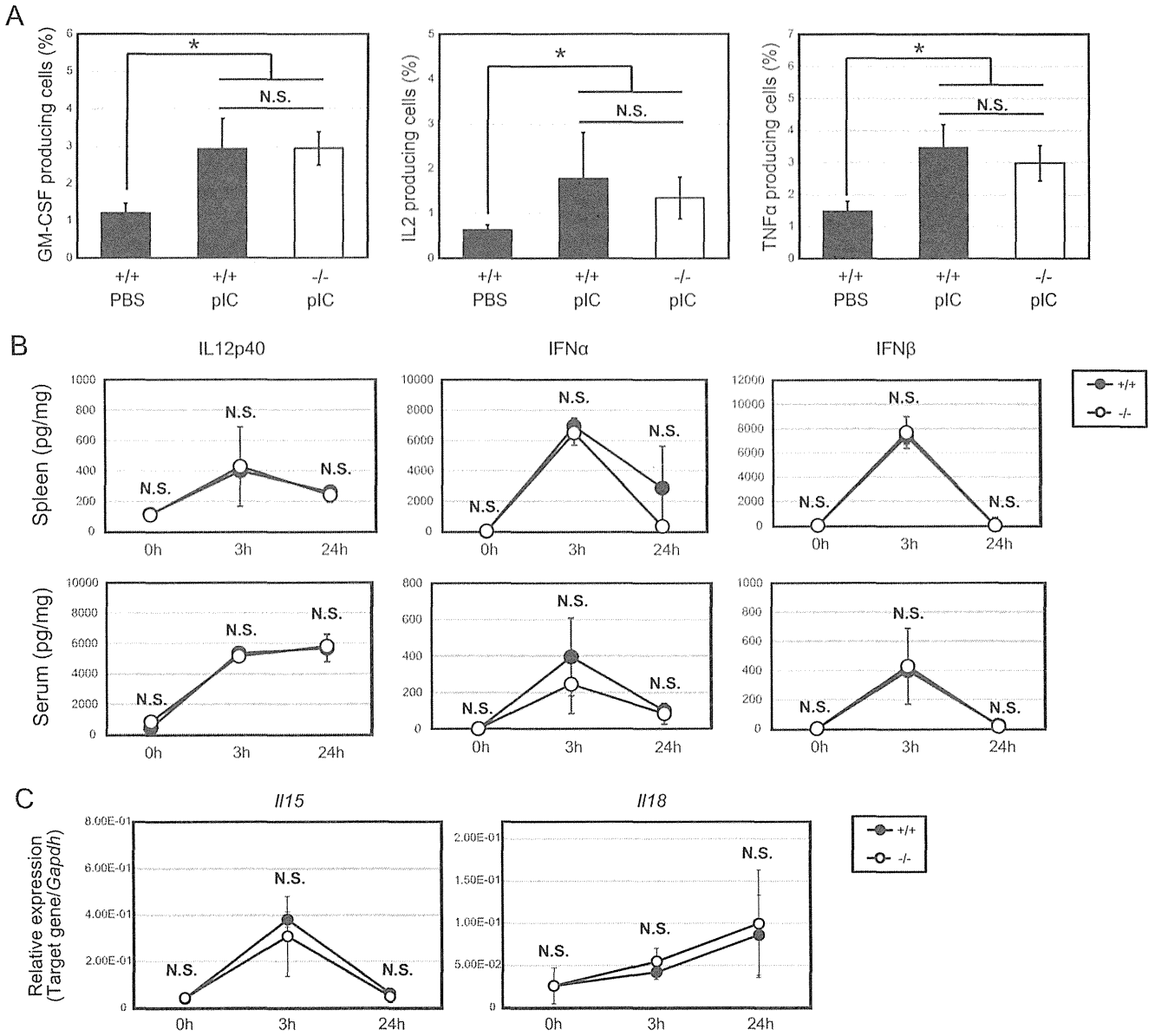
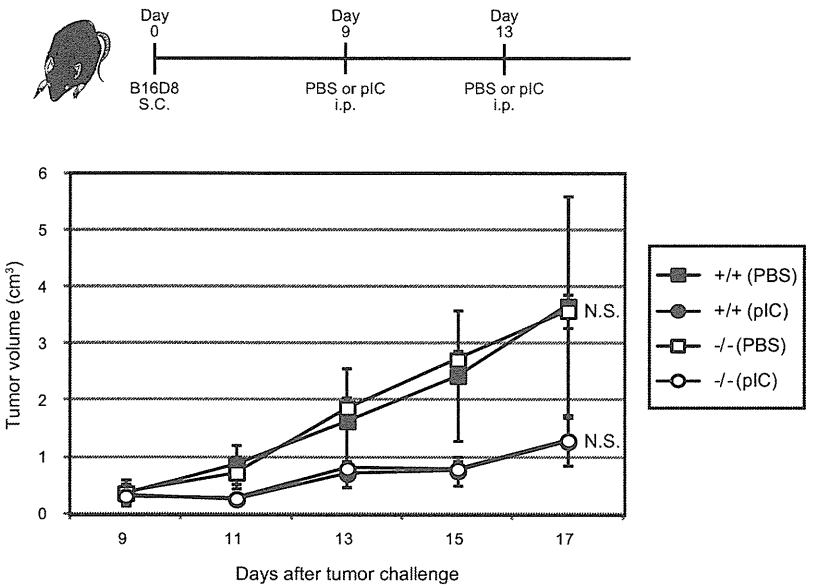


Supplementary Figure 2. Proinflammatory cytokines in WT and *Inam*$^{-/-}$ mice.

(A)Production of GM-CSF, IL2 and TNFα by NK cells. WT (+/+) and Inam-/- (−/−) mice were intraperitoneally injected with 200 μg polyI:C or PBS. After 3 h, splenocytes were isolated, cultured with brefeldin A for an additional 4 h, and analyzed for intracellular content of GM-CSF, IL2 and TNFα by FACS, gating on CD3ε-/NK1.1+ cells (n = 3). (B) Protein levels of IL12p40, IFN-α and IFN-β after polyI:C stimulation. (B) Messenger RNA expression of *Il15* and *Il18* genes in spleen. WT (+/+, n = 3) and *Inam*$^{-/-}$ (−/−, n = 3) mice were intraperitoneally injected with 200 μg polyI:C or PBS. After 0h, 3h and 24h, mouse sera and spleen extracts were collected and protein levels of IL12p40, IFN-α and IFN-β were evaluated by ELISA. At the same time, total RNA was isolated from spleen and subjected to quantitative PCR to determine *Il15* and *Il18* expression. The data shown are representative of at least two independent experiments. Data are means ± SD of three independent samples. *p < 0.05.



Supplementary Figure 3. INAM-independent B16D8 tumor regression by polyI:C treatment.

WT (+/+) and *Inam*$^{-/-}$ (−/−) mice were subcutaneously injected with 6 × 105 B16D8 melanoma cells at day 0. At day 9 and 13, WT and *Inam*$^{-/-}$ mice were intraperitoneally injected with 200 μg polyI:C or PBS (n = 3 or 4). At day 9, 11, 13, 15 and 17, tumor volumes were measured using a caliper. Tumor volume was calculated by using the formula: Tumor volume (cm3) = (long diameter) × (short diameter) × (thickness) × 0.4. The data shown are representative of at least two independent experiments.

