collected and concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, United Kingdom). Concentrated virus then was diafiltered using phosphate-buffered saline, and the viral fluid was layered on top of a preformed continuous 10%–60% sucrose gradient in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The level of the HCV core protein, HCV RNA, infectivity toward naive Huh7.5.1 cells, total protein, and buoyant density were determined for each fraction. Infectivity is indicated as specific infectivity, which was calculated as the infectivity titer (focus-forming unit [FFU]) divided by the HCV core protein (fmol).

approximately 7-fold higher infectivity than Frac 6 (Table 1). After sucrose cushion purification, the total protein level of the solution of purified virus particles was reduced to less than 0.03% of that of the initial cell culture supernatant (Supplementary Table 1).

Immunization of Mice With HCV Particles

We next immunized BALB/c mice with 2 or 5 pmol of HCV particles that were purified through a sucrose cushion (termed cushion-2 and cushion-5, respectively) or with the HCV particles (2 pmol HCV core) from the earlier-described sucrose gradient fractions 6 or 8, and confirmed the immunogenicity of these particles. Before immunization, the purified HCV particles were inactivated by ultraviolet irradiation and conjugated with monophosphoryl lipid A plus trehalose-6,6-dimycolate. The HCV particles then were injected intraperitoneally into female BALB/c mice and blood samples were collected. Immunization was repeated 4 times at 2-week intervals, and the presence of anti-E1 and anti-E2 antibodies in immunized mouse serum was analyzed using an enzyme immunoassay. Both anti-E1 and anti-E2 antibodies were

induced in mice immunized with the HCV particles and the efficiency of antibody induction was the same regardless of the nature of the antigen (Figure 2A and B).

To confirm that the sera from these immunized mice could inhibit HCV infection, an HCVpp inhibition assay was performed. In this assay, sera from HCV-particle–immunized mice inhibited HCVpp infection of naive Huh7.5.1 cells by HCV strains H77 (1a), TH (1b), and J6CF (2a) (Figure 2C–E). Furthermore, the inhibitory effects of different amounts (2 or 5 pmol) of HCV core purified by the sucrose cushion method or of HCV particles from different fractions obtained by sucrose gradient purification were not significantly different. Inhibitory effects of the serum from immunized mice were detected against TH/JFH-1 (genotype 1b) and J6/JFH-1 (genotype 2a) HCVcc infection (Supplementary Figure 1). The 50% inhibitory dilution titer of Frac 6 vaccine-immunized mouse serum was 1/181 and 1/614, and that of Frac 8 vaccine-immunized mouse serum was 1/432 and 1/766 for TH/JFH-1 and J6/JFH-1 HCVcc, respectively. These results indicate that the neutralizing effects of the serum from immunized mice were intergenotypic, and that 2 pmol of the HCV core was a sufficient dose for HCV-particle immunization. In contrast, immunization with saline or bovine serum albumin and adjuvant did not induce any neutralizing effects in mice (Figure 2C–E). We used bovine serum albumin as a control because albumin was assumed to be the main contaminating protein in the purified HCV particles (Supplementary Figure 2).

The HCV-Particle Vaccine Induced Anti-HCV Antibody More Efficiently Than an HCV-Component Vaccine

For comparative study of HCV-component and HCV-particle vaccines, recombinant E1 and E2 proteins were produced and used to immunize mice using the same adjuvant as that used earlier for the HCV-particle vaccine. These recombinant FLAG-tagged J6E1 and J6E2 proteins (J6E1/FLAG and J6E2/FLAG) were expressed and secreted into the culture supernatant of COS-1 cells, and were purified by affinity chromatography using M2-agarose. Before injection into mice, the purified recombinant proteins, as well as the inactivated HCV particles obtained from the sucrose gradient fractionation described earlier, were analyzed by Western blot using anti-E1 and anti-E2 antibodies, and the amount of E1 or E2 protein present in the HCV particles was determined. Densitometric analysis of the immunoblot shown in Figure 3 indicated that an aliquot of HCV particles that contained 1 pmol of HCV core protein included approximately 10 ng each of

Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Core/RNA, fmol/copy	Infectivity/core, FFU/fmol	Infectivity/RNA, FFU/copy	Core/protein, fmol/μg
Frac 6	6.54×10^{-7}	129.8	8.50×10^{-5}	113.8
Frac 8	5.38×10^{-7}	987.9	5.32×10^{-4}	19.3
Ratio (Frac 8/Frac 6)	0.82	7.61	6.26	0.17
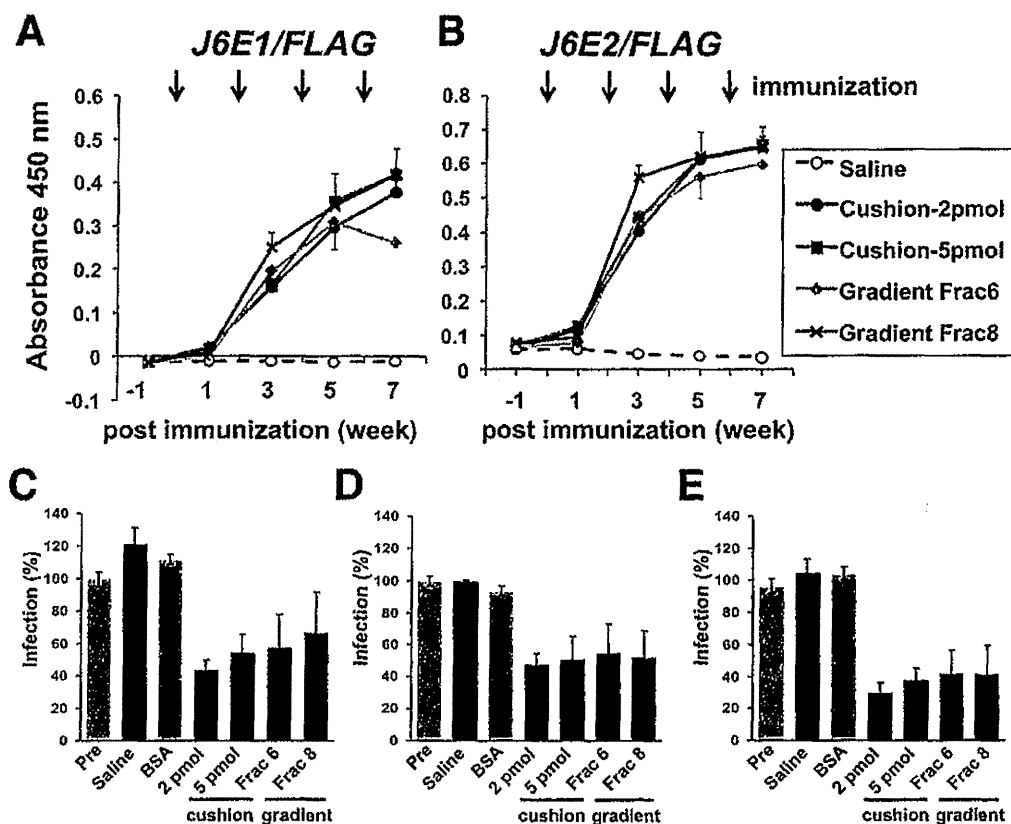
Figure 2. Cross-genotypic neutralizing effect of the sera from HCV-particle–immunized mice. HCV particles that were purified using a sucrose cushion (2 or 5 pmol HCV core) or sucrose gradient fractionation (Frac 6 and Frac 8, 2 pmol HCV core each) were inactivated by ultraviolet irradiation. The inactivated HCV -particles were conjugated with Sigma Adjuvant System (Sigma, St Louis, MO), and were injected intraperitoneally into BALB/c mice (5 weeks old, female, n = 3). Saline or bovine serum albumin (150 μg) with adjuvant was injected into mice as a control. Immunization was repeated 4 times at 2-week intervals (at weeks 0, 2, 4, and 6). Blood was collected at weeks 1, 3, 5, and 7 after immunization. Pre-immune sera and immunized mouse sera were prepared from these blood samples and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera were diluted (1000-fold) and their reactivity with (A) J6E1/FLAG and (B) J6E2/FLAG recombinant proteins was analyzed using an enzyme immunoassay. Assays were performed in triplicate. The y-axis indicates absorbance at 450 nm. Mean values ± SEM are shown. Mouse serum-mediated inhibition of HCV infection was assayed using HCVpp genotypes (C) 1a (H77), (D) 1b (TH), and (E) 2a (J6CF). The sera were diluted (100-fold) and HCVpp infection in naive Huh7.5.1 cells was examined. Assays were performed in triplicate and infection rates are expressed as mean ± SEM.

