は、それぞれEL-4/CN2、EL-4/N3-4細胞による刺激後、顕著にIFN-γを産生することを確認した（図３）。51Cr遊離法によるCTL誘導の検討ではHCV蛋白を発現しないEL4細胞をtarget細胞とした時、emp、HCV-CN2、HCV-N25を投与したマウスから採取したいずれの脾細胞においても細胞傷害活性はほとんど見られなかった。しかしtarget細胞として、EL-4/CN2またはEL-4/NS3-4細胞を用いたところ、emp投与群由来の脾細胞において、細胞障害活性は見られなかったが、HCV-CN2、HCV-N25投与群由来の脾細胞は、それぞれEL-4/CN2、EL-4/N3-4細胞による刺激後、細胞傷害活性がみられることを確認し、この活性はHCV蛋白を発現しないEL4細胞で刺激した場合は見られなかったことから、HCV蛋白特異的な細胞傷害活性がHCV-DNAワクチンにより誘導されていることが認められた（図４）。

（４）HCV 蛋白発現腫瘍細胞接種マウスを用いた in vivo における HCV 特異的細胞性免疫誘導能の評価

HCV-DNAワクチンを投与したC57BL/6マウスに、HCV蛋白発現腫瘍細胞（EL-4/CN2またはEL-4/N3-4細胞）をマウス側腹部皮下に移植し、７日後に腫瘍の大きさを評価したところ、腫瘍の大きさは、emp投与群に比べHCV-DNA ワクチン（HCV-CN2、HCV-N25）投与群で有意に減少し、マウス体内においてHCV抗原に対して強い細胞性免疫が誘導されていることが認められた（図５）。

D. 考察

HCV 感染症は肝炎から、肝硬変、肝癌へと進行する慢性感染症疾患である。現在用いられている治療法においても治療効果を得られない患者は多く、新規の治療法、治療薬の開発は急務となっている。一方、HCV が病態を進行させるには極めて長い期間が必要であり、生涯を通じて HCV をコントロールし、寛快される例も存在することから、適切な免疫反応を強く誘導すれば治療に結びつく可能性があると考えられる。これらのことから、本研究では HCV に対する免疫反応の解析ならびに免疫療法、治療用ワクチンの開発を試みた。治療用ワクチンは現在では主に癌において試みられており、実用化もされている。治療用ワクチンを考える場合、癌と HCV の大きな違いは癌においては標的となる抗原は限られており、目的とするのは抗原に対し、如何に強い細胞性免疫を誘導するかということである。一方、HCV のような感染症を考える場合は、まずどの抗原が治療の標的に適しているのか、何故、自然感染では標的抗原に対する免疫反応が十分でないのか等の新たな視点の研究が必要である。本研究ではベクターに対する反応等の複雑な免疫反応を排除し、より抗原特異的な細胞性免疫を主体とした免疫反応を誘導すべく HCV 各種遺伝子を用いた DNA ワクチンを開発した。現在、この DNA ワクチンと HCV 遺伝子組み込み Tg マウスを用いて HCV 抗原と免疫反応の検討を行っている。今後、これらの機構が解明されれば治療用ワクチンの開発に繋がること考えられる。

E. 結論

HCV 各遺伝子に対する DNA ワクチンを構築し、非常に強い HCV 特異的細胞性免疫を誘導した。

F. 研究発表

1. 論文発表

1) Yoshida,T., Saito,A., Iwasaki,Y.,Iijima,S., Kurosawa,T., Katakai,Y., Yasutomi,Y.,Reimann,K.A., Hayakawa,T. and Akari,H. Characterization of natural killer cells in tamarins: a technical basis for studies of innate immunity. Frontiers Microbiol. 2011 in press

2) Xing, Li., Wang, J.C., Li, T-C., Yasutomi,Y., Lara,J., Khurdyakaov,Y., Schofield D., Emerson,S., Purcell,R., Takeda,N., Miyamura,T. and Holland,R.C. Spatial configuration of hepatitis E virus antigenic domain. J.Virol. 2011;85:1117-1124.

3) Chono,H., Matsumoto,K., Tsuda,H., Saito,N., Lee,K.., Kim,S., Shibata,H., Ageyama,N., Terao,K., Yasutomi,Y., Mineno J., Kim,S., Inoue,M. and Kato,I. Acquistion of HIV-1 resistance in T lymphocytes using an ACA-specific E.coli mRNA interferase. Human Gene Ther. 2011;22:35-43.

4) Saito,A., Nomaguchi,M., Iijima,S., Lee,Y-J., Kono,K., Nakayama,E.E., Shioda,T., Yasutomi,Y., Adachi,A., Matano,T., Akari,H. A novel monkey-tropic HIV-1 derivative encoding only minimal SIV sequences can replicate in cynomolgus monkeys. Micorbes Infect. 2011;13:58-64.

5) Naruse,T.K., Zhiyong,C., Yanagida,R., Yamashita,T., Saito,Y., Mori,K., Akari,H., Yasutomi,Y., Matano,T. and Kimura,A. Diversity of MHC class I genes in Burmese-origine rhesus macaques. Immunogenetics 2010;62:601-611.

6) Okabayashi,S., Uchida,K., Nakayama,H., Ohno,C., Hanari,K., Goto,I. and Yasutomi,Y. Periventricular Leucomalacia (PVL)-like lesions in two neonatal cynomolgus monkeys (macaca fascicularis). J.Comp.Pathol. 2010 Epub

7) Yasuhiro Yasutomi. Establishment of Specific Pathogen-Free Macaque Colonies in Tsukuba Primate Research Center of Japan for AIDS research. Vaccine 2010:B75-B77.

8) Fujimoto,K., Takano,J., Narita,T., Hanari,K., Shimozawa,N., Sankai,T., Yoshida T., Terao,K., Kurata,T. and Yasutomi,Y. Simian Retrovirus type D infection in a colony of cynomolgus monkeys. Comp.Med. 2010;60:51-53.

9) Cueno,M.E., Karamatsu,K., Yasutomi, Y., Laurena,A.C. and Okamoto.T. Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant. Transgenic Res. 2010;19:889-895.

2. 学会発表

「国内」

1) 塩釜ゆみ子、松原明弘、河岡義裕、保富康宏：ヘルパーT細胞（Th）制御によるインフルエンザ感染病態とワクチン効果の検討第13回日本ワクチン学会　東京

2010年12月11日－12日

2) 保富康宏：アジュバント分子組み込みエイズウイルスの開発（シンポジウム）第24回日本エイズ学会、東京、2010年11月24日～26日

3) 齊藤暁、河野健、黒石歩、中山英美、塩田達雄、足立昭夫、野間口雅子、保富康宏、俣野哲朗、明里宏文：カニクイザルTRIM5 alleleがサル指向性HIV-1の増殖に与えるインパクト第24回日本エイズ学会、東京、2010年11月24日～26日

4) 下澤律浩、高橋一郎、柴田宏昭、伊奈田宏康、野阪哲哉、保富康宏：カニクイザル体細胞に由来する人工多能性幹細胞の作製第57回日本実験動物学会、京都、2010年5月12日～14日

5) 塩釜ゆみ子、松原明弘、河岡義裕、保富康宏：IL-4とそのアンタゴニストを用いたヘルパーT細胞反応調節によるインフルエンザ感染病態とアレルギー反応第58回日本ウイルス学会　徳島2010年11月7日－9日

「国際」

1)Yusuke TSUJIMURA, Yasuhiro YASUTOMI : Therapeutic effects of Ag85B in allergic asthma by inducing not only Th1 response but also Interleukin-17, -22 production.14th International Congress of Immunology. Kobe Japan, August 22-27,2010.

2) Akihiro Matsubara, Kenta Watanabe, Mitsuo Kawano, Satoru Mizuno, Yusuke Tsujimura, Hiroyasu Inada, Masayuki Fukumura, Isamu Sugawara, Tetsuya Nosaka, Kazuhiro Matsuo, Yasuhiro Yasutomi: Intranasal immunization with replication-deficient recombinant human parainfluenza type 2 virus-Ag85B showed protective effects against Mycobacterium tuberculosis infection. TB Vaccines. A Second Global Forum, Tallinn, Estonia September 21-24,2010.

3) Intranasal immunization with replication-deficient recombinant human parainfluenza type 2 virus-Ag85B showed protective effects against Mycobacterium tuberculosis infection. TB Vaccines. A Second Global Forum, Tallinn, Estonia September 21-24,2010.