＜症例報告＞

B型・D型肝炎ウイルス重複感染による肝障害に対してペグインターフェロンが有効であった1例

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要旨：症例は26歳，女性のモンゴル人．既往歴としてB型慢性肝炎を認める．今回，健診にて肝機能障害を指摘され，B型慢性肝炎急性増悪の診断で当科紹介となる．来院時採血で更に肝障害増悪を認めたが，HBV-DNA量は2.8 log copy/mLと低値だった．また他のウイルス感染やAIH，PBCも否定的だった．妊娠中と判明したため無治療で経過観察したところ，肝障害は徐々に自然軽快し，妊娠39週で出産した．出産後もHBV-DNA量上昇は認めず，肝障害は更に改善したが，出産から9カ月後に再度肝障害増悪を認めた．この段階でD型肝炎ウイルス（HDV）の重複感染を考え，保存血清を用いてHDV-RNAを検査したところ陽性と判明し，HBVキャリアに対するHDV重複感染と診断した．Peg-IFNα-2aの投与を開始したところ，肝障害は改善し，治療中のHDV-RNAも陰性化した．日本においてHDV感染は稀な疾患であるが，HBV-DNA量の増加を伴わないB型慢性肝炎急性増悪症例に遭遇した場合には，HDV重複感染を念頭に置くべきと思われた．

索引用語：　D型肝炎　　Peg-IFN　　重複感染

はじめに

D型肝炎ウイルス（HDV）は，1977年Rizzettoらにより初めて報告された外殻蛋白合成能に欠損がある不完全型のRNAウイルスであり[1)]，その発現・増殖にはB型肝炎ウイルス（HBV）をヘルパーウイルスとして必要とする[2)]．このHDV感染により，B型慢性肝炎の臨床経過が悪化したり，稀には劇症肝炎様の病態を引き起こすことが知られている[3)4)]．本邦ではHBVキャリアにおけるδ抗体陽性率は約1％と低いことが報告されており[5)]，実際のD型肝炎の診断・治療に関する報告は少ない．今回，我々はHBVキャリアであるモンゴル人に妊娠を契機とした肝障害悪化を認め，最終的にHBVとHDVの重複感染と診断し，ペグインターフェロン（Peg-IFN）による治療を導入したことで肝障害の改善を認めた1例を経験したので報告する．

症　例

26歳，女性．モンゴル人．

主訴：肝機能障害．

家族歴：父，母，弟に肝炎の既往なし．

既往歴：幼少児　痙攣（原因不明，無治療で自然軽快）でモンゴルの病院に入院．その際，採血検査などで注射器の回し打ちがなされていたとのこと．

内服薬：なし．

輸血歴：なし．

生活歴：飲酒・喫煙共になし．

職業歴：事務員．

現病歴：2003年にモンゴルで流産したが，その際，ALT 2000 U/Lまで一過性に上昇し，HBVキャリア（genotype D）であることが判明した．2004年にモンゴ

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＜受付日2014年6月28日＞＜採択日2014年8月16日＞

Table 1 Laboratory data on admission

Hematology		Biochemistory		Virus marker	
WBC	5900 /μl	TP	7.6 g/dl	HBsAg (CLEIA)	>2000.0 (+) C.O.I
Neut	61.9 %	Alb	4.5 g/dl	anti-HBs (CLEIA)	0.9 (−) mIU/mL
Lymph	29.7 %	AST	448 U/l	HBeAg (CLEIA)	0.1 (−) C.O.I
RBC	4.70×10^6 /μl	ALT	1029 U/l	anti-HBe (CLEIA)	>100.0 (+) %
Hb	13.9 g/dl	LDH	284 U/l	HBV-DNA (realtime PCR)	2.8 log copy/ml
Plt	22.0×10^4 /μl	ALP	339 U/l	HBV genotype	genotype D
		T.Bil	0.5 mg/dl	HBV preC/CP mutation	
Coagulation		CK	48 U/l	preCore wild type	100 %
PT	102.2 %	BUN	9 mg/dl	preCore mutant type	0 %
PT-INR	0.99	Cre	0.5 mg/dl	Core promoter	negative
APTT	92.2 %	Glu	91 mg/dl	anti-HCV (CLEIA)	0.1 (−) C.O.I
HPT	88.4 %	Na	137 mEq/l	HCV core protein	≦0.1 fmol/l
FDP	<2.5 μg/ml	K	4.7 mEq/l	IgM-HA (CLIA)	<0.80 (−)
DD dimer	0.9 μg/ml	Cl	104 mEq/l	HEV-RNA	negative
		CRP	0.10 mg/dl	CMV IgM (EIA)	0.44 (−)
Immunology				CMV IgG (EIA)	27.6 (−)
IgG	1379 mg/dl	TSH	1.582 μIU/mL	anti-EBNA (FAT)	40 ×
IgA	271 mg/dl	FT3	2.94 ng/dl	VCA IgM (FAT)	<10 ×
IgM	160 mg/dl	FT4	0.76 pg/mL	VCA IgG (FAT)	80 ×
ANA	40 ×				
AMA-M2	5 Index			Tumor marker	
				AFP	3.7 ng/ml
				PIVKA-II	22 mAU/ml

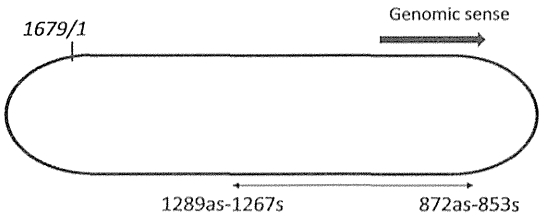
ルから日本へ移住. 今回, 2010年6月1日の検診でAST 227 U/L, ALT 468 U/Lと肝障害を指摘される. 6月10日に前医を受診し採血を行ったところ, AST 376 U/L, ALT 767 U/Lと肝障害の増悪を認めたため, B型慢性肝炎急性増悪として6月14日当院紹介となる. 同日施行した緊急採血でAST 448 U/L, ALT 1029 U/Lと肝障害の更なる増悪を認めたため, 同日緊急入院となる. なお, 6月13日に患者が市販の妊娠検査薬で調べたところ, 妊娠反応陽性であった.

入院時現症：体温36.3℃, 血圧125/70 mmHg, 脈拍70回/分・整. 表在リンパ節を触知せず. 咽頭に異常を認めず. 腹部は平坦・軟で圧痛を認めず. 肝・腎・脾は触知しない. 下腿浮腫を認めず.

入院時検査所見（Table 1）：白血球, 赤血球, 血小板数は正常であった. 一般肝機能検査ではAST448 U/L, ALT1029 U/LとALT優位のトランスアミナーゼ値の上昇を認めたが, PT 102.2%, HPT 88.4%と凝固系には異常を認めなかった. T.Bilも0.5 mg/dlと正常値であった. 免疫グロブリンにも異常は認められず, 抗核抗体などの自己抗体も陰性であった. HBs抗原陽性, HBe抗体陽性のHBVキャリアであったが, HBV-DNA量は2.8 log copy/mlと低値であった. なお採血結果からA型肝炎ウイルス, C型肝炎ウイルス, E型肝炎ウイルス, サイトメガロウイルス, EBウイルス感染はいずれも否定的であった. 甲状腺機能にも異常は認められなかった.

腹部エコー：軽度脂肪肝の所見を認めるのみであった.

入院後経過：入院当初はB型慢性肝炎急性増悪による肝障害を疑ったが, 入院後の産婦人科診察にて妊娠4〜5週であることが確認され, 更に入院時採血でPT 102.2%と良好であったことから, 肝障害に対する投薬は行わず, 安静・補液で経過観察となる. その後, 入院時採血でHBV-DNA量が2.8 log copy/mlと低値であることが判明し, B型慢性肝炎急性増悪による肝障害は否定的と考えた. 6月26日採血でAST 890 U/L, ALT 1731 U/Lまで増悪するが, その後は自然に軽快し7月8日退院となる. 外来でも投薬は行わず経過観察の



Name	Location	Sequence 5'→3'
853p	853-872	CGGATGCCCAGGTCGGACC
1267N	1267-1289	GAAGGAAGGCCCCTGGAGAACAAGA
Delta-F7	868-887	GCATGGTCCCAGCCTCC
Delta-R2	1268-1287	TCTTCGGGTCGGCATGG

