Saito A, Nomaguchi M, Kono K, Iwatani Y, Yokoyama M, Yasutomi Y, Sato H, Shioda T, Sugiura W, Matano T, Adachi A, Nakayama EE, Akari H.	TRIM5 genotypes in cynomolgus monkeys primarily influence inter-individual diversity in susceptibility to monkey-tropic human immunodeficiency virus type 1.	Journal of General Virology	94	1318-1324	2013
Saito A, Akari H.	Macaque-tropic human immunodeficiency virus type 1 (HIV-1mt): Break out of the host factors.	Frontiers in Microbiology	4	187	2013
Shime H, Kojima A, Maruyama A, Saito Y, H. Oshiumi H, Matsumoto M, Seya T.	Myeloid-derived suppressor cells confer tumor-suppressive functions on natural killer cells via polyinosinic:polycytidylic acid treatment in mouse tumor model.	J. Innate Immun.	DOI:10.1159/000355126		2013
Shiota T, Li TC, Yoshizaki S, Kato T, Wakita T, Ishii K.	Hepatitis E virus capsid C-terminal region is essential for the viral life-cycle: Implication in viral genome encapsidation and particle stabilization.	Journal of Virology	87	6031-3036	2013
Suzuki R, Matsuda M, Watashi K, Aizaki H, Matsuura Y, Wakita T, Suzuki T.	Signal peptidase complex subunit 1 participates in the assembly of hepatitis C virus through an interaction with E2 and NS2.	PLoS Pathog	9	e1003589	2013
Suzuki T, Oshiumi H, Miyashita M, Aly HH, Matsumoto M, Seya T.	Cell type-specific subcellular localization of phospho-TBK1 in response to cytoplasmic viral DNA.	PLos ONE	8	e83639	2013

Takaki H, Honada K, Atarashi K, Kobayashi F, Ebihara T, Oshiumi H, Matsumoto M, Shingai M, Seya T.	MAVS-dependent IRF3/7 bypass of interferon b-induction restricts the response to measles infection in CD150Tg mouse bone marrow-derived dendritic cells.	Mol Immunol.	57	100-110	2013
Takaki H, Takeda M, Tahara M, Shingai M, Oshiumi H, Matsumoto M, Seya T.	The MyD88 pathway in plasmacytoid and $CD4^+$ dendritic cells primarily triggers type I interferon production against measles virus in a mouse infection model.	J. Immunol.	191	4740-4747	2013
Tanaka Y, Suenaga T, Matsumoto M, Seya T, Arase H.	Herpesvirus 6 Glycoproteins B (gB), gH, gL and gQ are Necessary and Sufficient for Cell-to-Cell Fusion.	J. Virol.	87	10900-10903	2013
Tatematsu M, Nishikawa F, Seya T, Matsumoto M.	Toll-like receptor 3 recognizes incomplete stem structures in single-stranded viral RNA.	Nat. Commun.	4	1833	2013
Yoshida T, Suzuki S, Iwasaki Y, Kaneko A, Saito A, Enomoto Y, Higashino A, Watanabe A, Suzuki J, Inoue K, Kuroda T, Takada M, Ito R, Ito M, Akari H.	Efficient *in vivo* depletion of $CD8^+$ T lymphocytes in common marmosets by novel CD8 monoclonal antibody administration.	Immunology Letters	154	12-17	2013
Yoshida T, Omatsu T, Saito A, Katakai Y, Iwasaki Y, Kurosawa T, Hamano M, Higashino A, Nakamura S, Takasaki T, Yasutomi Y, Kurane I, Akari H.	Dynamics of cellular immune responses in the acute phase of dengue virus infection.	Archives of Virology	158	1209-1220	2013

Watashi K, Liang G, Iwamoto M, Marusawa H, Uchida N, Daito T, Kitamura K, Muramatsu M, Ohashi H, Kiyohara T, Suzuki R, Li J, Tong S, Tanaka Y, Murata K, Aizaki H, Wakita T.	Interleukin-1 and tumor necrosis factor-alpha trigger restriction of hepatitis B virus infection via a cytidine deaminase AID.	J Biol Chem.	288	31715-31727	2013
Kumeta H, Sakakibara H, Enokizono Y, Ogura K, Horiuchi M, Matsumoto M, Seya T, Inagaki F.	The N-terminal domain of TIR domain-containing adaptor molecule-1, TICAM-1.	J. Biomolec. NMR	DOI:10.1007/s10858-014-9819-1		2014
Okamoto M, Oshiumi H, Azuma M, Matsumoto M, Seya T.	IPS-1 is essential for type III interferon production by hepatocytes and dendritic cells in response to hepatitis C virus infection.	J. Immunol.			2014
Suzuki R, Ishikawa T, Konishi E, Matsuda M, Watashi K, Aizaki H, Takasaki T, Wakita T.	Production of single-round infectious chimeric flaviviruses with DNA-based Japanese encephalitis virus replicon.	J Gen Virol.	95	60-65	2014
Tatematsu M, Seya T, Matsumoto M.	Beyond double-stranded RNA: TLR3 signaling in RNA-induced immune responses.	Biochem. J.	45	195-201	2014
Li TC, Yang, T, Shiota T, Yoshizaki S, Yoshida H, Saito M, Imagawa T, Malbas F, Lupisan S, Oshitani H, Wakita T, and Ishii K.	Molecular detection of hepatitis E virus in rivers in the Philippines.	American Journal of Tropical Medicine and Hygine		In press	2014