4) Yasuhiro Yasutomi: Gene delivery of suppressor of cytokine signaling 1 (SOCS1) showed therapeutic effects to autoimmune myocarditis in mice.2md Annual International Congress of Cardiology, Shanghai, China, December 7-9,2010.

5) Y. Tsujimura and Y. Yasutomi. Therapeutic effects of Ag85B in allergic asthma by inducing not only Th1 response but also Interleukin-17, -22 production. Immunity in the Respiratory Tract : Challenges of the Lung Environment. Keystone Symposia, Vancouver, Canada, February 26 – March 3, 2011.

G. 知的所有権の出願・取得状況

1. 特許取得

なし

2. 実用新案登録

(1) パラミクソウイルスベクターを用いた経鼻噴霧型結核ワクチン

2010 年 11 月 1 日 (PCT/JP2010/069435)

(2) 遺伝子導入用ウイルスベクターの製造方法

2011 年 2 月 8 日 (特願 2011-025234)

厚生労働科学研究費補助金（肝炎等克服緊急対策研究事業）
分担研究報告書

C型肝炎ウイルスのトランスパッケージング型粒子を用いた感染機構の解析

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研究要旨　　複数の遺伝子型／株由来の構造蛋白質を用いて、C 型肝炎ウイルス（HCV）の１回感染性トランスパッケージング型粒子(HCVtcp)を産生させる事が出来た。これらのHCVtcpおよびレトロウイルス表面にHCVのエンベロープ蛋白質を被せた偽ウイルス（HCVpp)について、抗 CD81 抗体および抗 ApoE 抗体による感染中和を解析したところ、抗 CD81 抗体は HCVtcp および HCVpp の感染を中和したものの、HCVtcp は HCVpp に比べてその感受性が顕著に高かった。また抗 ApoE 抗体は HCVtcp の感染を中和したものの、HCVpp の感染には影響を及ぼさなかった。この事から、HCVtcp と HCVpp はその粒子構造や感染機構が異なる事が示唆され、HCVtcp は HCV 本来の性質を反映した感染過程の解析に有用であると考えられた。

A．研究目的

C 型肝炎ウイルス（HCV）感染は、持続感染化し肝臓癌に至る重大な感染症であり、現在のウイルス保留者数は世界で1.7億人、国内で 200 万人と言われている。その多くが慢性肝炎から肝硬変、肝癌へと移行し、肝癌による死亡者は国内で年間 3 万人を超えている。インターフェロンおよびリバビリンによる治療が行われているが、治療効果は十分とは言えず、また重い副作用もあるため、新しい治療薬や治療用ワクチンの開発が望まれている。

本研究では、現在開発中の HCV 遺伝子発現組換えワクチニアウイルスワクチンの治療効果における中和抗体誘導能の関与を明らかにする為に、１回感染性トランスパッケージング型 HCV 粒子(HCVtcp)を産生させた。この粒子の感染機構について、これまで汎用されているレトロウイルス表面に HCV のエンベロープ蛋白質を被せた偽ウイルス（HCVpp)と比較検討を行った。

B．研究方法

複数の遺伝子型／株の遺伝子配列を用い、HCV の core から NS2 領域を発現するプラスミドを作製した。一方で、JFH-1 株のレプリコン（構造蛋白質領域を欠損し、EMCV-IRES および F luc 遺伝子を挿入したもの）cDNA を polI promoter/terminater 間に挿入したレプリコンプラスミドを作製した。これらの２種類のプラスミドを Huh7.5.1 細胞へトランスフェクションして HCVtcp を作製した。また同じ遺伝子型由来の HCVpp も作製した。これらの粒子を用いて、Huh7.5.1 細胞への感染における抗 CD81 抗体および抗 ApoE 抗体の影響を調べた。

C．研究結果

遺伝子型 2a の JFH-1 株および J6 株のい

ずれの構造蛋白質を用いた場合でも、HCVtcp の産生が認められた。JFH-1 株に適合変異を導入すると、さらに高い感染価が得られた。遺伝子型 1b の構造蛋白質を用いた場合、Con1 株および TH 株においては E1 領域に適合変異を導入する事により、HCVtcp の産生が認められた。一方で構造蛋白質は発現しているにも関わらず、遺伝子型 1a の H77c 株およびその他２種類の遺伝子型 1b の株については、感染性粒子の産生は認められなかった。

さらに HCVtcp と HCVpp について、抗 CD81 抗体および抗 ApoE 抗体による感染中和を調べたところ、抗 CD81 抗体は HCVtcp および HCVpp の感染を中和したが、HCVtcp は HCVpp に比べて抗 CD81 抗体に対し著しく高い感受性を示した。また抗 ApoE 抗体は HCVtcp の感染を中和したものの、HCVpp の感染は中和しなかった。

D．考察

以上の結果から、HCVtcp と HCVpp は粒子構造や感染機構が異なり、このため細胞侵入過程においても違いがある可能性が示唆された。HCVtcp は HCVpp のように他のウイルス蛋白を利用しておらず、また HCV が増殖可能な肝臓由来の細胞で作製する事から、HCVpp に比べてより HCV 本来の性質を反映した解析ツールであると考えられる。これを用いる事により、候補ワクチンを免疫した動物の血清中の中和抗体価についての適切な評価が可能となる事が期待される。

E．結論

複数の遺伝子型／株由来の構造蛋白質を用いた HCVtcp を産生させる事が出来た。HCVtcp と HCVpp は粒子構造や感染機構が異なる事が示唆され、HCVtcp は HCVpp に比べて、より HCV 本来の性質を反映した解析ツールとして有用であると考えられた。

F．研究発表

1.論文発表

1) Moriishi K, Shoji I, Mori Y, Suzuki R, Suzuki T, Kataoka C, Matsuura Y. Involvement of PA28gamma in the propagation of hepatitis C virus. **Hepatology.** 52: 411-420 (2010)

2) Masaki T, Suzuki R, Saeed M, Mori K, Matsuda M, Aizaki H, Ishii K, Maki N, Miyamura T, Matsuura Y, Wakita T, Suzuki T. Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription. **J. Virol.** 84: 5824-5835 (2010)

2.学会発表

1) Suzuki R, Alazawa D, Ishii K, Matsuura Y, Wakita T, Suzuki T. Entry mechanisms of hepatitis C virus examined by trans-complemented particles. 9th International Symposium on Positive-Strand RNA Viruses. Atlanta, GA, USA 2010. 5. 17-23.

2) Suzuki R, Alazawa D, Ishii K, Matsuura Y, Wakita T, Suzuki T. Efficient production of trans-complemented hepatitis C virus particles: Use for study of viral entry process. 17th International Meeting on Hepatitis C Virus and Related Viruses. Yokohama, Japan 2010. 9. 10-14.

3) 鈴木亮介、斎藤憲司、赤澤大輔、石井孝司、松浦善治、脇田隆字、鈴木哲朗. C 型肝炎ウイルスの *trans*-packaging型粒子を用いた感染機構の解析. 日本ウイルス学会第58回学術集会, 徳島, 2010年11月7-9日.

4) 鈴木亮介、相崎英樹、脇田隆字、鈴木哲朗. 分割ユビキチン法を利用したHCV NS2と結合する宿主因子の探索およびウイルス粒子形成への関与. 第33回日本分子生物学会年会, 神戸市, 2010年12月7-10日.