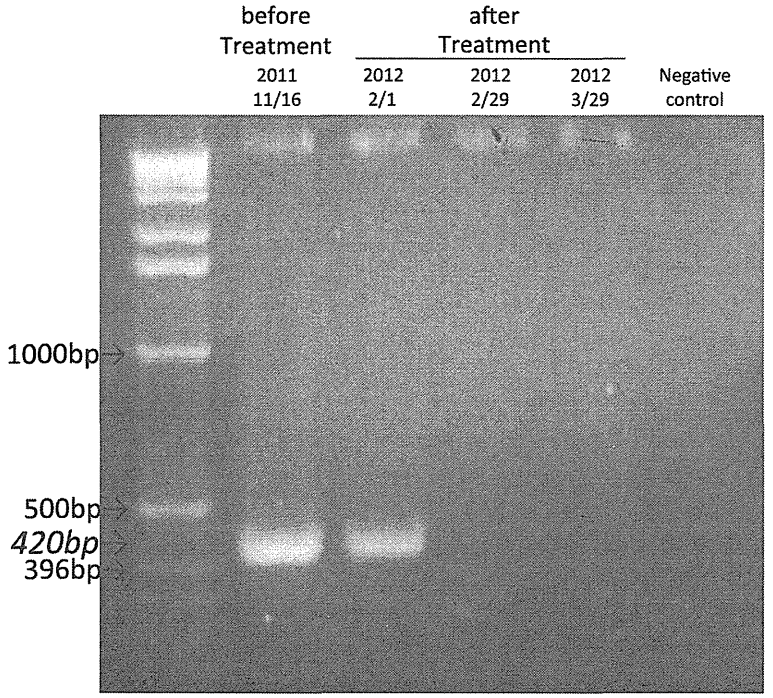
Fig. 1 Strategy for HDV genome amplification. Regions 853-1289 were indicated by arrows. The sequence and position of primers (853p, 1267N) were listed. In addition, the primers (Delta-F7, Delta-R2) were designed for real-time PCR quantification.

みであったが，出産直前にAST 220 U/L，ALT 268 U/Lと軽度の肝障害増悪を認めた以外はAST 50 U/L，ALT 100 U/L前後で推移し，2011年2月8日，妊娠39週で出産となる．出産後の肝機能はAST，ALTともに50 U/L前後まで低下し，肝障害の原因として妊娠が何らかの影響を及ぼしていたものと考えた．その後はモンゴルに一時帰国したため，フォローアップできなかったが，モンゴルから帰国した2011年11月中旬頃から再び肝障害増悪を認め，妊娠以外の原因によって肝障害増悪を来していると判断した．肝障害の増悪が続くため，12月28日再入院となる．再度，各種ウイルス感染や自己免疫疾患などについて検査するも前回同様すべて陰性であった．造影CTで器質的疾患の有無についても確認したが，明らかな異常を認めなかった．この時点で，1）HBVキャリアであるが経過中HBV-DNA量は低値で推移している，2）モンゴル人である，以上の2点からHDVの重複感染による肝障害を疑い，当院受診時からの保存血清（2010年8月25日，2011年2月2日，2011年11月16日の3ポイント）を用いてHDV感染の有無について確認した．HDVに対する特異的プライマーを設定（forward：853–872，reverse：1267–1289）し（Fig. 1）[6][7]，RT-PCRを行ったところ，これら3点全てでPCR陽性であることが判明し，本症例がHBVとHDV重複感染による肝障害と診断した．2012年1月3日からPeg-IFNα-2a 180 μgによる治療を開始した．これによりAST/ALTは50 U/L程度まで著明に低下し，治療中のHDV-RNAも検出限界未満となった．更にHDV-RNAの定量を行うため，既存の報告を参考にリアルタイムPCR検出系の確立も試みた[8]．治療開始前（2011年11月23日）の保存血清から得られたcDNAに対してDelta-F7，Delta-R2をプライマーとして設定し（Fig. 1），TaKaRa LA Taq（タカラバイオ株式会社，滋賀）を用いて検出領域を増幅した．その後，TAクローニング法を用いて検出領域のクローンを作成し，スタンダードサンプルとして使用した．リアルタイムPCRはDelta-F7，Delta-R2をプライマーとし，FastStart SYBR Green Master（Roche Diagnostics，Switzerland）を用いてLightCycler480®（Roche Diagnostics）により行った（95℃で60 secの後，95℃ 15 sec，60℃ 25 sec，72℃ 15 secを45 cycle）．同システムの自動解析により，Threshold cycleを定め，定量を行った．今回確立した系の検出感度は1.0×10^4 copies/mLであり，2011年11月16日のHDV-RNA量は1.2×10^6 copies/mL，2012年2月1日は1.0×10^4 copies/mL未満：シグナル陽性，それ以降は検出感度以下であった（Fig. 2）．HDV genotypeについて系統解析した結果はgenotype Iであった（Fig. 3）．治療中ではあったが，患者希望により2012年5月モンゴル帰国となる．最後に本症例の臨床経過をFig. 4に示す．

考　察

D型肝炎ウイルスは，B型肝炎ウイルスと共存することによってのみ増殖し，肝炎を起こす不完全ウイルスで，HBVとの同時感染かHBVキャリアへの重複感染で感染が成立する．同時感染ではB型急性肝炎を重症化させ，重複感染ではB型慢性肝炎の急性増悪や病期進行を早めることが知られている．

世界には4億2000万人のHBVキャリアが存在すると推定されているが，1980年代に世界の各地域のHBVキャリアを対象としたHD抗体を用いた調査報告によるとHD抗体陽性率は5%であったことから，世界のHDV感染者は約2000万人と推定されている[9]．HDV抗体陽性者は世界中に存在するものの，HBV感染者の分布とは異なり，地中海沿岸，中近東，中央アジア，南米（アマゾン川流域），南太平洋諸島の一部でその頻度が高く，一方HBVキャリア率が高い中国や韓国といった東アジアでは低いことが報告されている[10]．しかし，中国に近いモンゴルにおいてはHDV抗体陽性率が高く[11]，24～41%のHBVキャリアにHDV感染を認めたとの報告もある[12]．本邦では1989年に全国の国立病院



Real time PCR	2011/11/16	2012/2/1	2012/2/29	2012/3/29
HDV-RNA (copies/mL)	1.2×10^{6}	$<1.0\times10^{4}$	Not detected	Not detected

Fig. 2 The results of RT-PCR assay for serum HDV-RNA on each day. The 420-bp fragment indicates the amplified HDV genome. HDV-RNA level at baseline was 1.2×10^{6} copies/mL on November 16, 2011. After starting Peg-IFN treatment, HDV-RNA decreased ($<1.0\times10^{4}$ copies/mL: 2012/2/1) and finally became undetectable (2012/2/29, 2012/3/29).

31施設を対象に1306例のHBVキャリアでのHD抗体陽性者を検討したところ，8例のみ陽性でその頻度は0.61％と低値であったことから，本邦におけるHDV感染者の頻度は低いものと考えられている[13]．

HDVは大きく3つのgenotypeに分類されている[6]．GenotypeⅠは世界中に広く分布するタイプであり，本症例も系統解析した結果，genotypeⅠであった．GenotypeⅡは日本と台湾から報告されており，genotypeⅢは南米のアマゾン川流域に分布している．最近では新しいgenotype分類も報告されており，それによるとgenotypeⅠからⅧの8つのgenotypeが報告されている[14]．

HDV感染のスクリーニング法として，2003年4月まではHD抗体測定が保険適用であったが，同年5月に測定試薬の製造が中止されて以後，本邦でのHD抗体測定は困難となっている[15]．このため現在ではHDV感染診断はRT-PCR法により血中のHDV RNAを検出する方法が基本となるが，HDV RNA検出には保険適用はなく，各施設の研究室で測定するか，検査受託企業への外注検査に依頼する必要があり，現在，本邦におけるHDV感染検査は非常に困難なものとなっている．このような状況が本症例においてHBV・HDV重複感染による肝障害との診断に至るのに苦慮した要因の一つになったと思われる．

HDVの感染様式としては，HBVとの同時感染か，HBVキャリアへの重複感染の2つが考えられる．同時感染は通常一過性で，しばしば劇症肝炎や重症肝炎を起こすが，HDVの慢性化は約5％程度と稀である．一方，重複感染の場合は70～95％でHDV感染が持続し，D型慢性肝炎に移行する[16]．