the E1 and E2 proteins. Thus, HCV particles that contained 2 pmol of HCV core contained approximately 20 ng of each envelope protein.

Recombinant J6E2/FLAG was inoculated into mice at a concentration of 20, 200, or 2000 ng/mouse or was inoculated together with the same dose of J6E1/FLAG (20 or 200 ng/mouse). Inactivated HCV particles from Frac 6 or Frac 8 were injected into mice using 2 pmol (20 ng each of E1 or E2) of HCV core protein per mouse. Sera from immunized mice were collected and the presence of anti-E2 antibodies was analyzed using an enzyme immunoassay. Anti-E2 antibodies were induced in all immunized mice, and the highest level of anti-E2 antibodies was induced in the group of mice that were immunized with 200 or 2000 ng of the J6E2 protein per mouse. On the other hand, the level of anti-E2 antibodies that was induced by immunization with 20 ng of the inactivated HCV particles was higher than that induced by the same amount of recombinant envelope proteins (Figure 4A). To analyze the inhibitory effects of the sera from immunized mice against HCV infection, HCVpp was incubated with heat-inactivated mouse sera, and then was used to

inoculate Huh7.5.1 cells. Sera from HCV-particle–immunized mice were able to inhibit HCVpp infection; however, sera from mice immunized with 20 or 200 ng of the recombinant E2 or E2 plus E1 proteins did not show significant neutralization activity. Although sera from mice immunized with 2 mg of the recombinant E2 protein could neutralize HCVpp infection, the sera from mice immunized with only 20 ng of the HCV particles from sucrose gradient Frac 6 or Frac 8 also were able to induce similar levels of anti-envelope antibody and HCV neutralization (Figure 4B). These results suggest that HCV particles have a greater potential as vaccine candidates than recombinant envelope proteins in terms of their potency for antibody induction.

Vaccination With Cell-Cultured HCV Induces Anti-HCV IgG

As described earlier, inactivated HCV particles can induce an anti-HCV effect in the sera of immunized mice. To confirm that this anti-HCV effect was dependent on HCV-specific antibodies, IgG was purified from the sera of Saline- or HCV-particle-immunize mice, respectively, and was assayed in an HCV inhibition assay. The purity of the
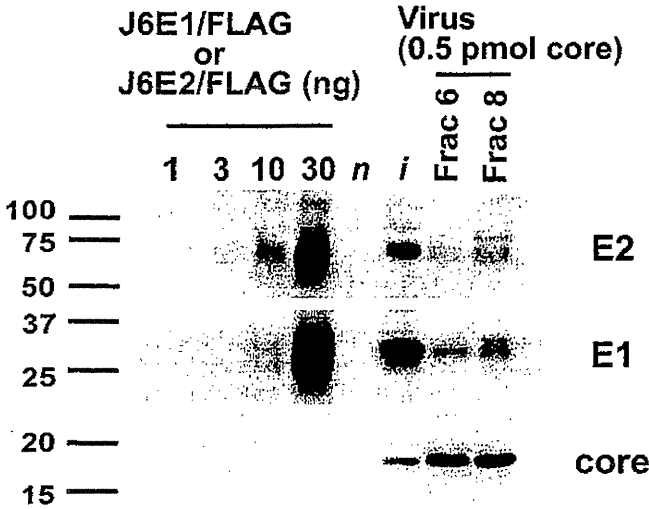
Figure 3. Western blot analysis of envelope proteins in purified HCV particles. The HCV core (0.5 pmol) of purified HCV particles (Frac 6 and Frac 8), as well as 1–30 ng of recombinant envelope proteins (J6E1/FLAG or J6E2/FLAG) were resolved on 12% sodium dodecyl sulfate-polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Naïve Huh7 cells and J6/JFH-1–infected Huh7 cells were lysed using Passive Lysis Buffer (Promega, Madison, WI), and were assayed similarly as negative (n) and positive (i) controls, respectively. The membrane was blocked and probed with primary antibodies against the virus core (2H9; 3 μg/mL), E1 (B7567; 10 μg/mL), and E2 (AP33; 3 μg/mL) proteins. The membrane then was probed with horseradish-peroxidase–conjugated secondary antibody, and bound antibody was detected using ECL-plus (GE Healthcare, Little Chalfont, UK).