Moi ML, Takasaki T, Omatsu T, Nakamura S, Katakai Y, Ami Y, Yuriko S, Saijo M, Akari H, Kurane I.	Demonstration of marmosets (*Callithrix jacchus*) as a non-human primate model for secondary dengue virus infection: high levels of viremia and serotype cross-reactive antibody responses consistent with secondary infection of humans.	Journal of General Virology		In press	2014
Naruse TK, Akari H, Matano T, Kimura A.	Divergence and diversity of ULBP2 genes in rhesus and cynomolgus macaques.	Immunogenetics		In press	2014
石井孝司、清原知子	A 型肝炎ワクチン	BIO Clinica	28	25-29	2013
鈴木亮介、小西英二	フラビウイルスのリバースジェネティクス	ウイルス	63(1)	13-22	2013
村山麻子、加藤孝宣	NS5B-RNA ポリメラーゼの構造と機能.	肝胆膵	67(6)	918-923	2013
石井孝司	A 型肝炎、E 型肝炎	臨床と微生物	41	72-78	2014

Ⅳ．研究成果の刊行物・別刷

GASTROENTEROLOGY 2013;145:447–455

BASIC AND TRANSLATIONAL—LIVER

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

DAISUKE AKAZAWA,[1,2] MASAKI MORIYAMA,[1,2] HIROSHI YOKOKAWA,[1,2] NORIAKI OMI,[1] NORIYUKI WATANABE,[2] TOMOKO DATE,[2] KENICHI MORIKAWA,[2,3] HIDEKI AIZAKI,[2] KOJI ISHII,[2] TAKANOBU KATO,[2] HIDENORI MOCHIZUKI,[1] NORIKO NAKAMURA,[1] and TAKAJI WAKITA[2]

[1]*Pharmaceutical Research Laboratories, Toray Industries, Inc, Kanagawa, Japan;* [2]*Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan;* [3]*Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan*

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

Abbreviations used in this paper: Cont, control; DMEM, Dulbecco's modified Eagle medium; Frac, fraction; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HCVpp, hepatitis C virus pseudoparticle; IC50, 50% inhibitory concentration; iHCV, vaccine immunized hepatitis C virus; rE1, recombinant E1; rE2, recombinant E2; RTD-PCR, real-time detection reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, albumin enhancer/promoter urokinase plasminogen activator.

0016-5085/$36.00
http://dx.doi.org/10.1053/j.gastro.2013.05.007

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 $\times$ 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 $\pm$ 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

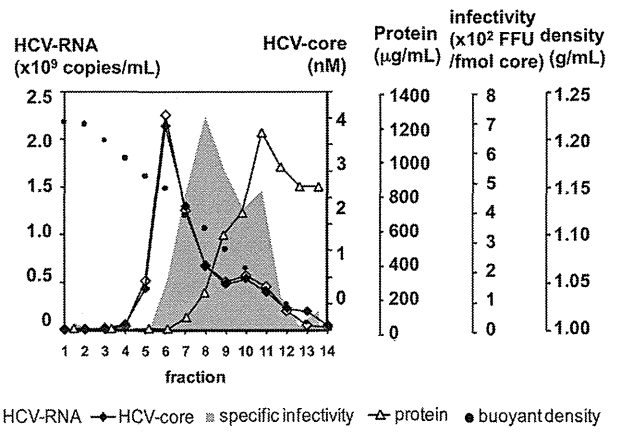

Figure 1. HCV-particle purification from cell cultures. Huh7 cells were inoculated with J6/JFH-1 chimeric HCVcc (multiplicity of infection, 0.2) and infected cells were passaged into CellSTACK (Corning, Corning, NY). The cell supernatant was collected and concentrated by ultrafiltration using a hollow 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 2*A* 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 2*C–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 2*C–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