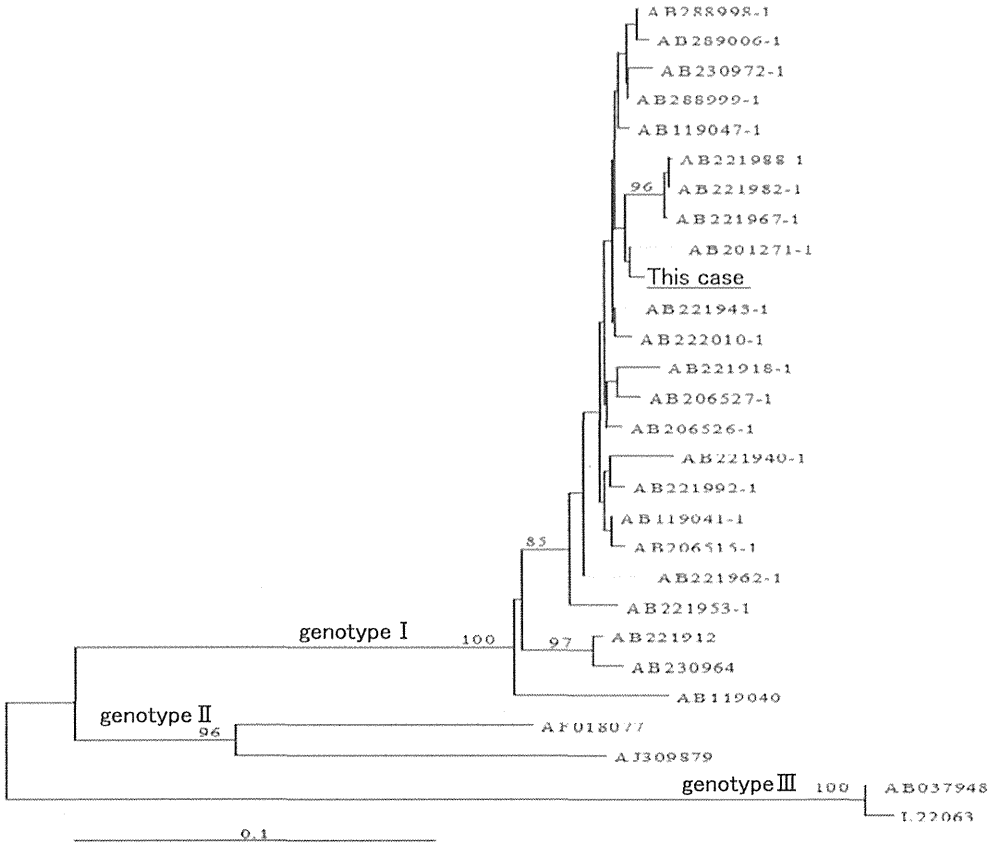


Fig. 3 Neighbour-joining phylogenetic tree constructed from region 853-1289 of HDV genome. The strain from the patient in this case was compared with the representative sequences including 23 genotype I, 2 genotype II and 2 genotype III. The strain from this patient belonged to genotype I.

D 型慢性肝炎の治療法としては，現在のところ有効性が認められているのは IFN のみである. Farci らは，IFNα2a 900 万単位を週 3 回 48 週投与し，ALT 値の正常化を 71% に，血清 HDV-RNA の陰性化を 50% の症例に認め，それらの症例では組織学的にも改善したと報告している[17]. しかし，IFN 投与終了後にほとんどの症例で HDV-RNA の再出現と ALT 値の再上昇を認め，再発率が高いとも報告している[17]. 近年では Peg-IFN による治療成績が報告されており，Niro らは Peg-IFNα-2b（1.5 μg/kg）を 72 週単独投与した群と Peg-IFNα-2b 72 週にリバビリン 48 週投与を併用した群との比較を行っているが，Peg-IFN 投与によって 21% の症例で SVR を達成したと報告している[18]. また Wedemeyer らは，Peg-IFNα-2a 180 μg 48 週投与により約 25% の症例で HDV RNA クリアランスを達成できると報告している[19]. 一方，HBV の治療で用いられる lamivudine に代表される核酸アナログ製剤は，その単独投与あるいは IFN との併用に関わらず HDV に対して効果がないことが報告されている[20)21)]. 前述の Wedemeyer らの研究でも Peg-IFNα-2a と adefovir との治療効果の検討がなされているが，核酸アナログ製剤である adefovir には HDV に対する効果はないと報告している[19]. これは核酸アナログ製剤によって HBV 複製が抑制されても，HBsAg 産生を抑えることができないためと推察される. また HDV と同じ RNA ウイルスである HCV 治療に対して用いられる ribavirin も，ribavirin 単独投与あるいは IFN との併用に関わらず，HDV RNA クリアランスに効果がないことが報告されている[18)22)23)]. 以上より，

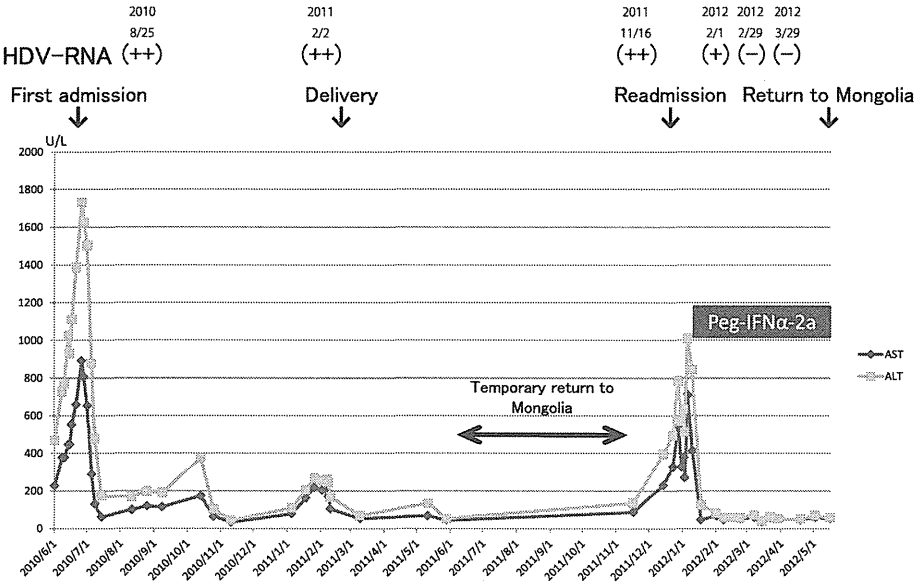


Fig. 4　Clinical course in this case. After starting treatment with Peg-IFN, AST and ALT levels became almost normal. Furthermore, HDV-RNA in serum was undetectable around 8 weeks after the treatment.

現在ではPeg-IFNの1年間投与がD型慢性肝炎に対する治療法として推奨されている[24]．本症例においてもPeg-IFNα-2aによる治療を行ったところ，治療開始前は高値であったAST，ALT値は投与開始後から速やかに改善し，Peg-IFNα-2a投与中の保存血清を用いたHDV-RNAのRT-PCRでは，治療開始後2カ月目からPCRバンドが検出されなくなった．以上より，治療途中で患者がモンゴルに帰国したため最終的な治療効果については不明だが，少なくともPeg-IFNによる治療期間においてはHDVに対する抗ウイルス効果があり，本症例における肝障害はHDV感染によるものと考えられた．

本症例は当初，妊娠を契機としたB型慢性肝炎急性増悪症例と考えていたが，経過中のHBV-DNA量は常に低値であることからB型慢性肝炎急性増悪は否定的となり，原因不明のまま確定診断に至らなかった症例である．しかし，患者背景などを改めて検討し直すことで最終的にHBV・HDV重複感染による肝障害と診断できた．本邦においてHBV・HDV重複感染症例は極めて稀であり，また現在，本邦で使用可能なコマーシャルベースのHDV測定系が存在しないことも災いして診断に苦慮したが，国際交流が盛んな今日において，このような症例は増加することが予想される．HBV-DNA量の増加を伴わないB型慢性肝炎急性増悪を認めた際には，HDVの重複感染を念頭に置くべきと思われる．

結　語

HBV・HDV重複感染による肝障害に対してPeg-IFNα-2aが有効であった1例を経験した．本邦においてHBV・HDV重複感染症例は稀であり，若干の文献的考察を加え報告した．

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A case of a HBV carrier with HDV superinfection treated by PEG-IFN

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A 26-year-old Mongolian woman was admitted to our hospital because of liver dysfunction. As she has been a HBV carrier, acute exacerbation of chronic hepatitis B seemed to be the cause of liver dysfunction at first. However, the loads of serum HBV-DNA on admission were low (2.8 log copy/ml). As she was pregnant, she was observed without treatment. Liver function once improved without treatment, but it became worse again after delivery. At this time, it was suspected HDV superinfection could affect the liver dysfunction. For HDV-RNA was positive in stored sera by RT-PCR, she was diagnosed as HBV and HDV superinfection and started treatment with Peg-IFN. Although HDV infection is rare in Japan, in case of acute exacerbation of a HBV carrier with low serum HBV-DNA level, HBV and HDV superinfection should be considered.