G. 知的所有権の出願・登録状況

1. 特許取得

C型肝炎ウイルス粒子高生産系

（脇田隆字、石井孝司、鈴木哲朗、鈴木亮介、宮村達男、田邉純一、曽根三郎）

特許番号：1956087、登録国：ＥＰ

2. 実用新案登録

なし。

3. その他

なし。

Ⅲ．　研究成果の刊行に関する一覧表

別紙５

研究成果の刊行に関する一覧表

雑誌

Amako Y. et al. （小原）	Pathogenesis of hepatitis C virus infection in *Tupaia belangeri*	*J. Virology*	84(1)	303-311	2010
Satoh K. et al. （小原）	Natural killer cells target HCV core proteins during the innate immune response in HCV transgenic mice	*J. Med. Virol.*	82(9)	1545-1553	2010
Nuriya H. et al. （小原）	Detection of hepatitis B and C viruses in almost all hepatocytes by modified PCR-based *in situ*	*J. Clin. Microviol.*	48(11)	3843-3851	2010
Kasama Y. et al. （小原）	Persistent expression of the full genome of hepatitis C virus in B cells induces spontaneous development of B-cell lymphomas *in vivo*	*Blood*	116(23)	4926-4933	2010
Weng L. et al. （小原）	Sphingomyelin activates hepatitis C virus RNA polymerase in a genotype specific manner	*J. Virology*	84(22)	11761-11770	2010
Arai M. et al. （小原）	Establishment of infectious HCV virion-producing cells with newly designed full-genome replicon RNA	*Arch. Virol.*	156	295-304	2011
Yoshikawa K. et al. （小原）	Incorporation of biaryl units into the 5′ and 3′ ends of sense and antisense strands of siRNA duplexes improves strand selectivity and nuclease resistance	*Bioconjugate Chemistry*	22	42-49	2011
Kimura K. et al. （小原）	Role of interleukin-18 in intrahepatic inflammatory cell recruitment in acute liver injury	*J. Leukocyte Biology*	89	433-442	2011
Takano T. et al. （小原）	Translocase of outer mitochondrial membrane 70 is induced by hepatitis C virus and is related to the apoptotic response	*J. Med. Virol.*	83	801-809	2011
Miyashita, M. et al. （瀬谷）	The SKI2-related helicase DDX60 is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling.	*Molec. Cell. Biol.*	in press		2011

Sawahata, R., et al. （瀬谷）	Failure of mycoplasmal lipoprotein MALP-2 to activate NK cells through dendritic cell TLR2.	*Microbes Infect.*	in press		2011
Watanabe A. et al. （瀬谷）	Raftlin mediates cell entry of poly(I:C) to induce TLR3 activation.	*J. Biol. Chem.*	in press		2011
Takaki, H. et al. （瀬谷）	Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-b-inducing potential.	*Molec. Immunol.*	48	497-504	2011
Yabu, M. et al. （瀬谷）	Lactic acid acts on macrophages to induce antigen-dependent IL-17 production from effector/memory helper T cells.	*Int. Immunol.*	23	29-41	2010
Oshiumi H. et al. （瀬谷）	Essential role of Riplet in RIG-I-dependent antiviral innate immune responses.	*Cell host microbe.*	8	496-509	2010
Oshiumi H. et al. （瀬谷）	Hepatitis C virus (HCV) core protein promotes viral replication by abrogating IFN-b-inducing function of DEAD/H BOX 3 (DDX3) helicase.	*PLoS ONE.*	5	e14258	2010
Ehira, N. et al. （瀬谷）	An embryo-specific expressing TGF-beta family protein, growth-differentiation factor 3 (GDF3), augments progression of B16 melanoma.	*J. Exp. Clin. Cancer Res.*	29	135	2010
Ebihara, T. et al. （瀬谷）	Identification of a polyI:C-inducible membrane protein, that participates in dendritic cell-mediated natural killer cell activation.	*J. Exp. Med.*	207	2675-2687	2010
Tatematsu, M. et al. （瀬谷）	Molecular mechanism for activation of Toll/IL-1 receptor domain-containing adaptor molecule-1 (TICAM-1).	*J. Biol. Chem.*	285	20128-20136	2010
Azuma, M. et al. （瀬谷）	The peptide sequence of diacyl lipopeptides determines dendritic cell TLR2-mediated NK activation.	*PLoS ONE*	5	e12550	2010
Kasamatsu, J. et al. （瀬谷）	Phylogenetic and expression analysis of Lamprey Toll-like receptors.	*Dev. Comp. Immunol.*	34	855-865	2010

Akazawa, T. et al. （瀬谷）	Adjuvant engineering for cancer immunotherapy: development of a synthetic TLR2 ligand with increased cell adhesion.	*Cancer Sci.*	101	1596-1603	2010
Oshiumi, H. et al. （瀬谷）	DEAD/H BOX 3 (DDX3) helicase binds the RIG-I adaptor IPS-1 to up-regulate IFN-beta inducing potential.	*Eur. J. Immunol.*	40	940-948	2010
Hirata, N. et al. （瀬谷）	Dendritic cell-derived TNF-a is responsible for development of IL-10-producing $CD4^+$ T cells.	*Cell. Immunol.*	261	37-41	2010
Kubota, N. et al. （瀬谷）	IL-6 and interferon-alpha induced by polyI:C-stimulated bone-marrow-derived dendritic cells regulate peripheral expansion of regulatory T cells.	*Biochem. Biophys. Res. Commun.*	391	1421-1426	2010
Sasai, M. et al. （瀬谷）	Direct binding of TRAF2 and TRAF6 to TICAM-1/TRIF adaptor of the Toll-like receptor 3/4 pathway.	*Molec. Immunol.*	47	1283–1291	2010
Matsumoto, M. et al. （瀬谷）	Antiviral responses induced by the TLR3 pathway. (review)	*Rev. Med. Virol.*	in press		2011
Seya, T. （瀬谷）	Innate immunity and vaccine. (preface)	*Vaccine*	28	8041-8042	2010
Seya, T. et al. （瀬谷）	Pattern-recognition receptors of innate immunity and their application to tumor immunotherapy. (review)	*Cancer Sci.*	101	313-320	2010
.Yoshida,T. et al. （保富）	Characterization of natural killer cells in tamarins: a technical basis for studies of innate immunity.	*Frontiers Microbiol.*	in press		2011
Xing, Li. et al. （保富）	Spatial configuration of hepatitis E virus antigenic domain.	*J.Virol.*	85	1117-1124	2011
Chono,H. et al. （保富）	Acquistion of HIV-1 resistance in T lymphocytes using an ACA-specific E.coli mRNA interferase.	*Human Gene Ther.*	22	35-43	2011
Saito,A. et al. （保富）	A novel monkey-tropic HIV-1 derivative encoding only minimal SIV sequences can replicate in cynomolgus monkeys.	*Micorbes Infect*	13	58-64	2011

Naruse,T.K. et al.（保富）	Diversity of MHC class I genes in Burmese-origine rhesus macaques.	*Immunogenetics*	62	601-611	2010
Okabayashi,S. et al.（保富）	Periventricular Leucomalacia (PVL)-like lesions in two neonatal cynomolgus monkeys (macaca fascicularis)	*J.Comp.Pathol.*	Epub		2010
Yasuhiro Yasutomi.（保富）	Establishment of Specific Pathogen-Free Macaque Colonies in Tsukuba Primate Research Center of Japan for AIDS research.	*Vaccine*	B	75-77.	2010
Fujimoto,K. et al.（保富）	Simian Retrovirus type D infection in a colony of cynomolgus monkeys.	*Comp.Med.*	60	51-53.	2010
Cueno,M.E. et al.（保富）	Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant.	*Transgenic Res.*	19	889-895	2010
Moriishi K. et al.（鈴木）	Involvement of PA28gamma in the propagation of hepatitis C virus.	*Hepatology.*	52	411-420	2010
Masaki T. et al.（鈴木）	Production of infectious hepatitis C virus by using RNA polymerase I-mediated transcription.	*J. Virol.*	84	5824-5835	2010

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Pathogenesis of Hepatitis C Virus Infection in *Tupaia belangeri*▽†

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The lack of a small-animal model has hampered the analysis of hepatitis C virus (HCV) pathogenesis. The tupaia (*Tupaia belangeri*), a tree shrew, has shown susceptibility to HCV infection and has been considered a possible candidate for a small experimental model of HCV infection. However, a longitudinal analysis of HCV-infected tupaias has yet to be described. Here, we provide an analysis of HCV pathogenesis during the course of infection in tupaias over a 3-year period. The animals were inoculated with hepatitis C patient serum HCR6 or viral particles reconstituted from full-length cDNA. In either case, inoculation caused mild hepatitis and intermittent viremia during the acute phase of infection. Histological analysis of infected livers revealed that HCV caused chronic hepatitis that worsened in a time-dependent manner. Liver steatosis, cirrhotic nodules, and accompanying tumorigenesis were also detected. To examine whether infectious virus particles were produced in tupaia livers, naive animals were inoculated with sera from HCV-infected tupaias, which had been confirmed positive for HCV RNA. As a result, the recipient animals also displayed mild hepatitis and intermittent viremia. Quasispecies were also observed in the NS5A region, signaling phylogenic lineage from the original inoculating sequence. Taken together, these data suggest that the tupaia is a practical animal model for experimental studies of HCV infection.