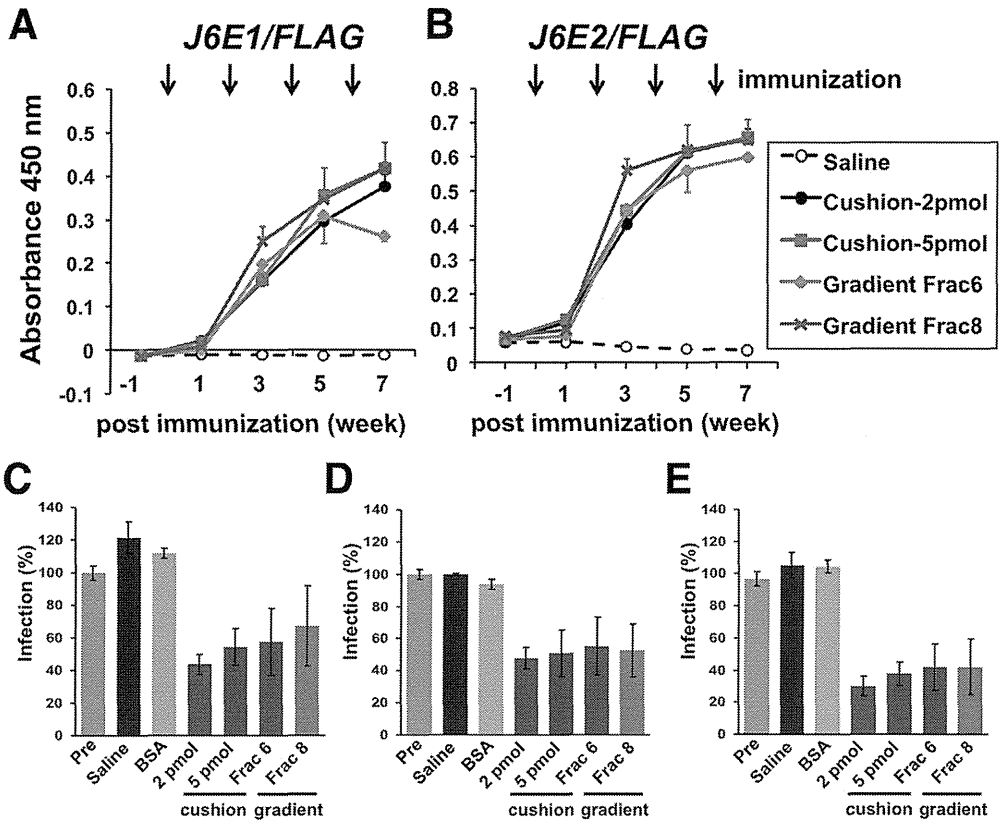

Figure 2. Cross-genotypic neutralizing effect of the sera from HCV-particle–immunized mice. HCV particles that were purified using a sucrose cushion (2 or 5 pmol HCV core) or sucrose gradient fractionation (Frac 6 and Frac 8, 2 pmol HCV core each) were inactivated by ultraviolet irradiation. The inactivated 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 4*A*). 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 4*B*). 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

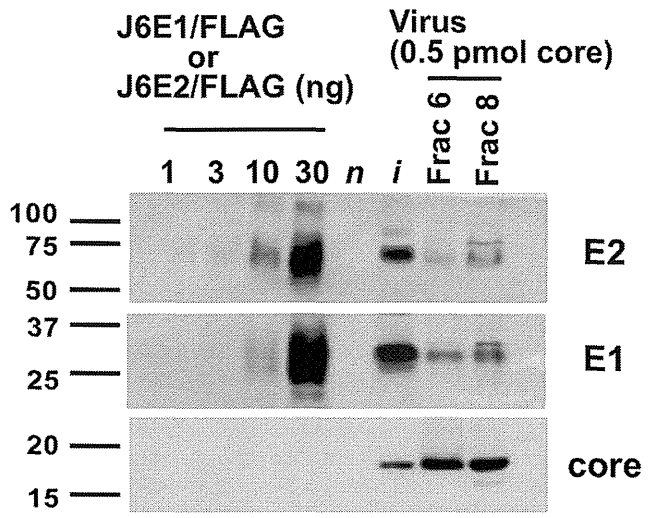

Figure 3. Western blot analysis of envelope proteins in purified HCV particles. The HCV core (0.5 pmol) of purified HCV particles (Frac 6 and Frac 8), as well as 1–30 ng of recombinant envelope proteins (J6E1/FLAG or J6E2/FLAG) were resolved on 12% sodium dodecyl sulfate–polyacrylamide gels and transferred to a polyvinylidene difluoride membrane. Naive 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 5*A*). 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 5*B*). 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 naive 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 5*C*). These data indicate that anti-HCV envelope neutralizing antibodies were induced in mice by injection of the HCV-particle vaccine.

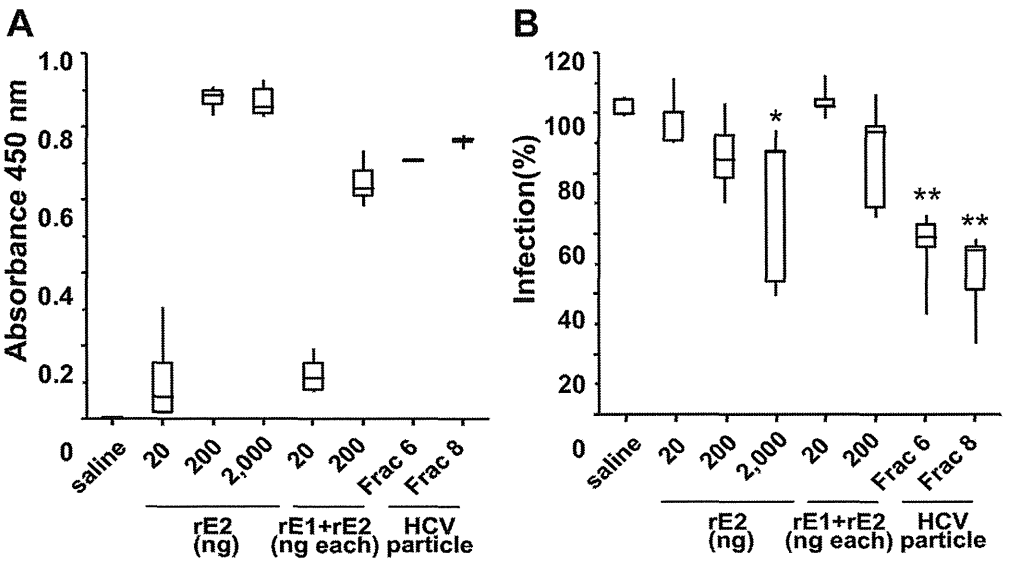

Figure 4. Neutralizing effects of sera from HCV-component and HCV-particle vaccine-immunized mice. BALB/c mice (n = 5) were immunized with recombinant proteins (20, 200, or 2000 ng of FLAG-tagged E2, 20 or 200 ng each of FLAG-tagged E1 and FLAG-tagged E2), or with purified HCV particles (2 pmol HCV core 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).