purified IgG was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis (Figure 5A). Purified IgGs were pre-incubated with J6/JFH-1 HCVcc and this mixture subsequently was used to inoculate Huh7.5.1
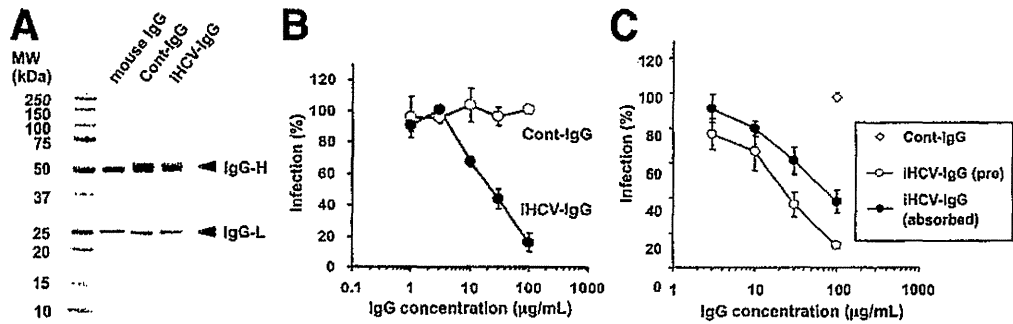
cells. The IgG from HCV-particle-immunized mice (iHCV-IgG) inhibited HCV infection in an iHCV-IgG dose-dependent manner, whereas the IgG from saline-immunized mice (Cont-IgG) did not show any neutralizing effects on this in vitro assay (Figure 5B). The 50% inhibitory concentration (IC50) of iHCV-IgG against HCVcc infection was 16.1 μg/mL. The iHCV-IgG from Frac 6- and Frac 8-immunized mice showed a neutralizing effect on TH (genotype 1b) and J6CF (genotype 2a) HCVpp infections (Supplementary Figure 3); the IC50 values were 18.0 (TH) and 30.4 (J6CF) μg/mL for Frac 6-IgG, and 15.1 (TH) and 33.1 (J6CF) μg/mL for Frac 8-IgG. Similarly, IgG from Frac 6- and Frac 8-immunized mice showed a neutralizing effect on HCVcc infection (Supplementary Figure 4). Thus, both fractions of the HCV particles appear to have similar immunogenicity. As a control, mice were immunized with naïve Huh7-cultured media that was processed similar to the method used for the purification of HCV particles or saline together with adjuvant. IgG was purified from the mice sera, and HCVcc inhibition assay was performed. The purified IgG did not show a significant inhibitory effect for HCV infection compared with saline-immunized mice (Supplementary Figure 5). However, iHCV-IgG significantly inhibited the HCV infection at a concentration of 30 μg/mL. The effect of adsorption of the anti-E1 and anti-E2 antibodies in iHCV-IgG by incubation with recombinant J6E1 and J6E2/FLAG proteins on the inhibitory effect of iHCV-IgG then was examined. The HCV neutralizing effect of iHCV-IgG was reduced by adsorption of the anti-envelope antibodies, and the IC50 value was increased to 52.9 μg/mL (Figure 5C). These data indicate that anti-HCV envelope neutralizing antibodies were induced in mice by injection of the HCV-particle vaccine.
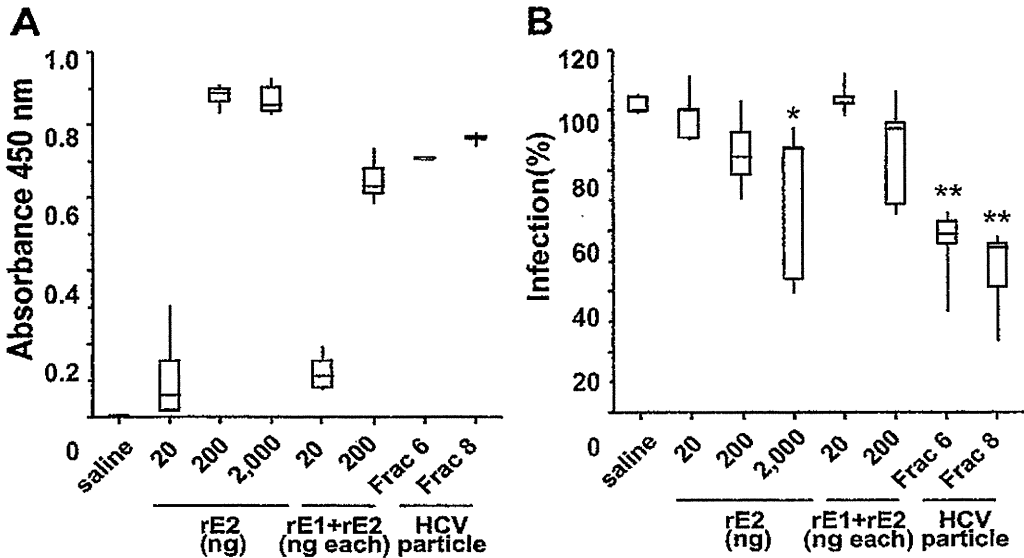
Figure 4. Neutralizing effects of sera from HCV-component and HCV-particle vaccine-immunized mice. BALB/c mice (n = 5) were immunized with recombinant proteins (20, 200, or 2000 ng of FLAG-tagged E2, 20 or 200 ng each of FLAG-tagged E1 and FLAG-tagged E2), or with purified HCV particles (2 pmol HCV core of Frac 6 or Frac 8) together with adjuvant. (A) The presence of anti-E2 antibodies in sera from immunized mice was analyzed using an enzyme immunoassay and the (B) ability of these sera to inhibit J6CF HCVpp infection of cultured Huh7.5.1 cells also was assayed. Each assay was performed in triplicate, and the data are presented as box plots. The statistical significance of differences between groups was analyzed using the Dunnett test (*P < .05, **P < .001 vs saline).

Figure 5. IgG purified from the sera of HCV-particle–immunized mice had a neutralizing effect against HCV infection in vitro. (*A*) IgG from the sera of mice at 7 weeks after immunization (iHCV-IgG) was purified using a KAPTIV-GY resin. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG). Each IgG was analyzed for purity by SDS–polyacrylamide gel electrophoresis and Coomassie brilliant blue staining. As a control, a purchased mouse IgG was used. (*B*) Serially diluted Cont-IgG or iHCV-IgG was mixed with J6/JFH-1 HCVcc (multiplicity of infection, 0.1) and this mixture was inoculated into naive Huh7.5.1 cells. HCV infection was determined by analysis of the intracellular concentration of the HCV core protein. Infectivity is expressed as the percentage of cells that were infected. The IC50 value of iHCV-IgG was 16.1 μg/mL. (*C*) Anti-E1 and anti-E2 antibodies in iHCV-IgG were adsorbed by recombinant J6E1/FLAG and J6E2/FLAG protein, and the neutralizing effect of the E1E2-adsorbed IgG on HCV infection of Huh7 cells was assessed using the HCVcc inhibition assay as described for panel *B*. The IC50 of the E1E2-adsorbed IgG shifted to 52.9 μg/mL.

HCV Vaccine Induced Anti-HCV Infection In Vivo

As shown earlier, anti-HCV envelope neutralizing antibodies were induced in HCV-particle–immunized mice. To confirm the effect of the HCV-particle vaccine in vivo, we next examined its ability to neutralize an HCV challenge to human liver chimeric uPA-SCID mice. Purified IgG (100 μg) from HCV-particle–immunized mice was injected intraperitoneally into these chimeric mice 1 week before HCV infection. The mice then were challenged with multiple doses of 10^3, 10^4, and 10^5 RNA copies of J6/JFH-1 HCVcc at 2-week intervals. Blood samples were collected and HCV RNA was measured using RTD-PCR. HCV infection was inhibited (no infection) in all 6 mice in the vaccine-treated mouse group (iHCV-IgG) when 10^3 copies of HCV were inoculated (minimal infectious dose), whereas infection was observed in 4 of the 6 mice in the Cont-IgG mouse group. However, the neutralizing effect of iHCV-IgG vaccination was not detected when 10^4 or 10^5 copies of HCV were used for infection (Table 2). These data indicated that an inactivated HCV-particle vaccine potentially could be used as a prophylactic vaccine for HCV.