Hepatitis C virus (HCV) is a small enveloped virus that causes chronic hepatitis worldwide (32). HCV belongs to the genus *Hepacivirus* of the family *Flaviviridae*. Its genome comprises 9.6 kb of single-stranded RNA of positive polarity flanked by highly conserved untranslated regions at both the 5′ and 3′ ends (4, 27, 29). The 5′ untranslated region harbors an internal ribosomal entry site (29) that initiates translation of a single open reading frame encoding a large polyprotein comprising about 3,010 amino acids (35). The encoded polyprotein is co- and posttranslationally processed into 10 individual viral proteins (15).

In most cases of human infection, HCV is highly potent and establishes lifelong persistent infection, which progressively leads to chronic hepatitis, liver steatosis, cirrhosis, and hepatocellular carcinoma (9, 16, 21). The most effective therapy for treatment of HCV infection is administration of pegylated interferon combined with ribavirin. However, the combination therapy is an arduous regimen for patients; furthermore, HCV genotype 1b does not respond efficiently (19). The prevailing scientific opinion is that a more viable option than interferon treatment is needed.

The chimpanzee is the only validated animal model for in vivo studies of HCV infection, and it is capable of reproducing most aspects of human infection (5, 18, 23, 28, 35, 36). The chimpanzee is also the only validated animal for testing the authenticity and infectivity of cloned viral sequences (8, 14, 35, 36). However, chimpanzees are relatively rare and expensive experimental subjects. Cross-species transmission from infected chimpanzees to other nonhuman primates has been tested but has proven unsuccessful for all species evaluated (1).

The tupaia (*Tupaia belangeri*), a tree shrew, is a small nonprimate mammal indigenous to certain areas of Southeast Asia (6). It is susceptible to infection with a wide range of human-pathogenic viruses, including hepatitis B viruses (13, 20, 31), and appears to be permissive for HCV infection (33, 34). In an initial report, approximately one-third of inoculated animals exhibited acute, transient infection, although none developed the high-titer sustained viremia characteristic of infection in humans and chimpanzees (33). The short duration of follow-up precluded any observation of liver pathology. In addition to the putative in vivo model, cultured primary hepatocytes from tupaias can be infected with HCV, leading to de novo synthesis of HCV RNA (37). These reports strongly support tupaias as a valid model for experimental studies of HCV infection. However, longitudinal analyses evaluating the clinical development and pathology of HCV-infected tupaias have yet to be exam-

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TABLE 1. Experimental HCV infections performed in this study

Tupaia no.	Inoculum		Biopsy/sacrifice[b]
	Type	Quantity (GE/tupaia)[a]	
Group I[c]			
Tup.4	RCV	1×10^7	84, 94/144 wk p.i.
Tup.5	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.6	HCR6	6×10^5	95, 105/155 wk p.i.
Tup.8	RCV	1×10^7	84, 94/144 wk p.i.
Group II[d]			
Tup.9	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.10	Tup.5 (5 wk p.i.)	1×10^2	NT
Tup.11	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.12	Tup.8 (10 wk p.i.)	1×10^2	NT
Tup.13	Tup.4 (8 wk p.i.)	1×10^2	NT
Tup.14	Tup.4 (8 wk p.i.)	1×10^2	NT
Group III[e]			
Tup.15	None		92/100 wk
Tup.17	None		92/100 wk
Tup.38	None		242 wk
Tup.39	None		242 wk

[a] Viral RNA GE/tupaia was estimated by Quantitative real-time RT-PCR (GE, genome equivalents; sensitivity > 10 GE/ml serum).
[b] Liver biopsy was performed at indicated time-point. p.i., postinoculation; NT, not tested.
[c] Group I, primary infection experiment in which 1-year-old animals were inoculated with two different types of inocula.
[d] Group II, reinfection experiment, where HCV RNA-positive sera from Group I experimental infections were passaged to naive animals.
[e] GroupIII, no-infection control.

ined. In the present study, we describe the clinical development and pathology of HCV-infected tupaias over an approximately 3-year time course.

MATERIALS AND METHODS

Animals. Table 1 summarizes the tupaias used in this study. Tupaias born in laboratory captivity were obtained from the Laboratory Animal Center at the Kunming Institute of Zoology (Chinese Academy of Sciences). Tupaias were imported with permission from the Convention on International Trade in Endangered Species of Wild Fauna and Flora (7), quarantined for medical inspection, and housed individually in standard rat cages supplied with filtered air. The animals were fed a daily regimen of eggs, fruit, and the CMS-1 commercial diet for marmosets (CLEA, Japan). Their appetites and feces were carefully monitored. Animal care and experimental handling conformed to study guidelines established by the Subcommittee on Laboratory Animal Care at the Tokyo Metropolitan Institute of Science.

Patient serum used for animal infection. HCV genotype 1b serum, designated HCR6, was obtained from a patient with chronic active hepatitis C. The infectious titer of HCR6 was determined in chimpanzee and Molt4 cells and denoted plasma K (HCR6) by Shimizu et al. (24). The HCR6 serum exhibited a PCR titer of 6×10^6 genome equivalents/ml and an infectious titer of 3.7×10^4 50% chimpanzee infectious doses/ml. Serum aliquots were frozen at −80°C until they were used.

Virion reconstitution of cloned HCV. As described previously, pHCR6 (genotype 1b; 9,611 nucleotides; GenBank accession no. AY045720) is a plasmid carrying HCV genomic cDNA cloned from HCR6 serum (30). pHCR6Rz was designed for precisely trimmed RNA expression, with the entire genomic region of pHCR6Rz recloned under the control of the T7 promoter and the 5′ and 3′ distal ends flanked by hammerhead- and hepatitis D virus ribozyme-encoding sequences, respectively (22, 25).

For molecular reconstitution of HCV particles, pHCR6Rz was transfected into IMY-N9 cells as described previously (12). Briefly, semiconfluent IMY-N9 cells in 100-mm plastic dishes were transfected with 15 μg of plasmid using 40 μl of cationic lipids (DMRIE-C reagent; Life Technology) in accordance with the manufacturer's instructions. Five hours after transfection, the cells were infected with AdexCAT7 (2) (kindly provided by Y. Matsuura) at a multiplicity of infection of 20. After infection, the culture medium was replaced with Hepato-STIM (Becton Dickinson). The culture supernatants were collected at 24 h postinfection and stored at −80°C.

Virus inoculation and collection of serum samples. Animals were infected at 6 months of age. The anesthetic agent, ketamine hydrochloride, was administered intramuscularly at 50 mg/kg body weight prior to virus inoculation and bleeding of the tupaias. The inocula were introduced intravenously at 6×10^5 genome equivalents/animal for patient serum HCR6 and 1×10^7 genome equivalents/animal for reconstituted virions derived from the pHCR6Rz inoculation. Blood samples were drawn from infected and control animals pre- and postinfection. Briefly, the animals were bled weekly for 20 weeks and biweekly thereafter. At each time point, 0.5 ml of blood was drawn from the thigh vein; the sera were separated, aliquoted, and stored for subsequent assays.

Reinfection experiments were performed by transmission of HCV RNA-positive serum from group I (Table 1) to naive animals.

Serum alanine aminotransferase (ALT) concentrations were determined using a Transnase Nissui kit (Nissui Pharmaceutical Co.), standardized, and displayed as IU/liter.

RNA isolation and quantitative RTD-PCR assay for HCV RNA. Serum samples (100 μl) were tested for circulating HCV RNA in vivo using quantitative real-time detection (RTD)-PCR (TaqMan). RNA was extracted from the sera and livers of sacrificed animals using the acid guanidium-phenol chloroform method with tRNA as a carrier (3). Two tupaias (Tup.5 and Tup.6) were inoculated with patient serum HCR6. Another two animals (Tup.4 and Tup.8) were inoculated with reconstituted viral particles (RCV). Tup.15 served as a mock-infected control. Liver specimens (3- to 4-mm^2 blocks) from these tupaias were homogenized with 1.5 ml of 5 M guanidine thiocyanate using a polytron-type homogenizer (Ultra-Turrax T25; IKA Laborteehnik, Staufen, Germany). RNA was then reextracted with 4 M guanidine thiocyanate.

RNA samples were subjected to RTD-PCR on an ABI 7700 sequence detector (Applied Biosystems) as described previously (26). The extracted RNA was dissolved in 200 μl of diethyl pyrocarbonate-treated water containing 10 mM dithiothreitol and 200 units/ml RNase inhibitor in a siliconized tube. RTD-PCR was performed using 1 μg of total RNA, one set of PCR primers, and a probe for a location within the 5′ noncoding region using the EZ r*Tth* RNA PCR kit (Perkin Elmer) and the ABI Prism 7700 sequence detector system. A standard curve was constructed using a 10-fold dilution series of in vitro-transcribed and previously titrated synthetic HCV RNA.