Key words: hepatitis D　Peg-IFN　superinfection

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RESEARCH ARTICLE

Validation of Cross-Genotype Neutralization by Hepatitis B Virus-Specific Monoclonal Antibodies by *In Vitro* and *In Vivo* Infection

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Abstract

Vaccines based on hepatitis B virus (HBV) genotype A have been used worldwide for immunoprophylaxis and are thought to prevent infections by non-A HBV strains effectively, whereas, vaccines generated from genotype C have been used in several Asian countries, including Japan and Korea, where HBV genotype C is prevalent. However, acute hepatitis B caused by HBV genotype A infection has been increasing in Japan and little is known about the efficacy of immunization with genotype C-based vaccines against non-C infection. We have isolated human monoclonal antibodies (mAbs) from individuals who were immunized with the genotype C-based vaccine. In this study, the efficacies of these two mAbs, HB0116 and HB0478, were analyzed using in vivo and in vitro models of HBV infection. Intravenous inoculation of HBV genotype C into chimeric mice with human hepatocytes resulted in the establishment of HBV infection after five weeks, whereas preincubation of the inocula with HB0116 or HB0478 protected chimeric mice from genotype C infection completely. Interestingly, both HB0116 and HB0478 were found to block completely genotype A infection. Moreover, infection by a genotype C strain with an immune escape substitution of amino acid 145 in the hepatitis B surface protein was also completely inhibited by incubation with HB0478. Finally, in vitro analysis of dose dependency revealed that the amounts of HB0478 required for complete protection against genotype C and genotype A infection were 5.5 mIU and 55 mIU, respectively. These results suggested that genotype C-based vaccines have ability to induce cross-genotype immunity against HBV infection.

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Introduction

Hepatitis B virus (HBV) is a blood-borne, hepatotropic virus that infects an estimated 350 million people worldwide. Besides the manifestations associated with acute hepatitis, chronic HBV infection constitutes a significantly high risk for the development of liver cirrhosis and hepatocellular carcinoma. HBV strains are classified into eight genotypes based on genetic diversity [1,2] and the prevalence of these genotypes varies geographically [3]. Hepatitis B surface antigen (HBsAg) is the key molecule for HBV entry into the hepatocyte [4] and HBV vaccination establishes host immunity by activating B lymphocytes that produce HBsAg-specific antibodies (anti-HBs) with neutralizing activities. The highly immunogenic region of HBsAg, known as the "a" determinant, comprises two peptide loops in which several amino acids vary among the HBV genotypes [5].

Vaccination of high risk individuals and universal infant/childhood vaccination programs have effectively decreased the incidence of acute HBV infection and consequent chronic hepatitis B [6]. Recombinant vaccines containing HBsAg generated from HBV genotype A2 (gt-A2) have been used worldwide. Although these A2-type vaccines are effective in preventing non-A2 HBV infections [7], investigation of cross-genotype protection is limited in the clinical setting. On the other hand, genotype B (gt-B) and genotype C (gt-C) strains are the most prevalent in east Asian countries [1] and some of these countries, including Japan and Korea, have used recombinant vaccines generated from gt-C for immunoprophylaxis against HBV endemic in these communities [8,9]. In the last decade, however, the spread of gt-A strains imported from foreign countries and the subsequent increase of hepatitis caused by HBV gt-A is a growing concern in Japan [10]. Until now, little is known about whether the gt-C HBV vaccine can induce effective immunity against non-C HBV infection.

Previously, we isolated human monoclonal antibodies (mAbs) against HBV from healthy volunteers who had been immunized with a gt-C type recombinant HBV vaccine (Biimugen), using a cell-microarray system [11–13]. A subsequent report revealed that among these mAbs, HB0116 and HB0478, recognize the first N-terminal peptide loop within the "a" determinant and have HBV-neutralizing activities [14]. In this report, whether these mAbs generated by the gt-C type vaccine can protect gt-A strain infections was investigated using in vitro and in vivo HBV infection models, including primary human hepatocytes (PHHs) and severe combined immunodeficient mice transgenic for urokinase-type plasminogen activator, whose livers were repopulated with human hepatocytes (hereafter referred to as chimeric mice) [15–17]. The neutralizing activities of these mAbs against the frequently isolated immune escape mutant, which has an amino acid substitution of arginine for glycine at residue 145 within the second, C-terminal loop of HBsAg (G145R) [18–20], were also investigated.

Materials and Methods

Ethics statement

This study conformed to the ethics guidelines of the 1975 Declaration of Helsinki as reflected by approval by the Ethics Committee of University of Toyama with written informed consent (Permit Number: 14–123). All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care Use of Laboratory Animals of the National Institute of Health. The animal protocol was approved by the Ethics Committees of PhoenixBio Co., Ltd (Permit Number: 0253). Chimeric mice were housed in specific pathogen—free facilities at the laboratory of PhoenixBio Co., Ltd. Food and water were delivered ad libitum. Chimeric mice were weighed and anesthetized using isofluorane prior to blood collection from the

orbital vein. The chimeric mice were anesthetized using isofluorane and sacrificed by exsanguination from the heart at the end of the experiment.

HBV-specific mAbs and recombinant peptides

Recombinant HB0116 and HB0478 in IgG form were generated as described previously [14]. Synthetic peptides for the first loop of HBsAg gt-C and gt-A (123–137 gt-C: TCTI-PAQGTSMFPSC; 123–137 gt-A: TCTTPAQGNSMFPSC) were generated also as described previously [14].

The binding activity of each mAb for recombinant peptides was examined by ELISA with streptavidin-coated plates (Nunc, Roskilde, Denmark). Plates were coated with the peptides at 10 μg/mL and nonspecific binding was blocked with PBS containing 3% bovine serum albumin (BSA). Each mAb was added to the wells for 2 hours, followed by washing and reaction with alkaline phosphatase-conjugated anti-human IgG (Sigma, Saint Louis, MO). The O.D. value at 405 nm was evaluated after addition of phosphate substrate (Sigma). Control human monoclonal IgG1 (cIgG, Athens Research & Technology, Athens, GA) was added at the same concentration as the control.

Immunoprecipitation assay

1×10^4 copies of HBV of gt-C, gt-A and G145R (gt-C with an amino acid substitution of arginine for glycine at position 145 of HBsAg) were incubated with 1 μg of mAbs diluted in 2% BSA/PBS or cIgG on a rotating wheel overnight at 4°C and then protein A-Sepharose beads (GE Healthcare) were added to the mixture and incubated for a further 4 hours. The beads were centrifuged briefly to remove the supernatants, washed four times with 1 mL 2% BSA/PBS and resuspended in 30 μL sample loading buffer (Tris/HCl (pH 6.8), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0·001% bromophenol blue). After boiling for 5 minutes, 15 μL aliquots were applied to 15% SDS-PAGE and the proteins were separated and transferred to a nitrocellulose membrane. HBsAg was detected using 1 μg/mL of a HB0116/HB0478 mixture, followed by anti-human IgG conjugates of horseradish peroxidase (1:5000, Sigma) as the secondary antibody. The bands were visualized with enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK).

HBV-neutralizing assay using HepaRG cells

The HBV-neutralizing capacities of HB0116 and HB0478 were investigated using the HepaRG cell line (supplied by Biopredic International, Rennes, France). The HepaRG cells were cultured and differentiated as described previously [21,22]. 1×10^4 copies of HBV and 1 μg of each mAb were preincubated for 1 hour at room temperature and then added to HepaRG cells in medium containing 4% polyethylene glycol (PEG) 8000 (Sigma-Aldrich, St. Louis, MO, USA). After overnight incubation, the HepaRG cells were washed gently three times with medium and then cultured with fresh medium. On day 7 after infection, cellular DNA was extracted and HBV DNA was quantified as described previously [14].