Discussion

There is no vaccine available for clinical use against HCV infection. Development of an HCV cell culture system has facilitated an understanding of the HCV life cycle, and the production of HCV prophylactic and therapeutic

Table 2. Neutralizing Effect of IgG Purified From the Sera of HCV-Particle–Immunized Mice Against HCV Infection in Human Liver Chimeric uPA-SCID Mice

Virus challenge, HCV RNA copies	Infected mice	
	Cont-IgG	iHCV-IgG
10^3	4/6	0/6
10^4	5/6	6/6
10^5	6/6	6/6

drugs. HCVcc mimics the native HCV particle, and thus has potential for use as an HCV-particle vaccine. In the present study, we established an efficient method for HCV particle production and purification, which allowed evaluation of the HCV particle as a vaccine candidate. HCV particles were purified from cell-cultured HCV of genotype 2a J6/JFH-1. The HCVcc in the culture supernatant was concentrated and partially purified by ultrafiltration using a 500-kilodalton cut-off membrane. Subsequently, HCVcc was purified by sucrose gradient ultracentrifugation. As reported previously,[12,13] the virus particles present in Frac 6 and Frac 8 after this sucrose gradient centrifugation displayed different characteristics. The virus in Frac 6 displayed low infectivity, but had a high concentration of viral components, whereas that in Frac 8 displayed high infectivity, but a low concentration of viral components. It is thought that the content of lipids or lipoproteins in these HCV particle fractions is different.[12,13] Finally, each viral fraction was purified and concentrated by ultrafiltration. When these HCV particles were inactivated, conjugated with a monophosphoryl lipid A plus trehalose-6,6–dimycolate adjuvant, and injected into mice, anti-E1 and anti-E2 antibodies were induced in the immunized mice, and the sera from these mice had a neutralizing effect against HCV infection in vitro. Because these sera displayed cross-reactivity for HCV strains 1a (H77), 1b (TH), and 2a (J6CF), it was considered that the HCV-particle vaccine had a broad neutralizing effect against HCV infection.

Western blot analysis of the envelope proteins in these HCV particles (Figure 3) indicated that the Frac 6 HCV particles contained approximately 10 ng each of the E1 and E2 proteins per 1 pmol HCV core protein. Because the molecular weight of the core, E1, and E2 proteins is approximately 20, 35, and 70 kilodaltons, respectively,[14] the molar ratio of the structural proteins in the purified HCV particles (core:E1:E2) was calculated to be 5:1:1 from Western blot analysis. Western blotting analysis also suggested that the amount of the E1 and E2 proteins in the Frac 8 HCV particle was likely to be higher than that

in the Frac 6 HCV particle. Indeed, densitometric analysis of the blot indicated that the level of both E1 and E2 proteins in Frac 8 was 1.5-fold higher than that in Frac 6. This higher level of E1 and E2 proteins in Frac 8 may be the reason why the Frac 8 HCV particles had higher infectivity. Interestingly, the expression level of the core protein relative to that of the envelope proteins was different in infected cells and in purified HCV particles (Figure 3). These data suggest that only a limited amount of the envelope protein was incorporated into HCV particles.

We compared the potential of the purified HCV particles to function as a vaccine with that of HCV-envelope components. For this purpose, recombinant HCV E1 and E2 proteins were prepared and different doses of recombinant E2 (rE2), or recombinant E1 (rE1) plus rE2, were injected into mice with adjuvant. Other groups of mice were immunized with the HCV-particle vaccine (Frac 6 or Frac 8) using a dose of 2 pmol of HCV core per mouse. Because it was calculated that HCV particles that included 2 pmol of HCV core protein contained approximately 20 ng of recombinant E1 and E2, we used 20–2000 ng of rE2 or 20 or 200 ng each of rE1 plus rE2 proteins for comparative purposes. Although the sera from all immunized mice contained anti-E2 antibodies, and immunization with 200 or 2000 ng of recombinant E2 protein induced antibody production with the highest efficiency, HCV-particle immunization displayed the highest virus-neutralizing effect. Based on these results, it was concluded that purified HCV particles may be a more potent prophylactic HCV vaccine candidate than a vaccine that is composed of recombinant viral components. There are several possible explanations for the observed differences between these 2 types of vaccines. First, HCV envelope proteins that are a part of HCV particles are properly folded, which would maintain their function, whereas the recombinant envelope proteins may not be properly folded, and thus may lose their antigenicity. Second, other factors associated with the HCV particles may be important for inducing neutralizing antibodies. To address these possibilities, we adsorbed the anti-envelope antibodies in iHCV-IgG with recombinant envelope proteins. This procedure reduced, but did not completely abolish, the neutralizing effect of iHCV-IgG toward HCVcc infection. It is possible that if the recombinant envelope proteins were not folded properly as mentioned earlier, then not all of the anti-envelope antibodies were removed in this adsorption process. However, this possibility is considered unlikely because virus-neutralizing antibodies were induced by both immunization with the HCV particles and with the recombinant envelope proteins. This result suggests that HCV particles may include other factors, such as lipoproteins, that are important for HCV infection.[15–17] It also is necessary to compare the immunogenic activity of the inactivated virus vaccine with the potential immunogenic activities of recombinant HCV-like particles and E1-E2 heterodimers. Furthermore, attention needs to be paid as to whether the inactivated purified vaccine may induce autoantibodies against human proteins associated with the viral particles.

To confirm the in vivo efficacy of HCV-particle–induced neutralizing antibodies, the ability of iHCV-IgG to protect infected animals against HCV challenge was examined using human liver chimeric uPA-SCID mice. Interestingly, iHCV-IgG could protect against HCV challenge, at least at the lower virus challenge dose, although the mice challenged with the higher virus doses became infected with HCV. This result indicated that the HCV-particle vaccine could exert a prophylactic anti-HCV effect in vivo by induction of virus-neutralizing antibodies. However, the therapeutic effect of this vaccine is not yet clear and an understanding of its effect will require detailed study of its effect on cellular immune responses such as the cytotoxic T lymphocyte response. In addition, the prophylactic effect was observed only partially in the in vivo experiment. The reason for this insufficient protection is unclear; however, induction with a higher titer of neutralizing antibody may be needed, using adjuvants or more effective forms of the vaccine such as recombinant HCV-like particles. It also may be important to induce protective cellular immune responses in addition to humoral immune responses; effective cytotoxic T-lymphocyte responses may be necessary for a prophylactic effect against infection at higher virus doses.

Prophylactic and therapeutic HCV vaccines have been developed previously and some of these vaccines have been tested in clinical trials.[5,18,19] An HCV vaccine should be able to induce humoral and cellular immune response with specific anti-HCV effects. Previous vaccine candidates that have been tested have included recombinant proteins,[20–24] peptides,[25–29] DNA,[30] and viral vectors[31,32] with or without appropriate adjuvants. Recombinant E1 and E2 proteins with an MF59 adjuvant were developed by Chiron/Novartis (Basel, Switzerland) as a prophylactic vaccine, and the immunogenicity of this vaccine was confirmed in mice and guinea pigs.[33] The recombinant protein vaccine was well tolerated in a phase I clinical study, and induction of anti-envelope antibodies and an envelope-specific T-cell response was observed.[24] In another study, Fab fragments of anti-E2 antibodies that were obtained using a phage display library generated from an HCV-infected donor showed a broad HCV-neutralizing effect in human liver chimeric uPA-SCID mice.[34] A separate study showed that retroviral pseudotype-derived HCV-like particles (HCVLPs) also induced broad neutralizing antibodies in mice and macaques.[35] This result indicated that a prophylactic vaccine may be achievable by activation of the humoral immune response. Moreover, it was reported that insect cell-derived virus-like particles containing HCV structural proteins induced a humoral immune response[36] and stimulated dendritic cells[37] or a cytotoxic T-lymphocyte response.[38] Thus, it is possible that an HCVcc-derived HCV-particle vaccine also has the capacity to function as a therapeutic vaccine.