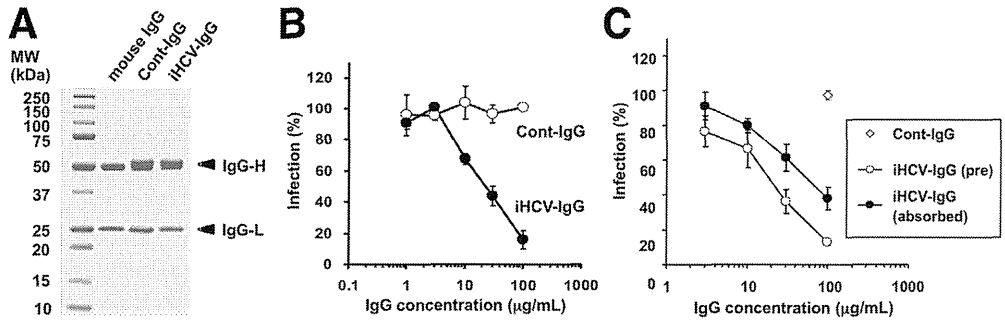

Figure 5. IgG purified from the sera of HCV-particle–immunized mice had a neutralizing effect against HCV infection in vitro. (*A*) IgG from the sera of mice at 7 weeks after immunization (iHCV-IgG) was purified using a KAPTIV-GY resin. Control mouse IgG was prepared similarly from saline-injected mice (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 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

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

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

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.