Consequently, the quantities represented by genome equivalents correspond to an absolute standard curve (26). All quantitative RTD-PCR assays were performed using duplicate samples, with both negative control serum and HCV-positive serum included. The control sera were diluted before use and were estimated to contain low copy numbers of HCV RNA (100 genome equivalents/ml serum). Samples were deemed positive for HCV RNA if both duplicates yielded PCR-amplified product. Averages of the two estimated values are shown in the figures.

Histological analysis. Tissue samples were carefully collected from anesthetized animals by abdominal incision, fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Silver and Sudan IV (Wako Pure Chemical Industries, Ltd.) staining were also carried out to visualize fiber generation and lipid degeneration, respectively. All histological staining was performed in accordance with conventional procedures. The histological status was determined using the modified hepatitis activity index scoring system, which grades necrosis and inflammation on a scale of 0 to 18 (periportal inflammation and necrosis, 0 to 10; lobular inflammation and necrosis, 0 to 4; portal inflammation, 0 to 4) (11). Fibrosis was scored using the Ishak fibrosis scale of 0 to 6 (0, no fibrosis; 1 or 2, portal fibrosis; 3 or 4, bridging fibrosis; and 5 or 6, cirrhosis). The values in each group (Table 2) represent the averages of the scores in five visual fields.

Statistical analysis. The statistical significance of differences between controls and HCV-infected animals was analyzed with the nonparametric Mann-Whitney U test. All comparisons were two tailed. The statistical analysis was conducted with SPSS 12.0 software (SPSS Inc., Chicago, IL).

RESULTS

Inoculation of HCV causes acute hepatitis and transient viremia in tupaias. To begin this study, two distinct but related inocula were chosen for infection of tupaias. Serum from a chronic hepatitis patient (designated HCR6) was chosen for its

TABLE 2. Grading: necroinflammatory scores and fibrosis

Group	Inoculum	Tupaia no.	Grade				Total	Avg	SD	Staging
			A	B	C	D				
94 wk p.i. (biopsy)										
I	HCR 6	Tup.5	0	0	0	0	0	1.3	1.5	0
		Tup.6	1	0	1	0	2			0
	RCV	Tup.4	0	0	0	0	0			0
		Tup.8	0	0	0	3	3			6
	Control	Tup.15	0	0	0	0	0	0	0	0
III		Tup.17	0	0	0	0	0			0
		Tup.38								
		Tup.39								
144 wk p.i. (sacrifice)										
I	HCR 6	Tup.5	1	0	2	3	6	5.5	3.7	0
		Tup.6	3	0	4	3	10			1
	RCV	Tup.4	0	0	0	1	1			0
		Tup.8	1	0	1	3	5			6
	Control	Tup.15						0	0	
III		Tup.17								
		Tup.38	0	0	0	0	0			0
		Tup.39	0	0	0	0	0			0

defined genotype (genotype 1b), and genetic heterogeneity was ascertained by the process of cloning consensus cDNA. The infectivity of this serum was also experimentally defined in chimpanzees; a 50% chimpanzee infectious dose was estimated at 3.7×10^4 50% chimpanzee infectious doses/ml. Furthermore, the consensus genomic sequence of HCV was cloned from the serum (pHCR6; 9,611 bases; GenBank AY045702.1). For the second inoculum (referred to as RCV), clonal viral particles were reconstituted as described in Materials and Methods. This inoculum was expected to be free of neutralizing antibodies and thus was considered potentially more infectious than patient sera. In the case of RCV infection, genetic diversification of viral RNA, also known as quasispecies, can be regarded as a direct indication of de novo synthesis of progenitor virus in vivo.

Either patient serum or cDNA-derived RCV was inoculated into tupaias (Table 1, group I). Two animals (one female and one male) were tested against each inoculum. Age-matched animals were bred as infection-free controls.

All experimental infections are described in Materials and Methods and Table 1. Prior to experimental infection, the normal serum ALT level in tupaias was measured at 22.3 IU/liter ($n = 23$).

Inoculation with patient serum HCR6 caused rapid fluctuations in the serum ALT concentrations, from two- to fivefold, in both inoculated tupaias, suggesting acute hepatitis in vivo (Fig. 1A and B). Correlative quantitative RTD-PCR revealed HCV viremia soon after serum inoculation in Tup.5, which continued to show transient viremia long term. The appearance of viremia sometimes coincided with a steep elevation in the serum ALT (Fig. 1A). Conversely, HCV RNA was not detected in the serum of Tup.6 up to 60 weeks postinoculation and only twice thereafter. Acute-phase ALT elevations (3 to 4 weeks postinoculation) in Tup.6 might represent tight control of HCV infection by the host immune system (Fig. 1B).

Distinct results were obtained for the two animals (Tup.4 and Tup.8) inoculated with RCV. Both animals displayed sustained viremia up to 10 weeks postinoculation (Fig. 1C and D), indicating persistent HCV infection and inability to eradicate the virus. Viremia was detected intermittently throughout the course of infection, sometimes accompanying the elevation of serum ALT. Humoral immune responses in Tup.5 and Tup.6 (see Fig. S1A in the supplemental material) and Tup.4 and Tup.6 (see Fig. S1B in the supplemental material) were indicated.

We performed RTD-PCR to confirm whether HCV could replicate in the tupaias' livers (Tup.4, Tup.5, Tup.6, and Tup.8) and obtained the following results (Fig. 1E): 310 ± 117 copies/μg total RNA in Tup.5, 80 ± 11 copies/μg in Tup.6, 199 ± 77 copies/μg in Tup.4, and 292 ± 48 copies/μg in Tup.8. In contrast, HCV RNA was not detected in the liver of the mock-infected animal (Tup.15).

HCV RNA was also not detected in samples from either preinoculation or age-matched, infection-free control tupaias (Table 1, group III), nor were significant elevations in serum ALT observed for any of the three infection-free controls (data not shown).

HCV causes chronic hepatitis in tupaia liver, leading to fibrosis and cirrhosis. Serum ALT and circulating HCV RNA levels in primary infected tupaias (Table 1, group I) were monitored for 3 years postinoculation. As described above, the magnitudes of serum ALT fluctuations varied substantially among infected animals (Fig. 1A, B, C, and D). Tupaia livers were examined for histological lesions in order to elucidate if HCV caused chronic hepatitis. Liver biopsies via abdominal incisions were performed at 2 years postinoculation. All animals were sacrificed at 3 years postinoculation (4.5 years for uninfected animals). H&E staining of liver specimens from HCV-infected tupaias showed infiltrating lymphocytes within sinusoids and around portal areas, indicating chronic hepatitis in the tupaia livers (Fig. 2B, D, and H). Infiltrating lymphocytes were also observed in limiting plates, indicating ongoing inflammation (Fig. 2G and H). Furthermore, a comparison of liver samples at 2 and 3 years postinoculation revealed that the