The HCV vaccine described in this study could be used for protection against HCV infection in high-risk patient groups. Given that the HCV particles are used as the vaccine, it will be necessary to improve the production and

purification of these particles before they can be used. We recently established a serum-free HCV culture system for efficient production and purification of virus particles that may be useful for this purpose.[39] It also will be necessary to examine other cell types for the production of HCV particles because the Huh7 cells used in this study are cells of a hepatoma cell line. Once HCV particle production using an industrial method is established and its safety is evaluated and confirmed, then it is expected that a purified HCV-particle vaccine will be of prophylactic use. Alternatively, a novel vaccine composed of HCV components should be developed using recombinant E1 and E2 proteins that can induce viral neutralizing antibodies with similar or even higher efficiency as HCV particles.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.05.007.

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Supplementary Materials and Methods

RNA Synthesis

RNA synthesis was performed as described previously.[1] Briefly, the pJ6/JFH1 plasmid was digested with *Xba* I and then treated with Mung Bean Nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment then was purified and used as a template for RNA synthesis. HCV RNA was synthesized in vitro using a MEGAscript T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNase I, followed by acid phenol extraction to remove any remaining template DNA.

RNA Transfection

RNA transfection was performed as described previously.[1] Briefly, trypsinized Huh7 cells were washed with Opti-MEM I reduced-serum medium (Invitrogen, Carlsberg, CA) and were resuspended at a density of 7.5×10^6 cells/mL in Cytomix buffer.[2] RNA (10 μg) that was synthesized from pJ6/JFH1 was mixed with 400 μL of the cell suspension and the mixture then was transferred into an electroporation cuvette (Precision Universal Cuvettes; Thermo Hybrid, Middlesex, UK). The cells then were pulsed at 260 V and 950 μF using the Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were transferred immediately to a 10-cm dish containing 8 mL of culture medium. Cell culture supernatants were collected 72 hours after transfection and were passed through a 0.45-μm filter. The filtered culture media then was pooled and concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore, Billerica, MA). The viral infectious titer was determined by immunofluorescence detection of infected foci using naive Huh7.5.1 cells, as described later.

HCV Particle Purification

Huh7 cells were infected with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2). After the infected cells were passaged into CellSTACK (Corning, Corning, NY) and cultured in 2% fetal bovine serum-supplemented medium, the cell supernatant was collected and passed through a 0.45-μm filter. The supernatant was concentrated by ultrafiltration using a hollow fiber UFP-500-C-3MA (GE Healthcare, Little Chalfont, UK) and then was diafiltered with 5 times the volume of phosphate-buffered saline. The concentrated and diafiltered cell supernatants were layered on top of a preformed continuous 10%–60% sucrose gradient or a 20%/60% sucrose cushion in 10 mmol/L Tris, 150 mmol/L NaCl, 0.1 mmol/L EDTA. The gradients were centrifuged using an SW28 rotor (Beckman Coulter, Fullerton, CA) at 28,000 rpm (141,000 $\times$ g) for 4 hours at 4°C, and 1-mL fractions were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100-μL decrease from fractions of a gradient run. The HCV core protein and HCV RNA in each fraction were quantified as described later. Purified HCV was prepared by collecting the peaks of HCV core and HCV RNA, which then were concentrated using an Amicon Ultra-15 (100,000 molecular weight cut-off; Millipore).

Quantification of HCV Core Protein and RNA

The concentration of HCV core proteins in sample aliquots was measured using the HCV Core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction Kit (Qiagen, Tokyo, Japan). Copy numbers of HCV RNA were determined by RTD-PCR using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan).[3]

HCV Pseudo-Particle Production

Murine leukemia virus pseudotypes were generated according to methods described previously.[4] Briefly, DNA corresponding to the Gag-Pol packaging construct (3.1 μg), the transfer vector construct (3.1 μg), and the HCV glycoprotein-expressing construct (1 μg) was transfected into 2.5×10^6 293T cells, which had been seeded the day before in 10-cm dishes using the FuGENE6 transfection reagent (Roche Diagnostics, Tokyo, Japan). For construction of a vector that expresses the HCV glycoprotein, the coding region corresponding to aa 132-746 (H77 strain), 132-747 (TH strain), or 132-750 (J6CF strain) was cloned into the pcDNA3.1 vector (Invitrogen). For the negative control, all constructs except the glycoprotein-expressing construct were transfected. The medium (8 mL/dish) was replaced 6 hours after transfection. Supernatants containing the pseudotypes were collected 48 hours later, and were passed through a 0.45-μm filter. The supernatants were stored at -80°C until use.

Preparation of Recombinant Proteins

Each E1 (aa 192-353) and E2 (aa 384-720) coding region of the J6CF strain was cloned into the p3×FLAG–CMV-13 (Sigma, St Louis, MO) vector. Each expression vector then was transfected into COS-1 cells using the Diethylaminoethanol (DEAE)-dextran method. The culture supernatant of the transfected cells was collected and FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins were purified using an anti-FLAG M2-agarose (Sigma) column under native conditions. The buffer containing the purified proteins was changed and the proteins were concentrated by ultrafiltration using Amicon Ultra-15 (Millipore).

Immunization of Mice With the HCV Vaccine

All animal procedures were approved by the Committees on Biosafety and Animal Handling Regulations of the National Institute of Infectious Diseases (Japan). Animal research was performed in compliance with the "Fundamental guidelines for proper conduct of animal experiment and related activities in institutions under jurisdiction (June 2006)" issued by the Ministry of Health, Labour and Welfare. Our animal work also adhered to the principles stated in the guidelines. BALB/c mice (4 weeks old, female) were purchased from SLC Japan (Shizuoka, Japan). Mice were tamed for 1 week before the start of experiments. HCV particles in virus-containing fluid were inactivated with ultraviolet irradiation and were conjugated with the same volume

of a solution containing monophosphoryl lipid A and trehalose-6,6–dimycolate using the Sigma Adjuvant System (Sigma). The conjugate (0.2 mL) was injected intraperitoneally into 5-week-old female BALB/c mice, and blood samples were collected. The same volume of saline was injected as a control. Mice were injected at weeks 0, 2, 4, and 6, and blood was collected at weeks 1, 3, 5, and 7 after injection. Blood samples were collected in a BD Vacutainer SST II (Becton Dickinson, Franklin Lakes, NJ) and centrifuged at 1200 × g for 10 minutes at room temperature. The supernatants were collected as sera and were heat-inactivated at 56°C for 1 hour. The heat-inactivated sera was used in viral inhibition assay experiments and for IgG purification.