In vivo HBV-neutralizing assay using chimeric mice

The chimeric mice were purchased from PhoenixBio Co, Ltd (Hiroshima, Japan). The HBV inocula used in this experiment were prepared as follows: culture supernatants from cells transfected with plasmids expressing HBV gt-C, gt-A, and G145R contained immature HBV virions [16] and chimeric mice were inoculated with these culture supernatants to obtain the

monoclonal and intact infectious virions. After establishing viremia in these mice, the sera were collected and used as inocula after titration in another experimental chimeric mouse.

Firstly, $1x10^4$ copies of the sera of chimeric mice infected with gt-A, gt-C, G145R were incubated at 37°C for 2 hours in the presence of HB0116 and/or HB0478 and injected intravenously into chimeric mice. Five weeks after injection, serum HBV DNA was measured by quantitative polymerase chain reaction (PCR) as reported previously [23].

In vitro HBV-neutralizing assay using PHHs isolated from chimeric mice

Freshly isolated PHHs were purchased from PhoenixBio Co., Ltd (Higashihiroshima, Japan). Briefly, human hepatocytes were collected from the livers of chimeric mice by collagenase perfusion and plated on collagen-coated 96-well multiplates at a density of 6.7×10^4 cells per well. The cells were then grown in dHCGM medium (Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 1 μg/mL of penicillin, 1 μg/mL of streptomycin, 20 mM HEPES, 15 μg/mL of L-proline, 0.25 μg/mL of human recombinant insulin, 50 nM dexamethazone, 5 ng/mL of human recombinant epidermal growth factor, 0.1 mM ascorbic acid, and 2% DMSO).

To investigate HBV kinetics, PHHs were inoculated with serum from HBV gt-C chimeric mice at 5 genomes per cell for 24 hours in the presence of 4% PEG 8000. The sera from chimeric mice contained excess subviral particles including HBs proteins. The cells were then washed three times with the medium to remove the inoculum, and the culture supernatants were collected and replenished with fresh medium on 2, 3, 5, 7, and 12 days post infection (dpi).

To optimize the infectious condition for the analysis of antibody neutralization, HBV gt-C at 10, 3, 1, and 0.3 genomes per cell was preincubated with or without 100 mIU of hepatitis B immune globulin (HBIG) for 2 hours and PHHs were inoculated with the HBV-HBIG mixture for 24 hours with PEG or for 48 hours without PEG. The cells were washed and the supernatants were collected as described above.

Antibody neutralization experiments were performed as follows. HBV gt-C or gt-A inocula at 10 genomes per cells (6.7×10^5 genomes/well) were preincubated with 670, 67, 6.7, or 0.67 ng of HB0478 (corresponding to 550, 55, 5.5, or 0.55 mIU) and exposed to PHHs for 48 hours without PEG. The cells were then washed and the supernatants were collected as described above.

Southern blot analysis of HBV DNA

Southern blot analysis was performed with full-length probes for HBV as described previously [24].

Quantification of HBV DNA, pregenomic RNA and HBsAg

Total RNA and total DNA were extracted from PHHs using ISOGEN (Nippon Gene Co. Ltd., Tokyo, Japan) and SMITEST EX R&D Kit (Genome Science Laboratories, Tokyo, Japan), respectively. Purified total RNA was then reverse-transcribed using a High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. Extracellular HBV DNA, intracellular HBV DNA and pregenomic RNA were quantified by real-time quantitative PCR using StepOne Plus and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The samples were denatured by incubating for 10 minutes at 95°C and amplified for 45 cycles (95°C 15 seconds, 60°C 60 seconds) with specific primers and TaqMan fluorescent probes. HBV DNA was amplified using primers HBV-F (5′-CACATCAGGATTCCTAGGACC-3′), HBV-R (5′-AGGTTGGTGAGTGATTGGAG-3′), and TaqMan probe HBV-FT (5′-FAM-CAGAGTCTAGACTCGTGGTGGACTTC-TAMRA-3′).

Primers HBV-PC-F (5′-GGTCTGCGCACCAGCACC-3′), HBV-DN-R (5′-GGAAAGAAGT-CAGAAGGCAA-3′) and TaqMan probe HBV-FM (5′-FAM-TCCAAGCTGTGCCTT-MGB-3′) specifically amplify cDNA from precore RNA. Primers HBV-PG-F (5′-CACCTCTGCC-TAATCATC-3′), HBV-DN-R and TaqMan probe HBV-FM amplifies cDNA from both precore RNA and pregenomic RNA. The amount of pregenomic RNA was calculated by subtracting the copy number of precore RNA amplification from that of precore/pregenomic RNA amplification [25]. Extracellular HBsAg was quantified by automated ELISA (Fujirebio Inc., Tokyo, Japan). The detection limits are 2 × 103 copies for HBV DNA, 2 × 102 copies for pregenomic RNA and 0.005 IU/mL for HBsAg.

Results

Influence of genotype and amino acid substitutions on recognition by HBV-specific mAbs

The mAbs HB0116 and HB0478 bind to the first loop (amino acids 123–137) of the "a" determinant and strongly inhibit HBV gt-C infection [14]. Therefore, whether the binding capacity of each mAb is affected by amino acid variation within the first loop was examined using recombinant peptides; there is amino acid variation between genotypes C and A at positions 126 (gt-C: I, gt-A: T) and 131 (gt-C: T, gt-A: N). Both HB0116 and HB0478 bound peptides not only corresponding to the first loop with the gt-C sequence but also corresponding to those with the gt-A sequence, indicating their cross-genotype recognition on binding in vitro (Fig. 1A). The binding capacities to the native HBs proteins of gt-A, gt-C, together with gt-C with the substitution G145R located within the second loop of HBsAg extracellular domain, were also examined. Interestingly, immunoprecipitation assays revealed that HB0116 bound to HBsAg of HBV gt-C and gt-A, but not to G145R, whereas HB0478 could bind to all three proteins (Fig. 1B).

Next, the HBV-neutralizing activity of these mAbs was evaluated using HepaRG cells, which support HBV infection, by inoculating them with a high dose of HBV. Fig. 2 shows that HB0116 suppressed the increase of HBV DNA after inoculation of both HBV gt-C and gt-A, but could not inhibit infection by G145R. However, HB0478 could prevent infection by HBV gt-C, gt-A, and also G145R. These results are consistent with the immunoprecipitation results shown in Fig. 1B and indicate that HB0478 can bind to the first loop, regardless of genotype, and also bind to the G145R substituted protein, which is seen as an antibody escape variant in clinical practice.

HB0116 and HB0478 protect against HBV gt-C and gt-A infections but only HB0478 protects against G145R mutant infection in vivo

The in vivo neutralizing activity of the mAbs was investigated using chimeric mice with human hepatocytes. After 1×10^4 copies of HBV gt-C or gt-A were incubated with HB0116 and/or HB0478, the mixtures were injected intravenously into naïve chimeric mice and serum HBV DNA concentrations were measured for the evaluation of HBV infection at five weeks after injection. Although HBV gt-C infection was confirmed in the control experiment (Group 1, 9.8×10^3 and 1.1×10^4 copies/ml) (Table 1), preincubation of the inoculum with either 1 μg or 10 μg of HB0116 or HB0478 completely blocked HBV infection with both gt-C and gt-A (Groups 2–5 for gt-C, Groups 6–9 for gt-A). Meanwhile, inoculation of the HBV G145R strain into naïve chimeric mice resulted in the establishment of infection (Group 10, 1.0×10^4 and 1.4×10^4 copies/ml) and incubation with 10 μg of HB0116 had no impact on infection by G145R (Group 11, 1.1×10^4–4.4×10^4 copies/ml), whereas as little as 1 μg of HB0478 completely blocked G145R infection (Groups 12 and 15). Apparently, a combination of HB0116 and