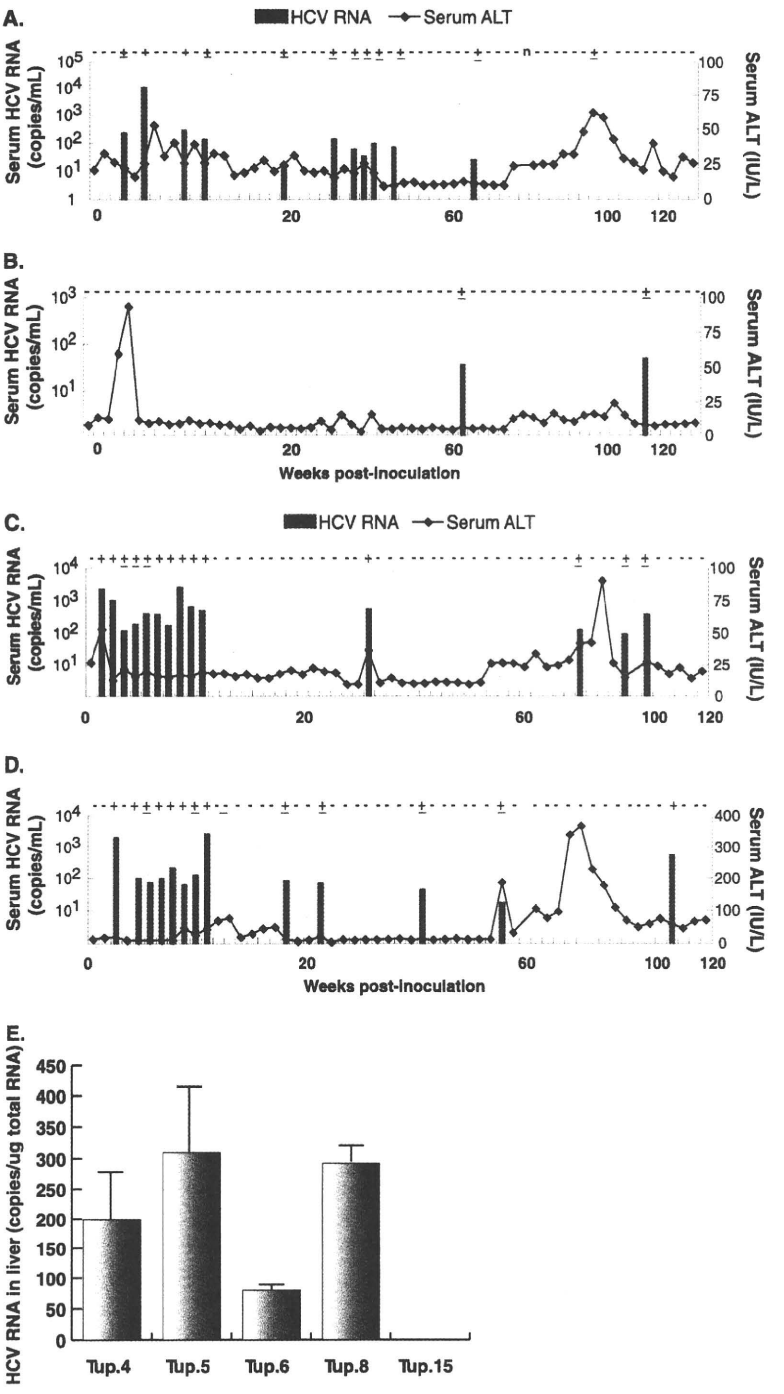

FIG. 1. Course of infection with patient serum HCR6 and RCV. (A) The results of quantitative RTD-PCR for HCV RNA and serum ALT concentrations were combined and plotted to show the course of infection in Tup.5. The bars and the ordinates on the left represent HCV RNA as genome equivalents/ml of serum. The curved line and the ordinates on the right represent serum ALT concentrations as IU/liter serum. (B) Serum HCV RNA and ALT concentrations for infection of Tup.6. (C) The graph for Tup.4. (D) The graph for Tup.8. The vertical axis for serum ALT in this graph is scaled differently from the others because of significant ALT elevation. (E) Quantification of HCV RNA in tupaia liver. HCV RNA in hepatocytes from tupaia (Tup.4, Tup.5, Tup.6, Tup.8, and Tup.15) livers was isolated 172 weeks after HCV infection and quantified by RTD-PCR. As few as 10 copies of the genome were detected, and the quantification range was between 10^1 and 10^8 copies (26).

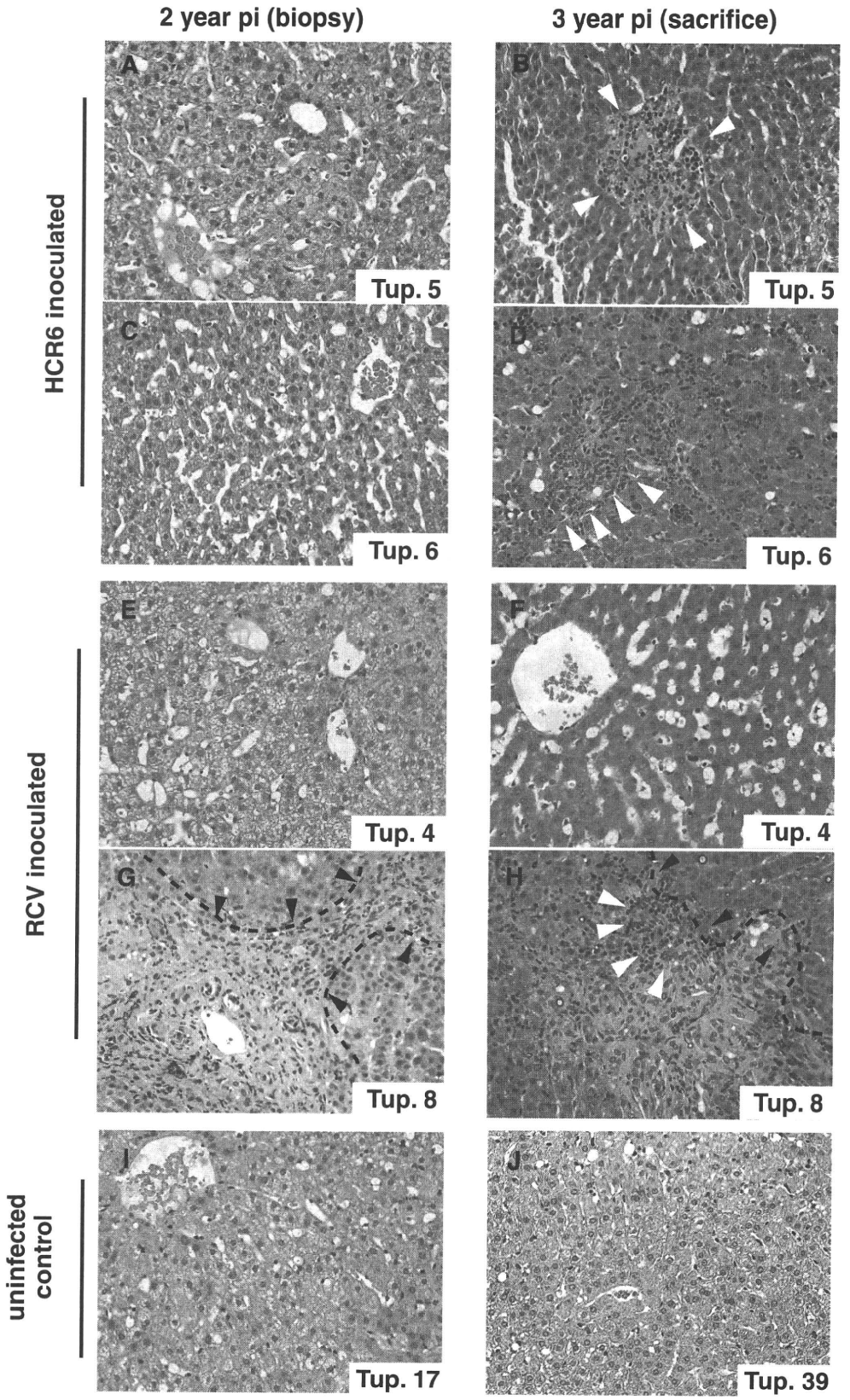

FIG. 2. Micrographs of liver specimens stained with H&E. Liver tissue from HCR6-inoculated tupaias (A to D) and RCV-inoculated tupaias (E to H) was obtained at 2 and 3 years postinoculation (pi). (I and J) Liver specimens from uninfected animals age matched to each inoculated animal were also obtained. The HCV-infected tupaia livers harbored infiltrating lymphocytes (white arrowheads) and fibrosis (broken lines and black arrowheads), which indicate chronic hepatitis.