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. Naive Huh7.5.1 cells (2 × 10^4) were seeded into a 48-well plate. HCVpp or HCVcc was mixed with serum that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. For the HCVpp assay, cells were washed once with phosphate-buffered saline and lysed with 40 μL/well of Cell Culture Lysis Reagent (Promega, Madison, WI), and luciferase activity was quantified using a Luciferase Assay System (Promega) as described previously.[5] For the HCVcc assay, cells were washed once with phosphate-buffered saline and lysed with 100 μL/well of Passive Lysis Buffer (Promega), and HCV core protein content was quantified using the HCV core enzyme-linked immunosorbent assay kit (Ortho Clinical Diagnostics). Assays were performed in triplicate and infectivity was calculated from the value of the luciferase activity or the HCV core content.

Enzyme Immunoassay

Recombinant J6E1/FLAG or J6E2/FLAG protein (50 ng) was coated onto a 96-well plate (Nunc, Roskilde, Denmark) at 4°C overnight. Each well was blocked with Blocking One (Nacalai Tesque, Kyoto, Japan) for 1 hour at room temperature, and then was washed with phosphate-buffered saline. Sera from immunized mice were diluted 1000-fold in PBS, added to each well, and the plate was incubated for 1–2 hours at room temperature. The sera was discarded and each well was washed twice with phosphate-buffered saline. Horseradish-peroxidase–conjugated anti-mouse IgG antibody (GE Healthcare) was diluted 3000-fold with Blocking One, added into each well, and the plate then was incubated for 1 hour at room temperature. Nonbound antibody was discarded, and peroxidase activity of the horseradish-peroxidase–bound antibody was detected using a peroxidase detection kit (Sumitomo Bakelite, Tokyo, Japan).

HCV Inhibition Assay

Inhibition of HCV infection of cultured cells was assayed using HCVpp and HCVcc for infection. HCVpp or

HCVcc was mixed with serum or immunoglobulin that was collected from vaccine-immunized mice and the mixtures then were incubated for 30 minutes at room temperature. Naive Huh7.5.1 cells were inoculated with the virus-antibody mixtures, and after 3 hours the mixtures were removed and the cells were washed once with phosphate-buffered saline. DMEM-10 was added to each well, and the cells were cultured for 72 hours. The cells were lysed and the lysates were assayed as described later.

Western Blot Analysis

The naive Huh7 cells that were used as a negative control and the J6/JFH-1–infected Huh7 cells that were used as a positive control were lysed using Passive Lysis Buffer (Promega), and then centrifuged to remove debris. Protein concentration was quantified using the BCA Protein Assay Kit (Pierce, Rockford, IL). The HCV core of purified HCV particles (0.5 pmol each of Frac 6 and Frac 8) and cell lysate samples (5 μg protein) then were denatured by boiling, resolved on 12% sodium dodecyl sulfate–polyacrylamide gels, and transferred to an Immobilon-P membrane (Millipore, Billerica, MA). Recombinant FLAG-tagged E1 (J6E1/FLAG) and FLAG-tagged E2 (J6E2/FLAG) proteins also were analyzed using 1, 3, 10, and 30 ng protein/lane. The membrane was blocked with BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan), and probed with primary antibodies against HCV core (2H9, 3 μg/mL), E1 (B7567[6], 10 μg/mL), and E2 (AP33, 3 μg/mL; a generous gift from Genentech, Inc, South San Francisco, CA) proteins in Tris-buffered saline containing Tween-20 (20 mmol/L Tris, pH 7.4, 150 mmol/L NaCl, 0.1% [vol/vol] Tween-20 [polyoxyethylene sorbitan monolaurate]) containing 10% (vol/vol) BlockAce. After several washes with Tris-buffered saline containing Tween-20, the membrane was probed with horseradish-peroxidase–conjugated secondary antibody (GE Healthcare, 1:5000 dilution), washed repeatedly, and bound antibodies were detected using ECL-plus (GE Healthcare) and visualized using LAS3000 (Fujifilm, Tokyo, Japan). After antigen detection, the antibody on the membrane was stripped using WB stripping solution (Nacalai Tesque) and the membrane was re-probed with another antibody.

IgG Purification

Mouse IgG was purified using the KAPTIV-GY resin (Technogen). Briefly, approximately 0.5 mL of the KAPTIV-GY resin was loaded onto an empty column (Bio-Rad) and was washed with loading buffer (50 mmol/L bis-Tris, pH 6.8). Sera from mice at 7 weeks after immunization were applied onto the column and the column was washed with loading buffer. IgG then was eluted with glycine-HCl (pH 3.0) and the eluates were neutralized with Tris-HCl (pH 9.0). The eluted IgG (iHCV-IgG) was concentrated using an Amicon Ultra-15 (molecular weight cut-off 30,000) and IgG purity was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and subsequent Coomassie brilliant blue staining. Control mouse IgG was prepared similarly from saline-injected mice (Cont-IgG).

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Supplementary Table 1. Composition of Purified HCV Particles After Ultracentrifugation

	Volume, mL	Core, pmol	RNA, copies	Protein, mg	Infectivity, FFU/mL
Culture supernatant	5720	164.0 (100%)	5.0×10^{11} (100%)	4.2×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose cushion purification	1.00	106.5 (65.0%)	2.5×10^{11} (49.5%)	1.4 (0.03%)	7.2×10^{7} (41.2%)
Culture supernatant	10,500	301.7 (100%)	9.1×10^{11} (100%)	7.8×10^{3} (100%)	3.0×10^{4} (100%)
Sucrose gradient purification (Frac 6)	1.05	103.0 (34.1%)	1.8×10^{11} (19.8%)	0.7 (0.01%)	3.6×10^{7} (11.9%)
Sucrose gradient purification (Frac 8)	1.05	56.6 (18.8%)	9.0×10^{10} (9.9%)	4.3 (0.06%)	5.7×10^{7} (18.8%)

大学病院の非肝臓内科における HBs 抗原および HCV 抗体陽性者に対する肝疾患診療の実態

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水田　敏彦　　藤井　進　　髙﨑　光浩　　尾崎　岩太　　杉岡　隆
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＜原　　著＞

大学病院の非肝臓内科における HBs 抗原および HCV 抗体陽性者に対する肝疾患診療の実態

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要旨：ウイルス性肝炎対策において，医療機関における肝臓内科と非肝臓内科間の院内連携の実態は不明である．そこで，当院における非肝臓内科での HBs 抗原および HCV 抗体の測定状況，ならびに陽性者における肝炎診療の実態を明らかにすることを目的に，本研究を実施した．2010 年 1 月〜12 月，肝臓内科以外の 27 診療科で HBs 抗原または HCV 抗体を測定した症例（重複例を除外，HBs 抗原 6,648 例，HCV 抗体 6,612 例）を対象とした．HBs 抗原陽性 126 例（1.9％），推定 B 型慢性肝炎 66 例（1.0％），HCV 抗体陽性 487 例（7.4％），推定 HCV キャリア 369 例（5.6％），推定 C 型慢性肝炎 244 例（3.7％）であった．高い感染率にもかかわらず，HBV キャリアの 79％，HCV キャリアの 82％ は肝臓内科との連携がなく，そのうち，前者の 89％，後者の 97％ においてウイルス性肝炎に関する診療方針の記載もみられなかった．肝炎ウイルスキャリア率が高く，かつ肝臓専門医を擁する医療機関において，適切な肝炎診療を行うための院内連携システムの構築が早急に必要である．