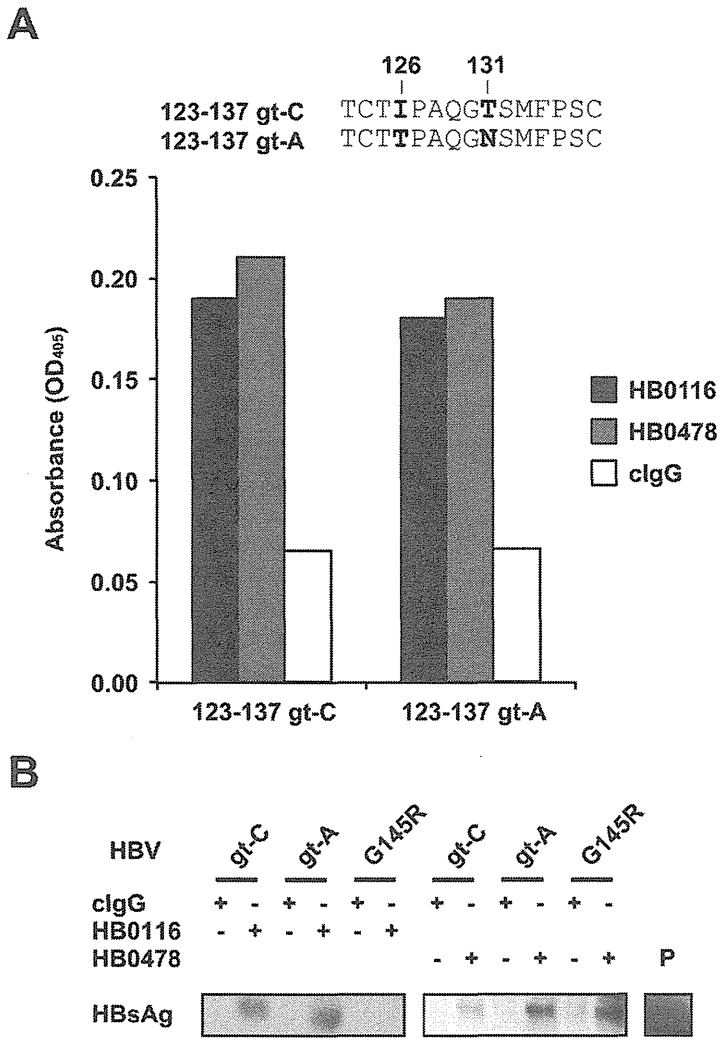

Fig 1. Binding capacity of mAbs HB0116 and HB0478 against with gt-C and gt-A HBsAg and the G145R variant. (A) Binding of mAbs HB0116 and HB0478 to synthetic peptides covering the first external loop of small-HBsAg was demonstrated by ELISA. The sequences of the recombinant peptides used in the analysis are shown above: amino acids which vary between genotype C (gt-C) and genotype A (gt-A) are indicated in bold. The absorbance at 405 nm is shown on the Y axis. Average data of three independent experiments are shown. **(B)** The gt-C, gt-A, and G145R virions were immunoprecipitated with HB0116 or HB0478 and HBsAg in the precipitates was detected by Western blotting. Recombinant HBsAg protein was used as the positive control (P lane). Representative data of three independent experiments are shown.

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HB0478, either at 1 μg or 10 μg protected the chimeric mice from HBV infection (Groups 13 and 14).

Evaluation of PHHs isolated from chimeric mice with human hepatocytes as an in vitro HBV infection model

PHHs isolated from the chimeric mice with human hepatocytes were used to characterize further the neutralizing activity of mAb HB0478. In vitro HBV infection of the PHHs was

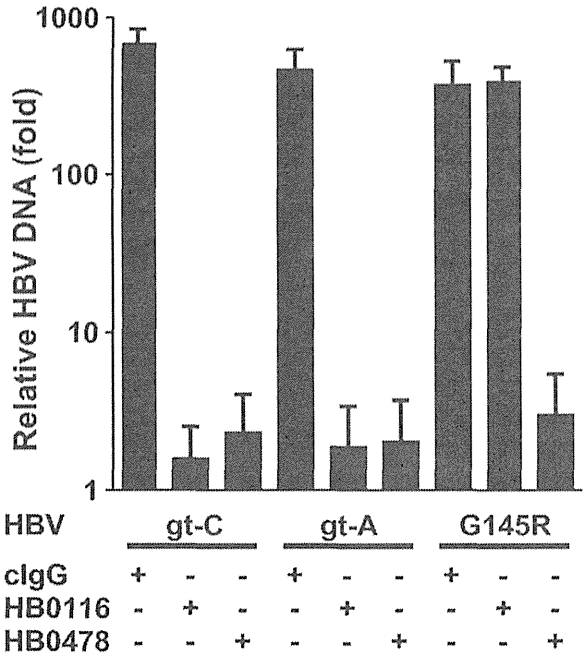

Fig 2. Relative HBV DNA concentrations in the total DNA extracted from HepaRG cells at 7 days after HBV infection. The Y-axis depicts the relative HBV DNA concentrations in the cells, with the concentrations on day 1 set at 1. Mean ± SD of three independent experiments are shown. cIgG, control human monoclonal IgG.

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confirmed by inoculating HBV gt-C at 5 HBV genomes per cell in the presence of 4% PEG 8000. The levels of pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA, and extracellular HBsAg were monitored and it was found that all these viral products gradually increased from 3 to 12 dpi (Fig. 3A). Southern blot analysis of cell lysates revealed the presence of single-stranded HBV DNA as a replication intermediate in the infected PHHs, confirming HBV replication in the cells (Fig. 3B). Furthermore, culture supernatants from HBV-infected donor PHHs were inoculated into newly prepared PHHs. An increase of HBsAg production from the PHHs was observed following exposure of the cells to another culture supernatant containing HBV DNA (Fig. 3C), indicating that the donor PHHs produced infectious HBV virions (also known as Dane particles).

Next, to investigate whether this model can be adapted for the study of neutralizing activities against HBV infection, the effect of HBIG on HBV infection was evaluated in vitro. Fig. 3D shows that HBIG strongly reduced HBV infection but residual infection was detected in the presence of PEG, whereas, in the absence of PEG, the HBV infection was completely blocked by HBIG. These results indicate that, when neutralizing activities against HBV infection were investigated using this PHH system, inoculation without PEG is appropriate for the specificity of the establishment of HBV infection. However, because inoculation without PEG would be less efficient for HBV infection, the efficacy of HBV infection in the absence of PEG was also examined. Various titers of HBV (10, 3, 1, and 0.3 genomes per cell) were inoculated into PHHs and the HBsAg titers in the supernatants were monitored for 22 days (Fig. 3E). Although the HBsAg levels from PHHs infected without PEG were lower than those with

Table 1. In vivo neutralization of HBV infection by monoclonal antibodies (mAbs).

HBV genotype	Group	HB0116 (μg/body)	HB0478 (μg/body)	HBV DNA (copies/mL)
C	Group 1	-	-	9.8×10^3
	(n = 2)	-	-	1.1×10^4
	Group 2	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 3	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 4	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 5	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
A	Group 6	1	-	n.d.
	(n = 3)	1	-	n.d.
		1	-	n.d.
	Group 7	10	-	n.d.
	(n = 3)	10	-	n.d.
		10	-	n.d.
	Group 8	-	1	n.d.
	(n = 3)	-	1	n.d.
		-	1	n.d.
	Group 9	-	10	n.d.
	(n = 2)	-	10	n.d.
G145R	Group 10	-	-	1.4×10^4
	(n = 2)	-	-	1.0×10^4
	Group 11	10	-	4.4×10^4
	(n = 3)	10	-	1.1×10^4
		10	-	3.3×10^4
	Group 12	-	10	n.d.
	(n = 3)	-	10	n.d.
		-	10	n.d.
	Group 13	1	1	n.d.
	(n = 2)	1	1	n.d.
	Group 14	10	10	n.d.
	(n = 2)	10	10	n.d.
	Group 15	-	1	n.d.
	(n = 2)	-	1	n.d.

n.d.: not detected.