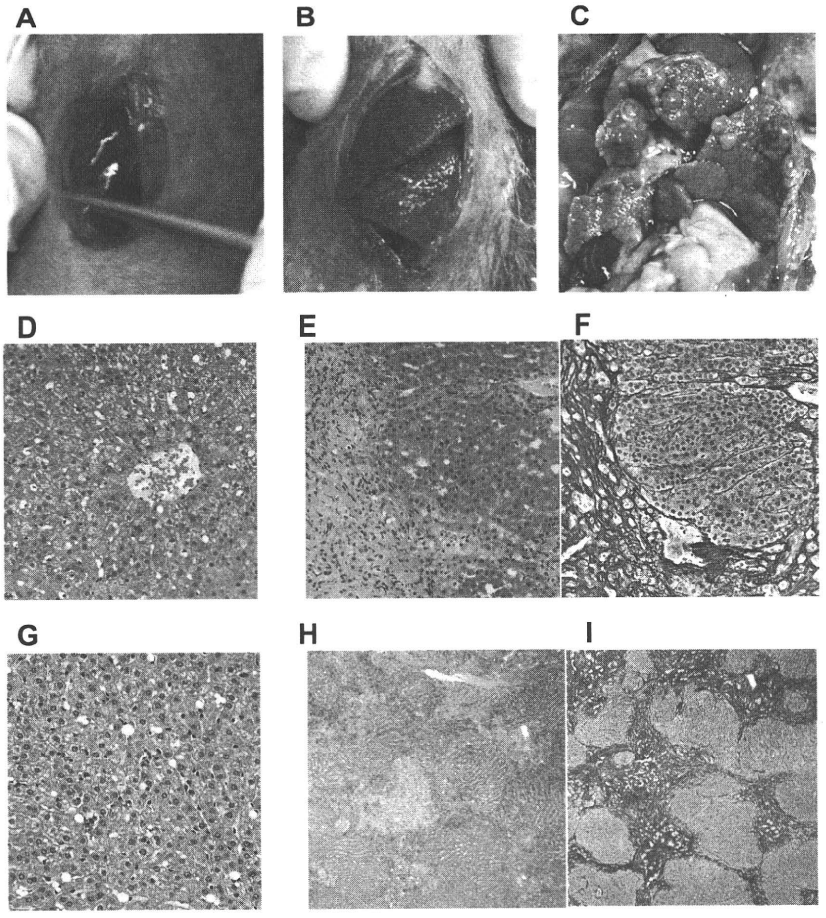

FIG. 3. Macro- and microscopic features of tupaia liver. (A) Infection-free control tupaia (Tup.15; 92 weeks). (B) RCV-infected animal displaying liver cirrhosis (Tup.8; 84 weeks postinoculation). (C) RCV-infected animal with massive surface nodules (Tup.8; 144 weeks postinoculation). (D and G) H&E staining of the uninfected Tup.15 at 92 weeks (D) and the uninfected Tup.39 at 242 weeks (G). (E, F, H, and I) H&E and silver staining of Tup.8 at 84 weeks postinoculation (E and F) or at 144 weeks postinoculation (H and I).

hepatitis had worsened with time in all HCV-infected tupaias (Fig. 2A to H and Table 2).

Fibrosis and cirrhosis were also examined. Mild fibrosis was seen in Tup.6, while severe fibrosis was seen in Tup.8. Cirrhosis was histologically investigated in all animals (Table 2). There was no significant difference between groups I and III at 94 weeks postinfection (P • 0.194), but at 144 weeks postinfection, a slight difference was observed (P • 0.059; SPSS 12.0). Macroscopic observation of the liver biopsy specimens (taken 2 years postinoculation) indicated liver cirrhosis in Tup.8 (Fig. 3B) compared with Tup.15 (uninfected control) (Fig. 3A), while silver staining of histology samples revealed fibrosis and cirrhotic nodules (Fig. 3E and F). Macroscopic observation upon sacrifice (3 years postinoculation) indicated that liver cirrhosis in Tup.8 had worsened (Fig. 3C). In contrast, age-matched infection-free negative control tupaias displayed none of these pathologies (Fig. 3A, D, and G).

Progressive lipid degeneration was noted in infected tupaias throughout the course of infection (Fig. 4). In particular, Tup.5 displayed microvesicular lipid droplets in the first biopsy specimens (at 2 years), which developed into macrovesicular droplets and foamy degeneration in biopsy specimens at 3 years (Fig. 4C and D). Liver specimens from other infected animals displayed intracellular micro- and macrovesicular lipid droplets in hepatocytes at 3 years postinoculation (Fig. 4F, H, and J). These anomalies were not present in liver specimens from infection-free control animals (Fig. 4A and B).

Transmission of viral-RNA-positive serum to naive animals reproduces acute hepatitis and viremia. To confirm virion regeneration in vivo, and to exclude the possibility of false-positive serum HCV RNA results due to amplification of the original inocula, HCV RNA-positive sera from primary inoculated tupaias were used to inoculate naive tupaias. Three different sera were tested in this passage experiment, with two naive tupaias used as recipient animals for each trial (see Materials and Methods) (Table 1, group II).

In the first reinfection experiment, serum from Tup.5 (originally infected with patient serum HCR6) was collected at 5 weeks postinoculation and used to infect two naive animals. The recipient animals showed intermittent viremia over the subsequent 3 months (Fig. 5A). In the second and third cases of reinfection, sera from Tup.8 at 10 weeks postinoculation and from Tup.4 at 8 weeks postinoculation also induced viremia in the naive inoculated animals, similar to the first reinfection experiment (Fig. 5B and C). Furthermore, the PCR titers of the recipient tupaias were significantly greater than the inoc-

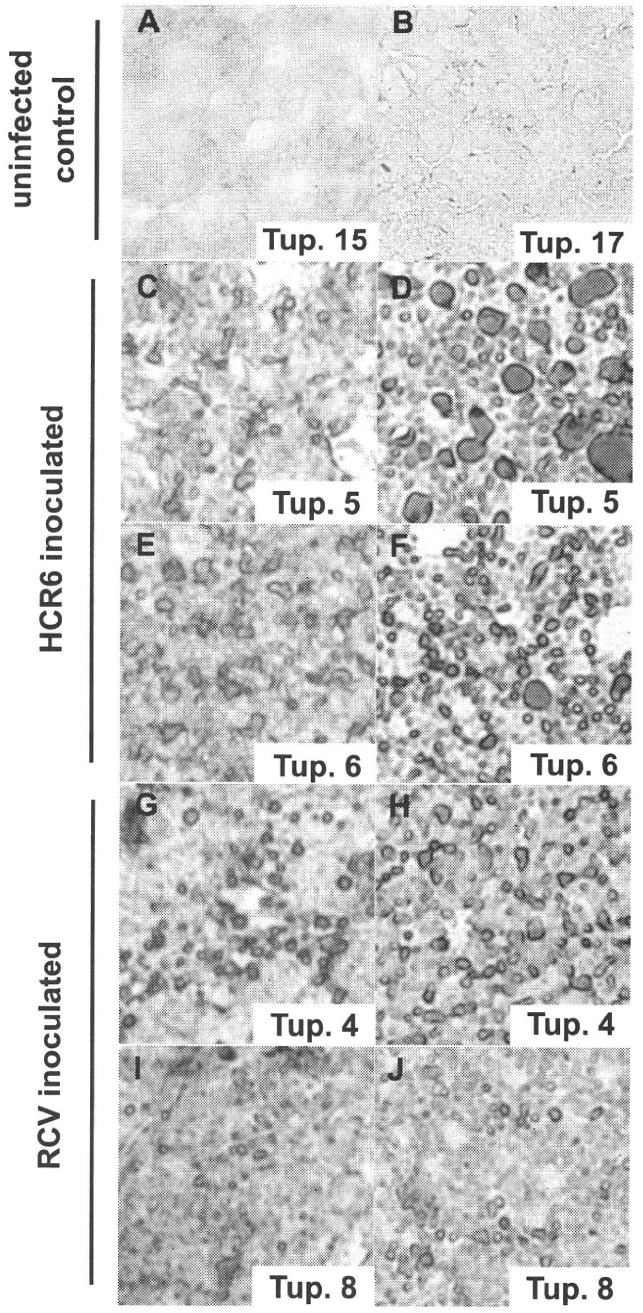

FIG. 4. Sudan IV-stained liver specimens exhibiting fatty liver degeneration. Cryosections of liver stained by Sudan IV as described in Materials and Methods show fatty liver degeneration. The left and right columns display biopsy specimens of infected animals (2 years postinoculation) and animals sacrificed at 3 years postinfection, respectively. (A and B) Uninfected controls at 2 years (Table 1 shows sample timing). (C to F) Patient serum HCR6-infected animals. (G to J) RCV-infected animals.