索引用語：　肝炎ウイルス　　HBs抗原　　HCV抗体　　スクリーニング
　　　　　　院内連携システム

緒　　言

我が国における HCV 感染者は，諸外国に比して高齢化が進んでおり，肝癌発症のリスクが高いことが指摘されている[1)]．国内における肝癌死亡率は HCV 感染率に一致して西高東低の分布を示すが，なかでも佐賀県は全国有数の C 型肝炎高罹患地区であり，非常に高い肝癌死亡率を有している[2)~4)]．平成 23 年度の肝および肝内胆管の悪性新生物の粗死亡率（10 万人対）は全国の 25.3 に対し佐賀県は 44.0 であり[5)]，依然，全国ワース

ト 1 位の状況が続いている．B 型肝炎については，C 型肝炎ほど全国的な罹患率の差はないが，佐賀県は比較的高率である[6)]．佐賀県のこれまでの検診データより，既に肝癌死亡は減少に転じてきているものの，今後 10 年間は依然として現在の全国平均よりも高い肝癌死亡率が予想されており，更なるウイルス性肝炎対策が急務である[7)]．

肝炎対策の問題点として，検診での肝炎ウイルス検査受検率が低いこと，そのうち要精密と判断された受検者の医療機関受診率が低いこと，また，たとえ医療機関を受診しても必ずしも適切な医療が提供されていないこと等が既に指摘されており，各方面で対策が講じられつつある[8)9)]．一方で，大学病院などの医療機関においては，術前スクリーニング等で肝炎ウイルス検査を行う機会が多いが，その陽性者が適切な肝炎診療を受けているかは不明である．平成 23 年 5 月 16 日に厚生労働省から出された「肝炎対策の推進に関する基

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2）佐賀大学医学部総合診療部
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4）佐賀大学医学部附属病院医療情報部
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＜受付日2012年10月6日＞＜採択日2013年4月1日＞

a)

```
        Testing for HBsAg
            n=7,712
              │
   ┌──────────┤
Duplication   │
  n=837       │
              │
          n=6,875
              │
          department
       ┌──────┴────────┐
   hepatology      non-hepatology
   n=227 (3%)      n=6,648 (97%)
                       │
                     HBsAg
               ┌───────┴───────┐
              (+)             (−)
          n=126 (1.9%)    n=6,522 (98%)
               │
      probable chronic hepatitis
          ┌────┴─────┐
         (+)        (−)
      n=66 (52%)  n=60 (48%)
          │
   hepatologist consultation
   ┌──────┴──────┐  ┌──────┴──────┐
  (+)          (−) (+)          (−)
 n=13        n=53  n=13        n=47
 (20%)      (80%) (22%)       (78%)
```

b)

```
        Testing for anti-HCV
            n=7,607
              │
   ┌──────────┤
Duplication   │
  n=800       │
              │
          n=6,807
              │
          department
       ┌──────┴────────┐
   hepatology      non-hepatology
   n=195 (3%)      n=6,612 (97%)
                       │
                    Anti-HCV
               ┌───────┴───────┐
              (+)             (−)
          n=487 (7.4%)    n=6,125 (93%)
               │
      ┌────────┴────────┐
   <4.0 S/CO         ≥4.0 S/CO
   n=118 (24%)       n=369 (76%)
                         │
              probable chronic hepatitis
                 ┌───────┴───────┐
                (+)             (−)
            n=244 (66%)      n=125 (34%)
                 │
        hepatologist consultation
     ┌──────┴──────┐  ┌──────┴──────┐
    (+)          (−) (+)          (−)
   n=46        n=198 n=21        n=104
   (19%)      (81%) (17%)       (83%)
```
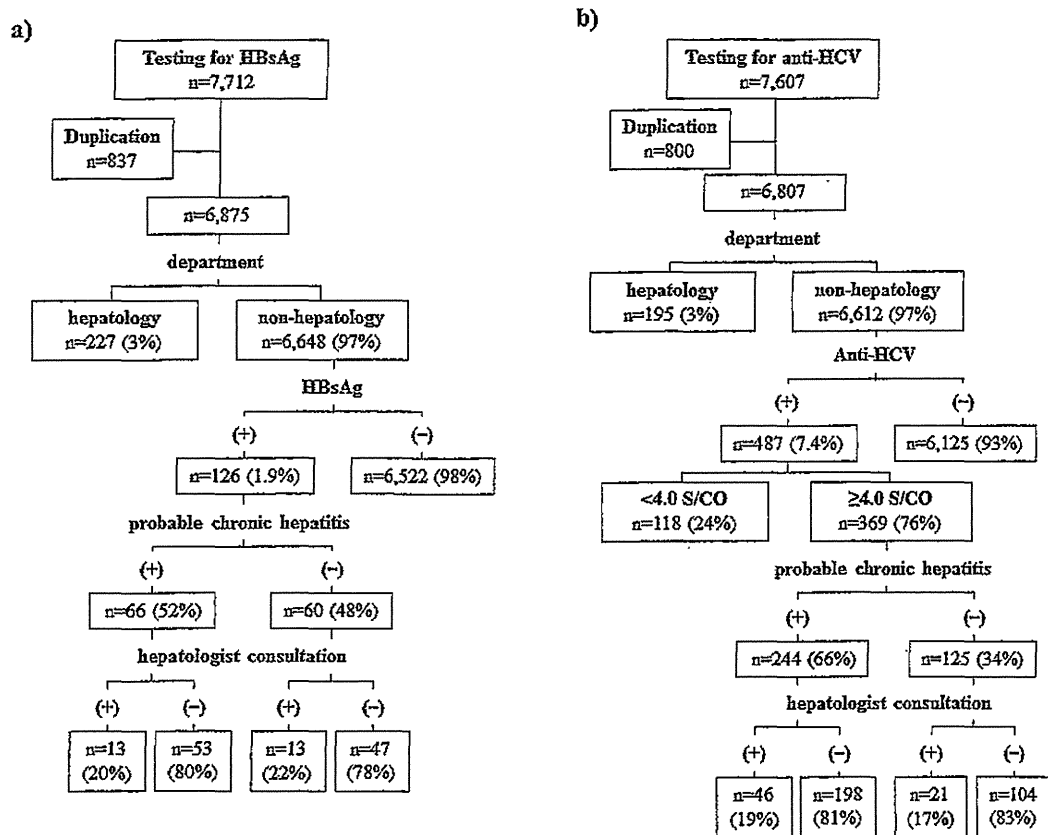
Fig. 1　Flow chart of the study subjects who underwent screening tests for a) HBsAg and b) anti-HCV.