References

1. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med 2005;11:791-796.
2. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci U S A 2005;102:9294-9299.
3. Lindenbach BD, Evans MJ, Syder AJ, et al. Complete replication of hepatitis C virus in cell culture. Science 2005;309:623-626.
4. Mercer DF, Schiller DE, Elliott JF, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.
5. Halliday J, Klenerman P, Barnes E. Vaccination for hepatitis C virus: closing in on an evasive target. Expert Rev Vaccines 2011; 10:659-672.
6. Murayama A, Date T, Morikawa K, et al. The NS3 helicase and NS5B-to-3'X regions are important for efficient hepatitis C virus strain JFH-1 replication in Huh7 cells. J Virol 2007;81:8030-8040.
7. Akazawa D, Date T, Morikawa K, et al. Characterization of infectious hepatitis C virus from liver-derived cell lines. Biochem Biophys Res Commun 2008;377:747-751.
8. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complex. J Exp Med 2003;197:633-642.
9. Kolykhalov AA, Agapov EV, Blight KJ, et al. Transmission of hepatitis C by intrahepatic inoculation with transcribed RNA. Science 1997; 277:570-574.
10. Wakita T, Wands JR. Specific inhibition of hepatitis C virus expression by antisense oligodeoxynucleotides. J Biol Chem 1994; 269:14205-14210.
11. Yanagi M, Purcell RH, Emerson SU, et al. Hepatitis C virus: an infectious molecular clone of a second major genotype (2a) and lack of viability of intertypic 1a and 2a chimeras. Virology 1999;262:250-263.
12. Miyanari Y, Atsuzawa K, Usuda N, et al. The lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 2007;9:1089-1097.
13. Aizaki H, Morikawa K, Fukasawa M, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. J Virol 2008;82:5715-5724.
14. Hijikata M, Kato N, Ootsuyama Y, et al. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc Natl Acad Sci U S A 1991;88:5547-5551.
15. Chang KS, Jiang J, Cai Z, et al. Human apolipoprotein e is required for infectivity and production of hepatitis C virus in cell culture. J Virol 2007;81:13783-13793.
16. Jiang J, Luo G. Apolipoprotein E but not B is required for the formation of infectious hepatitis C virus particles. J Virol 2009; 83:12680-12691.
17. Benga WJ, Krieger SE, Dimitrova M, et al. Apolipoprotein E interacts with hepatitis C virus nonstructural protein 5A and determines assembly of infectious particles. Hepatology 2010; 51:43-53.
18. Houghton M, Abrignani S. Prospects for a vaccine against the hepatitis C virus. Nature 2005;436:961-966.
19. Torresi J, Johnson D, Wedemeyer H. Progress in the development of preventive and therapeutic vaccines for hepatitis C virus. J Hepatol 2011;54:1273-1285.
20. Nevens F, Roskams T, Van Vlierberghe H, et al. A pilot study of therapeutic vaccination with envelope protein E1 in 35 patients with chronic hepatitis C. Hepatology 2003;38:1289-1296.
21. Leroux-Roels G, Depla E, Hulstaert F, et al. A candidate vaccine based on the hepatitis C E1 protein: tolerability and immunogenicity in healthy volunteers. Vaccine 2004;22:3080-3086.
22. Haller AA, Lauer GM, King TH, et al. Whole recombinant yeast-based immunotherapy induces potent T cell responses targeting HCV NS3 and core proteins. Vaccine 2007;25:1452-1463.
23. Drane D, Maraskovsky E, Gibson R, et al. Priming of CD4+ and CD8+ T cell responses using a HCV core ISCOMATRIX vaccine: a phase I study in healthy volunteers. Hum Vaccin 2009;5:151-157.
24. Frey SE, Houghton M, Coates S, et al. Safety and immunogenicity of HCV E1E2 vaccine adjuvanted with MF59 administered to healthy adults. Vaccine 2010;28:6367-6373.
25. Klade CS, Wedemeyer H, Berg T, et al. Therapeutic vaccination of chronic hepatitis C nonresponder patients with the peptide vaccine IC41. Gastroenterology 2008;134:1385-1395.
26. Wedemeyer H, Schuller E, Schlaphoff V, et al. Therapeutic vaccine IC41 as late add-on to standard treatment in patients with chronic hepatitis C. Vaccine 2009;27:5142-5151.
27. Yutani S, Komatsu N, Shichijo S, et al. Phase I clinical study of a peptide vaccination for hepatitis C virus-infected patients with different human leukocyte antigen-class I-A alleles. Cancer Sci 2009; 100:1935-1942.
28. Firbas C, Boehm T, Buerger V, et al. Immunogenicity and safety of different injection routes and schedules of IC41, a hepatitis C virus (HCV) peptide vaccine. Vaccine 2010;28:2397-2407.
29. Gowans EJ, Roberts S, Jones K, et al. A phase I clinical trial of dendritic cell immunotherapy in HCV-infected individuals. J Hepatol 2010;53:599-603.
30. Alvarez-Lajonchere L, Shoukry NH, Grá B, et al. Immunogenicity of CIGB-230, a therapeutic DNA vaccine preparation, in HCV-chronically infected individuals in a phase I clinical trial. J Viral Hepat 2009; 16:156-167.
31. Barnes E, Antonella F, Aston S, et al. Phase I trial of a highly immunogenic T cell vaccine for hepatitis c virus based on novel adenoviral vectors from rare serotypes. Hepatology 2009;50(Suppl 4):105A.
32. Honnet G, Veron L, Olivier D, et al. Phase I clinical trial with a novel HCV therapeutic vaccine tg4040: interim results of biomarker and immunomonitoring analyzes. Hepatology 2009;50(Suppl 4):413A.
33. Stamataki Z, Coates S, Evans MJ, et al. Hepatitis C virus envelope glycoprotein immunization of rodents elicits cross-reactive neutralizing antibodies. Vaccine 2007;25:7773-7784.
34. Law M, Maruyama T, Lewis J, et al. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. Nat Med 2008;14:25-27.
35. Garrone P, Fluckiger AC, Mangeot PE, et al. A prime-boost strategy using virus-like particles pseudotyped for HCV proteins triggers broadly neutralizing antibodies in macaques. Sci Transl Med 2011; 3:94ra71.
36. Steinmann D, Barth H, Gissler B, et al. Inhibition of hepatitis C virus-like particle binding to target cells by antiviral antibodies in acute and chronic hepatitis C. J Virol 2004;78:9030-9040.
37. Barth H, Ulsenheimer A, Pape GR, et al. Uptake and presentation of hepatitis C virus-like particles by human dendritic cells. Blood 2005; 105:3605-3614.

38. Elmowalid GA, Qiao M, Jeong SH, et al. Immunization with hepatitis C virus-like particles results in control of hepatitis C virus infection in chimpanzees. Proc Natl Acad Sci U S A 2007;104: 8427–8432.
39. Akazawa D, Morikawa K, Omi N, et al. Production and characterization of HCV particles from serum-free culture. Vaccine 2011; 29:4821–4828.

Received March 9, 2012. Accepted May 5, 2013.

Reprint requests

Address requests for reprints to: Takaji Wakita, MD, PhD, Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan. e-mail: wakita@nih.go.jp; fax: (81) 3-5285-1161.

Acknowledgments

The Huh7.5.1 cell line was a kind gift from Dr Francis V. Chisari; AP33 was a generous gift from Genentech, Inc; and hepatitis C virus complementary DNA of the H77 and J6CF strains were kindly provided by Dr Robert Purcell and Dr Jens Bukh. The authors thank Dr Junichi Tanabe for his much appreciated contribution to this work.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; from the Ministry of Health, Labour and Welfare of Japan; from the Ministry of Education, Culture, Sports, Science and Technology of Japan; from the National Institute of Biomedical Innovation; and from the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

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).

References

1. Kato T, Date T, Murayama A, et al. Cell culture and infection system for hepatitis C virus. Nat Protoc 2006;1:2334–2339.
2. van den Hoff MJ, Moorman AF, Lamers WH. Electroporation in 'intracellular' buffer increases cell survival. Nucleic Acids Res 1992; 20:2902.
3. Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. Gastroenterology 1999;116:636–642.
4. Bartosch B, Dubuisson J, Cosset FL. Infectious hepatitis C virus pseudo-particles containing functional E1-E2 envelope protein complex. J Exp Med 2003;197:633–642.
5. Akazawa D, Date T, Morikawa K, et al. CD81 expression is important for the permissiveness of Huh7 cell clones for heterogeneous hepatitis C virus infection. J Virol 2007;81:5036–5045.
6. Murakami K, Ishii K, Ishihara Y, et al. Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b. Virology 2006;351:381–392.