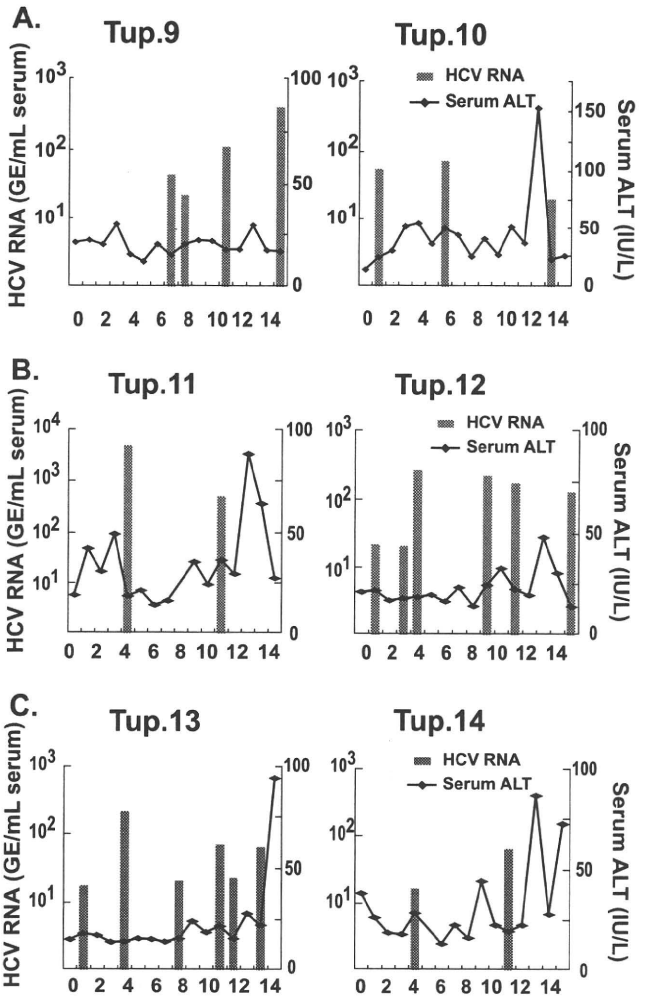

FIG. 5. Results of a reinfection experiment. (A) Quantitative RTD-PCR for HCV RNA and serum ALT levels are shown. Two naive animals were inoculated with tupaia serum (using serum taken at 5 weeks postinoculation from Tup.5, originally inoculated with patient serum HCR6) containing 100 genome equivalents (GE)/ml and were monitored for 15 weeks postinoculation (Table 1). (B) Tupaia serum (taken at 10 weeks postinoculation from Tup.8, originally inoculated with RCV) that was positive for HCV RNA was passaged into two naive animals. The animals were inoculated with tupaia serum at 100 GE/animal and monitored for 15 weeks postinoculation. (C) Tupaia serum (taken at 8 weeks postinoculation from Tup.4, originally inoculated with RCV) that was positive for HCV RNA was passaged into naive animals. The animals were inoculated with serum at 100 GE/animal and monitored for 20 weeks postinoculation.

ulation titers (10^2 genome equivalents/animal) (Table 1). For Tup.11, serum from 4 weeks postinoculation contained almost 10^4 genome equivalents/ml of HCV RNA (Fig. 5B). In addition, significant increases in serum ALT accompanied detection of serum HCV RNA. These results indicate that HCV RNA-positive sera from group I actually contained infectious virion particles. They also suggest that reconstituted HCV particles made from cDNA are infectious in tupaias.

We amplified a portion of the NS5A sequence, which is known as the interferon sensitivity determining region, by reverse transcription-PCR as described in the supplemental material. Each PCR product was subcloned and sequenced to compare the encoded amino acid sequences. For the purposes of this study, animals were inoculated with a molecular clonal virus consisting of a unique viral sequence of cDNA. The interferon sensitivity determining region sequences recovered from an animal infected with clonal inoculum (Tup.8 at 103 weeks postinoculation) were found to be heterogeneous, with

a few amino acid substitutions (K2212M for 2/10 cases, L2232P for 1/10 cases, and L2253S for 6/10 cases) (see Fig. S2E in the supplemental material). Interestingly, the codon for amino acid 2224 encodes valine, but it was found to be variant for alanine and valine in sequences from the original patient serum (HCR6). Tupaias infected with patient serum also exhibited variability at position 2224; valine occupancy was rare, as was seen in the original HCR6 population (see Fig. S2B and C in the supplemental material). On the other hand, this position was occupied solely by valine for sequences recovered from Tup.8 (see Fig. S2E in the supplemental material), indicating that genetic variations shown for Tup.8 originated from the pHCR6 cDNA sequence. Taken together, quasispecies detection of circulating virus represents further evidence demonstrating intrinsic replication of HCV in tupaias despite low levels and infrequent detection of viremia.

DISCUSSION

In the present study, we described persistent HCV infection in tupaias. Long-term follow-up was performed and revealed histological progression of HCV-related liver disorders in infected tupaias, including steatosis, fibrosis, and cirrhosis, in addition to acute and chronic hepatitis. HCV genomic RNA was detected in animal sera intermittently throughout the entire course of infection. However, HCV RNA was detected in the liver upon sacrifice (3 years postinoculation). Furthermore, HCV RNA in serum contained genomic variants that had diverged from the inoculated virus (see Fig. S1 and S2 in the supplemental material). These data strongly indicate an established persistent infection in the tupaias studied. All animals exhibited HCV viremia soon after inoculation, yet the viremia was intermittent and accompanied by relatively low RTD-PCR titers compared with equivalent human and chimpanzee infections. The discrepancy between humans and tupaias might be due to host-dependent differences in replication efficiency. Over the course of HCV infection in these tupaias, serum ALT profiles indicated repeated liver injury, probably due to host immune responses mediated by agents such as cytotoxic T lymphocytes rather than direct viral cytopathic effects.

In cases of tupaia infection, experimental inoculations rarely led to sustained viremia, which for most human cases lasts for the entire course of infection. Even the course of infection appeared transient and self-resolved. It seems likely that HCV replication is less compatible with the tupaia host environment. This possibility was substantiated by a previous report by Xu et al. (34), where tissue-cultured virions of cloned genotype 1b, referred to as HCVcc in the paper, could not cause chronic infection with sustained viremia in tupaias. Although HCVcc actually infected most of the inoculated tupaias (83%; 10/12), chronic infection was seen for only a fraction of them (20%; 2/10). In this study, we also tried to detect a humoral response to HCV core antigen. We found that tupaia sera were HCV positive for antibodies only at occasional time points, observable as intermittent steep responses (data not shown). Overall, sustained seroconversion was not seen in this study, probably because HCV propagation in vivo was so limited or well controlled by host immunity. Given that models of HCV propagation are severely limited, the most important and interesting finding of this study is the successful detection of HCV RNA in livers of infected tupaias 3 years after inoculation, indicating that HCV persists in tupaias. Although the limited propagation of HCV in tupaias is a drawback of this model at the present time, the isolation of tupaia-adapted HCV may be feasible by performing multiple infection passages. This possibility is supported by both quasispecies development and successful reinfection.

The chimpanzee is the animal species most closely related to humans, and as a model, it has contributed significantly to our understanding of HCV infection and pathogenesis. However, reproducing HCV pathogenesis in humans or chimpanzees can take as long as 10 to 20 years. The chronically infected tupaias in the present study developed complicated liver disorders in a much shorter time. Using tupaias, with their relatively short life span (3 to 5 years in the laboratory), as a model of HCV infection, we can evaluate HCV pathogenesis and correlate senescence and duration of infection.

The recent development of a primary human hepatocyte xenograft-uPA/SCID mouse model opened up opportunities to test putative antivirals against HCV replication in vivo (10, 17). In this innovative model, human hepatocytes, which are transplanted into the lobe of a mouse liver, can support HCV replication effectively. As a result, the level of circulating HCV RNA is comparable to that of a human patient. However, this mouse model is immunodeficient, and thus, it lacks the interplay between host immunity and viral infection. Therefore, it does not provide a suitable platform for characterizing immune responses to HCV infection.

HCV infection in tupaias represents an important model of HCV infection, particularly for the study of key determinants controlling virus propagation in vivo. The pathogenesis of HCV infection can be substantially different among humans, chimpanzees, and tupaias, and the mechanisms governing these differences are of great interest. Comparative studies of HCV infection in these different species will help us to understand the basic mechanisms of persistent infection.

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REFERENCES

1. **Abe, K., T. Kurata, Y. Teramoto, J. Shiga, and T. Shikata.** 1993. Lack of susceptibility of various primates and woodchucks to hepatitis C virus. J. Med. Primatol. **22:**433–434.
2. **Aoki, Y., H. Aizaki, T. Shimoike, H. Tani, K. Ishii, I. Saito, Y. Matsuura, and T. Miyamura.** 1998. A human liver cell line exhibits efficient translation of HCV RNAs produced by a recombinant adenovirus expressing T7 RNA polymerase. Virology **250:**140–150.
3. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
4. **Choo, Q. L., G. Kuo, A. J. Weiner, L. R. Overby, D. W. Bradley, and M. Houghton.** 1989. Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. Science **244:**359–362.
5. **Dash, S., G. Kalkeri, H. M. McClure, R. F. Garry, S. Clejan, S. N. Thung, and K. K. Murthy.** 2001. Transmission of HCV to a chimpanzee using virus