本的な指針」[10]では，国および地方公共団体が医療機関に対し，手術前等に行われる肝炎ウイルス検査の結果について受検者に適切に説明を行うよう要請されており，その実態調査を行うことが目標として掲げられている．

そこで，大学病院における肝臓内科以外でのHBs抗原およびHCV抗体の測定状況，ならびに陽性者への肝炎診療の実態を明らかにすることを目的に，本研究を実施した．

対象および方法

2010年1月から12月まで,佐賀大学医学部附属病院にてHBs抗原（HBsAg：hepatitis B surface antigen）またはHCV抗体(anti-HCV：anti-hepatitis C virus antibody)検査を実施された症例（HBsAg 7,712例，anti-HCV 7,607例）のうち，重複例を除き肝臓内科以外の27診療科（非肝臓内科）で測定された症例（HBsAg 6,648例，anti-HCV 6,612例）を対象とした（Fig.1）．HBsAg は ARCHITECT-HBsAg QT（Abbott Japan,

Tokyo, Japan)で測定し，0.05 IU/ml 以上を陽性と判定した．Anti-HCV は ARCHITECT-HCV(Abbott Japan, Tokyo, Japan) で測定し，1.0 S/CO 以上を陽性と判定し，1.0～4.0 S/CO を低力価，4.0～12.0 S/CO を中力価，12.0 S/CO 以上を高力価と判別した[11]．HBsAg 陽性例においては,血小板数<15×10^4/μL, AST≦ALT, ALT≧31 IU/L のいずれかを満たす症例は慢性肝炎の可能性があるものと仮定し，推定B型慢性肝炎と定義した．Anti-HCV 陽性例においては,中・高力価を HCV キャリアと仮定し，そのうち血小板数<15×10^4/μL, AST≦ALT, ALT≧31 IU/L のいずれかを満たす症例を推定C型慢性肝炎と定義した．

ウイルスキャリアにおいて肝臓内科へのコンサルトがあった群（介入群）となかった群（非介入群）の背景因子を比較し,有意差検定は Mann-Whitney U 検定，χ^2検定または Fisher の直接確率検定を用い，$p<0.05$ を有意とした．ウイルるキャリアにおいて肝臓内科へのコンサルトに寄与する因子について，単変量および多変量のロジスティック回帰分析を施行した．多変量

a)

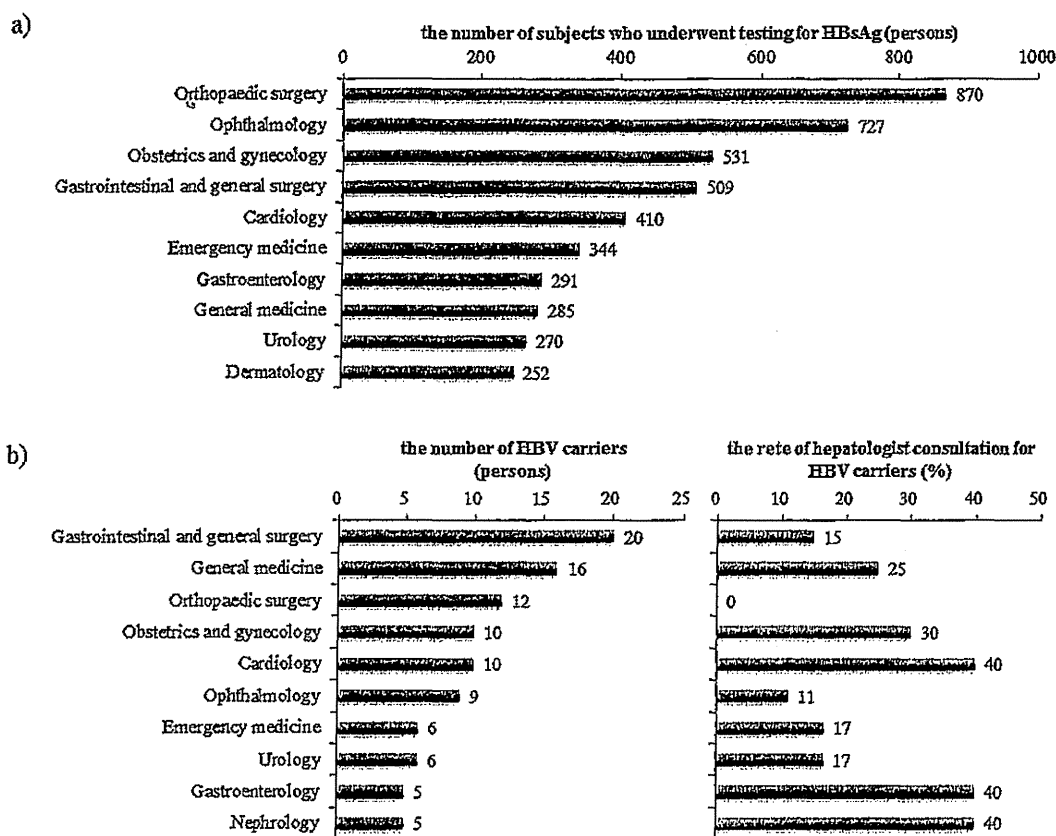the number of subjects who underwent testing for HBsAg (persons)

b)

Fig. 2　a) The number of subjects who underwent HBsAg screening test in the top 10 non-hepatology departments. b) The number of HBV carriers in the top 10 non-hepatology departments, and the rate of hepatologist consultation.

解析の因子は，単変量解析で $p<0.15$ の因子を選択した．診療科は内科系（総合診療科，消化器内科，循環器内科，腎臓内科，呼吸器内科，リウマチ・膠原病内科，血液・腫瘍内科，神経内科，感染症内科）と非内科系（一般・消化器外科，心臓血管外科，呼吸器外科，脳神経外科，整形外科，形成外科，眼科，耳鼻咽喉科，産婦人科，泌尿器科，皮膚科，歯科口腔外科，精神科，小児科，麻酔科，救急科，放射線科，検査科）に分類した．また，同様の解析を，肝炎の有無別に層別化して施行した．

本研究は佐賀大学医学部附属病院の倫理委員会の許可を得て実施し，対象者の個人情報保護に十分配慮した上でデータの抽出を行った．

結　　果

a）HBsAg 検査

HBsAg 測定者 6,648 例のうち，陽性者（HBV キャリア）は 126 例（1.9％）であった．そのうち血小板数＜

15×10^4/μL，AST≦ALT，ALT≧31 IU/L を満たすものはそれぞれ 26 例，39 例，37 例であり，推定 B 型慢性肝炎は 66 例（1.0％）であった（Fig. 1a）．27 診療科のうち測定数の多い上位 10 科は，整形外科 870 例，眼科 727 例，産婦人科 531 例，一般・消化器外科 509 例，循環器内科 410 例，救急科 344 例，消化器内科 291 例，総合診療科 285 例，泌尿器科 270 例，皮膚科 252 例であった(Fig. 2a)．陽性者は，一般・消化器外科 20 例，総合診療科 16 例，整形外科 12 例，産婦人科 10 例，循環器内科 10 例，眼科 9 例，救急科 6 例，泌尿器科 6 例，消化器内科 5 例，腎臓内科 5 例の順で多く，これら上位 10 科のうち肝臓内科へのコンサルト率は内科系診療科で比較的高い傾向がみられ，整形外科や眼科は低率であった(Fig. 2b)．陽性率では総合診療科 5.6％，腎臓内科 4.0％，呼吸器内科 4.0％，一般・消化器外科 3.9％，循環器内科 2.4％，泌尿器科 2.2％，リウマチ・膠原病内科 2.2％，産婦人科 1.9％，救急科 1.7％，消化器内科 1.7％ の順で高率であった．