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%)

Structures and interface mapping of the TIR domain-containing adaptor molecules involved in interferon signaling

Yoshiaki Enokizono[a,1], Hiroyuki Kumeta[a,1], Kenji Funami[b,1], Masataka Horiuchi[a], Joy Sarmiento[c], Kazuo Yamashita[c], Daron M. Standley[c], Misako Matsumoto[b], Tsukasa Seya[b,2], and Fuyuhiko Inagaki[a,2]

[a]Department of Structural Biology, Faculty of Advanced Life Science, Hokkaido University, N-21, W-11, Kita-ku, Sapporo 001-0021, Japan; [b]Graduate School of Medicine, Hokkaido University, N-15, W-7, Kita-ku, Sapporo 060-8638, Japan; and [c]Systems Immunology Lab, Immunology Frontier Research Center, Osaka University, Suita, Osaka 565-0871, Japan

Edited by Shizuo Akira, Osaka University, Osaka, Japan, and approved October 29, 2013 (received for review January 3, 2013)

Homotypic and heterotypic interactions between Toll/interleukin-1 receptor (TIR) domains in Toll-like receptors (TLRs) and downstream adaptors are essential to evoke innate immune responses. However, such oligomerization properties present intrinsic difficulties in structural studies of TIR domains. Here, using BB-loop mutations that disrupt homotypic interactions, we determined the structures of the monomeric TIR domain-containing adaptor molecule (TICAM)-1 and TICAM-2 TIR domains. Docking of the monomeric structures, together with yeast two hybrid-based mutagenesis assays, reveals that the homotypic interaction between TICAM-2 TIR is indispensable to present a scaffold for recruiting the monomeric moiety of the TICAM-1 TIR dimer. This result proposes a unique idea that oligomerization of upstream TIR domains is crucial for binding of downstream TIR domains. Furthermore, the bivalent nature of each TIR domain dimer can generate a large signaling complex under the activated TLRs, which would recruit downstream signaling molecules efficiently. This model is consistent with previous reports that BB-loop mutants completely abrogate downstream signaling.

innate immunity | TLR signaling | MyD88-independent pathway | TRAM | TRIF

The extracellular domain of toll-like receptor 4 (TLR4) specifically binds lipopolysaccharides (LPSs) from Gram-negative bacteria, inducing dimerization and leading to the dimerization of cytosolic Toll/interleukin-1 receptor (TIR) domains. This activated conformation of TLR4 recruits the TIR domain of a downstream adaptor molecule, TIR domain-containing adaptor molecule-2 (TICAM-2) [also known as TRIF-related adaptor molecule (TRAM)], that subsequently recruits the TIR domain of another adaptor molecule, TIR domain-containing adaptor molecule-1 (TICAM-1) [also known as TIR domain-containing adaptor inducing IFN-β (TRIF)] (1–3) at endosomes. Eventually this process activates IFN response factors and generates type-I interferons (IFNs) (4–7). Elucidation of the homotypic and heterotypic interactions between TICAM-1 and TICAM-2 is essential for understanding of TLR4-mediated type-I IFN generation (8).

A large number of TIR domain structures, including receptors and adaptors, have been determined by X-ray crystallography and NMR. The receptors include TLR1 (9), TLR2 (10), and IL-1R accessory protein-like (IL-1RAPL) (11). Adaptors include myeloid differentiation factor 88 (MyD88) (12) and MyD88 adaptor-like (Mal) (13, 14). In addition, AtTIR (15, 16) derived from *Arabidopsis thaliana* and PdTIR (17) from bacteria have been solved. Each of these TIR domain structures has a ferredoxin fold with five β-strands (βA–βE), five α-helices (αA–αE), and loops connecting β-strands and α-helices (9). Although homotypic interactions of the TIR domains have been proposed based on the crystal structures, most proposed models have small interacting surfaces, possibly due to crystal contacts. Recently, however, a crystal structure of the TLR10 TIR domain was reported that forms a homotypic dimer mediated by the loop connecting βB and αB (designated "BB-loop") (18). Interestingly, BB-loop mutations in TLR4 were reported to be dominant-negative and abrogated downstream signaling (19). TICAM-1 and TICAM-2 harboring BB-loop mutations are also dominant-negative and unable to form homotypic interactions (1, 2), reinforcing the importance of BB-loop–mediated homotypic dimer formation in signal propagation.

Despite extensive structural studies, it is not known why homotypic interactions are essential for downstream signaling (20–27). To address this issue, it is necessary to discriminate residues required for homotypic and those required for heterotypic interactions. Here, we first determine the structures of the monomeric BB-loop mutants of the TICAM-1 and TICAM-2 TIR domains using NMR. Then, based on the solution structures of the BB-loop mutants, coupled mutagenesis/yeast two-hybrid experiments, and restrained docking calculations, we show that the homotypic interaction of TICAM-2 TIR is essential to form a scaffold for recruiting the TICAM-1 TIR domain.

Results

Monomerization of the TICAM-1 and TICAM-2 TIR Domains by BB-Loop Mutations. The TIR domains of TICAM-1 (387–545) and TICAM-2 (75–235) (Fig. 1*A*) oligomerized and precipitated in aqueous solution at ~200 μM concentration, so monomerization was

Significance

Toll/interleukin-1 receptor (TIR) homology domains mediate the downstream signaling of Toll-like receptors (TLRs), but the molecular mechanism of the signal transduction is elusive on the structural basis. Here, we determined the structures of TIR domain-containing adaptor molecule (TICAM) 1 and TICAM-2 TIR domains and demonstrated their homotypic and heterotypic interaction surfaces. Both TICAM-1 and TICAM-2 TIR domains form a BB-loop–mediated homodimer. The dimerization of TICAM-2 TIR presents an interaction surface for TICAM-1 TIR. The present result is consistent with the notion that the BB-loop mutant is dominant negative and that dimerization of upstream TIRs is crucial for recruiting downstream TIRs.

Author contributions: F.I. designed research; Y.E., K.F., M.H., M.M., and T.S. performed research; Y.E., H.K., J.S., K.Y., D.M.S., and F.I. analyzed data; and D.M.S., T.S., and F.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The chemical shift assignments and NOE and dihedral restraint data have been deposited in the BioMagResBank, www.bmrb.wisc.edu [accession nos. 18883 (TICAM-1) and 18882 (TICAM-2)]. The atomic coordinates for the ensemble have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 2mlx (TICAM-1) and 2mlw (TICAM-2)].

[1]Y.E., H.K., and K.F. contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: seya-tu@pop.med.hokudai.ac.jp or finagaki@pharm.hokudai.ac.jp.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1222811110/-/DCSupplemental.

indispensable for structure determination by NMR. BB-loop mutants of TICAM-1 and TICAM-2 are known to be dominant-negative and unable to form homotypic interactions (1, 2). Thus, we prepared P434H and C117H (P116H) mutants of the TICAM-1 and TICAM-2 TIR domains, respectively (Fig. 1*B*) [hereafter designated TICAM-1 P434H and TICAM-2 C117H (TICAM-2 P116H)] and analyzed their homotypic interactions using yeast two-hybrid experiments. Yeast two-hybrid experiments showed that the homotypic interaction is disrupted by the BB-loop mutation (Fig. 1*C*), consistent with previous reports (1, 2) and NMR observation of both wild types and BB-loop mutants (SI Text and Fig. S1).

NMR Structures of the TICAM-1 P434H and TICAM-2 C117H Mutants. The solution structures of the TICAM-1 P434H and TICAM-2 C117H mutants were determined based on distance and dihedral angle constraints. Structural statistics for the final 20 conformers of each protein are summarized in Table S1. The core structures, consisting of residues other than the BB-loops and N-terminal and C-terminal regions, were well defined. The root-mean-square-deviation (rmsd) of the core backbone atoms (Cα, N, C′) of TICAM-1 P434H (395–427 and 442–527) and TICAM-2 C117H (83–110, 132–215) were 0.45 Å and 0.50 Å, respectively (Fig. 2 *A* and *B*). The global structures of both mutants were comprised of five parallel β-strands surrounded by six or seven α-helices and loops that connect β-strands and α-helices. Following the conventional nomenclature for TIR domains, the five strands in TICAM-1 were designated βA(397–400), βB(424–427), βC(451–455), βD (486–491), and βE(511–514), and the six helices in TICAM-1 were designated αA(406–419), αB(441–449), αC(462–474), αD (501–507), αE(520–528), and αE′(530–538) with a kink at residue 529 (Fig. 2*C*). Similarly, TICAM-2 C117H also contained five strands designated βA(81–85), βB(109–112), βC(134–138), βD(169–173), and βE(193–195) and seven α-helices, designated αA(90–101), αB(125–129), αC(142–152), αC′(156–161), αD (186–191), αE(202–210), and αE′(212–232) (Fig. 2*D*). In both structures, the conformation of the BB-loop was not well defined due to broadening of the NMR signals, resulting in insufficient NOE distance restraints. (Fig. 2 *A* and *B*).

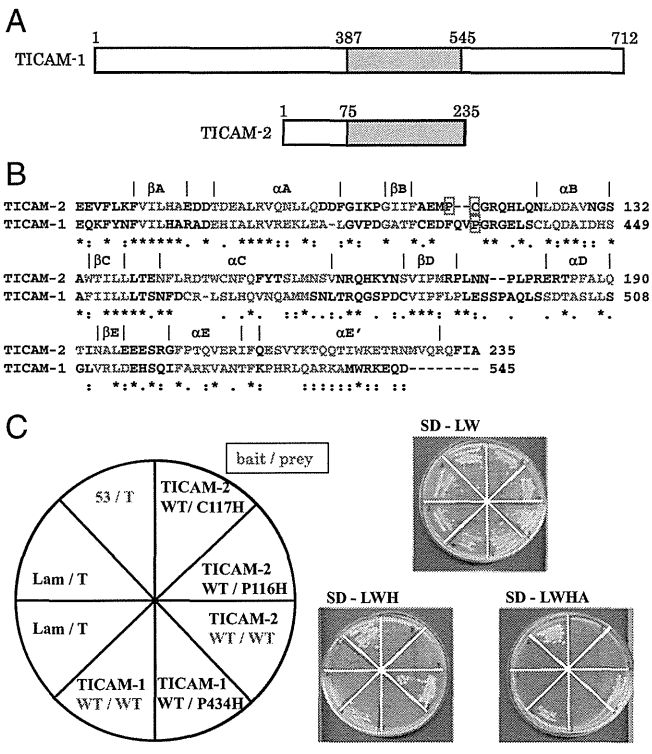

Fig. 1. Homotypic interaction of TICAM-1 and TICAM-2 TIR domains. (*A*) TIR domains of TICAM-1 and TICAM-2. The gray boxes represent the TIR domains of TICAM-1 and TICAM-2. (*B*) Sequence alignment of the TIR domains of human TICAM-1 and human TICAM-2. Amino acid residues involved in the β-sheet and α-helix are shown in blue and red, respectively. The residues on the BB-loop enclosed by a red line were substituted by His for the solution structure determination in this study. Numbers at the right side of the sequences correspond to the residue number in human TICAM-1 and TICAM-2. (C) Yeast two-hybrid analysis of homotypic interaction in TICAM-1 and TICAM-2. Pro434 of TICAM-1, Pro116 and Cys117 of TICAM-2 in the BB-loop were substituted by His residue. These mutants disrupted homotypic interaction of the TIR domain in TICAM-1 and TICAM-2. p53/T-antigen and Lamine/T-antigen were used as positive and negative controls in the yeast two-hybrid assay, respectively. SD-LW, SD-LWH, and SD-LWHA indicate a synthetic dropout medium lacking Leu and Trp, lacking Leu, Trp, and His, and that further lacking Ade, respectively.

Electrostatic surface potentials of TICAM-1 P434H and TICAM-2 C117H are shown in Fig. 2 *E* and *F*, respectively. TICAM-1 P434H has an extensive basic surface comprised of αE (Arg522, Lys523) and αE′ (Lys529, Arg532, Arg536, Lys537, Arg541, Lys542). In contrast, TICAM-2 C117H has an extensive acidic surface comprised of the AA-loop (Glu87, Asp88, Asp89) and the αA-helix (Asp91, Glu92, Asp102, Asp103).

A Dali search (28) revealed that the structure of the TIR domain of TICAM-1 is most similar to that of TICAM-2, with a z-score of 9.6 and an rmsd of 3.8 Å for the structured region (Cα 122 atoms), followed by the TIR domains of TLR2 (z-score 9.2), TLR1 (z-score 8.8), TLR10 (z-score 8.7), IL-1RAPL (z-score 8.5), and MyD88 (z-score 7.5). A structural superposition was made to align the secondary structures and functionally important residues in TICAM-1, TICAM-2, and other TIR domains using the MATRAS program (29, 30) (Fig. S2). Intriguingly, the residues that form an extensive acidic surface in the TICAM-2 TIR domain and an extensive basic surface in the TICAM-1 TIR domain are not conserved in other TIR domains, suggesting that these residues might be responsible for specific interaction between TICAM-1 and TICAM-2.

Acidic Region of TICAM-2 and Basic Region of TICAM-1 Are Essential for Heterotypic Interaction. To investigate heterotypic interactions between the TICAM-1 and TICAM-2 TIR domains, further yeast two-hybrid experiments were carried out (31). Because previous studies showed that oligomerization of the TICAM-2 TIR domain is essential for its interaction with the TICAM-1 TIR domain (1, 2, 27), the wild-type TICAM-2 TIR domain was used as bait, and the TICAM-1 TIR domain mutants were used as prey. To search for residues that are essential for the interaction with TICAM-2, basic residues within TICAM-1 αE and αE′-helices were selected and mutated to alanine in the first round of two-hybrid experiments. The TICAM-1 mutants harboring the BB-loop mutation, P434H/R512A, P434H/K529A, and P434H/R532A, could interact with TICAM-2, but the mutants R522A/K523A and P434H/R522A/K523A could not (Fig. 3*A*). These results indicate that Arg522 and Lys523 of TICAM-1, but not Pro434, Arg512, Lys529, and Arg532, are crucial for direct interaction with TICAM-2, consistent with the observation that TICAM-1 oligomerization is not required for interaction with the TICAM-2 TIR domain. We designated the region involving Arg522 and Lys523 the "RK site." Actually, Pro434 is located on the opposite side of the RK site in TICAM-1, indicating that the homotypic and heterotypic interaction sites in the TICAM-1 TIR domain are distinct (Fig. 3*B*). Interestingly, the BB-loop mutant could still interact with wild-type TICAM-2, implying that monomeric TICAM-1 retains its ability to bind to the TIR domain of the TICAM-2 wild-type dimer.

After finding two basic residues in the TICAM-2 binding surface of TICAM-1, we searched for acidic residues in TICAM-2 that complemented the interaction. Arrays of two or three acidic residues from the TIR domain of TICAM-2 (E87/D88/D89 in the AA-loop, D91/E92 in the N-terminal side of the αA-helix, D102 / D103 in the C-terminal side of the αA-helix, D126/D127 in the αB-helix, and E197/E198 in the EE-loop) were substituted with alanine.