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PEG, the HBsAg levels in the supernatants were well correlated with the initial input of HBV (10 to 0.3 genomes per cells) in the absence of PEG. These results suggest that, albeit with somewhat lower infectivity, inoculation without PEG is available for neutralization assays using the PHH system.

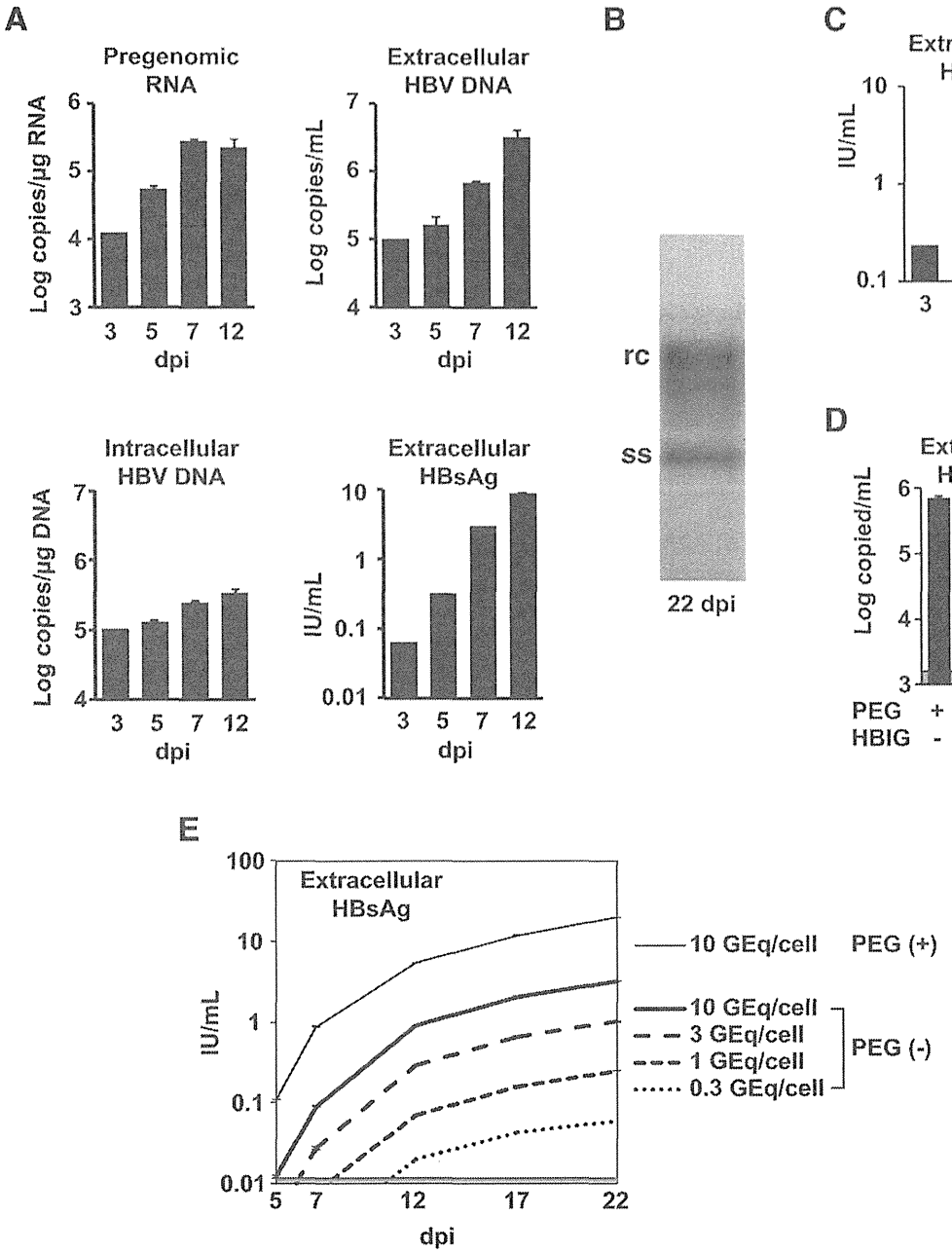

Fig 3. In vitro HBV infection model using PHHs isolated from chimeric mice with human hepatocytes. (A) PHHs were inoculated with HBV gt-C at 5 genomes per cell in the presence of PEG and intracellular pregenomic RNA, intracellular HBV DNA, extracellular HBV DNA and extracellular HBsAg were monitored by real-time quantitative PCR, or by automated ELISA. dpi, days post infection. **(B)** 20 μg of total DNA was extracted from PHHs 22 days after infection with HBV and analyzed by Southern blotting. Single-stranded HBV DNA (ss), a replication intermediate, and relaxed circular HBV DNA (rc) were detected. **(C)** Freshly prepared PHHs were inoculated with the day 52 supernatant from other HBV-infected PHHs. HBsAg secretion was monitored. **(D)** The use of PEG on HBV infection could mask the specificity of neutralization of HBV infection. Residual HBV infection was observed when PHHs were inoculated with a mixture of HBV and HBIG in the presence of PEG. An asterisk indicates a value below detection limit. **(E)** The efficacy of HBV infection without PEG was proportional to the size of the inoculum.

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HB0478 efficiently blocks HBV infection by both gt-C and gt-A

To evaluate the neutralizing activity of HB0478 against HBV infection, various amounts of HB0478 were preincubated with HBV gt-C or gt-A at 10 HBV genomes per cell (6.7×10^5 genomes/well) for 2 hours and exposed to PHHs for 48 hours without PEG (Fig. 4A). Fig. 4B shows the levels of HBV DNA in the supernatants harvested at 22 dpi. HB0478 in the amounts of 550 and 55 mIU completely blocked the infection by both gt-C and gt-A (HBV DNA was never detected in the supernatant). 5.5 mIU of HB0478 also completely inhibited gt-C infection, while it strongly reduced but did not completely inhibit gt-A infection. These results indicate that mAb HB0478 has powerful neutralizing activity against HBV infection and that HB0478 generated by the gt-C type vaccine could protect against HBV infection by both gt-C and gt-A, although less effectively against gt-A.

Discussion

Although the HBV vaccine strain used predominantly worldwide is genotype A2, genotype C strains are prevalent in Japan, where a selective vaccination program for high risk individuals with a gt-C-based vaccine is ongoing. A potential problem is that genotype A2 has been increasing recently as a cause of acute hepatitis B in Japan [10] and little is known about the efficacy of the gt-C-based vaccine against non-C HBV infection. In this report, we demonstrated that two mAbs, HB0478 and HB0116, derived from individuals immunized with the gt-C vaccine (Biimugen) that has been approved in Japan, neutralized HBV infections by both gt-C and gt-A in vitro and in vivo, suggesting that immunization with the gt-C vaccine could prevent infection by non-C HBV strains.

Epidemiological studies have shown that, in countries operating universal childhood vaccination programs using the gt-A2 vaccine, vertical transfer and/or incident infection of non-A2 were prevented efficiently [7]. Some studies have produced data supporting cross-genotype protection by immunization. An analysis of 221 mAbs isolated from volunteer HB vaccinees showed that 97% of them recognized common epitopes shared by all HBV genotypes [5]. The C(K/R)TC motif (amino acids 121–124), located in the N-terminal portion of the first loop of the "a" determinant of HBsAg, is conserved among all HBV genotypes (except for residue 122, K or R determining the serological subtype d or y, respectively) and highly immunogenic [26]. Moreover, a single mouse monoclonal Ab protected chimpanzees from infection by both adr (gt-C) and ayw (genotype D) strains [27].

Along with these findings, our results showed that the mAbs HB0478 and HB0116, generated following immunization with the gt-C type vaccine, neutralized the infectivity of both gt-C and gt-A HBV. In vitro experiments investigating dose dependency using freshly isolated PHHs also demonstrated that HB0478, at doses above 55 mIU, completely protected against both gt-C and gt-A infection, whereas HB0478, at a lower dose, 5.5 mIU, protected against gt-C infection only. It has been reported that analysis of nine HBV DNA positive blood donors in the United States revealed that 5 individuals who had been immunized with an A2-type vaccine were not protected against infections by non-A2 HBV [28]; however, the serum anti-HBs levels of these individuals (3–96 mIU/mL) were relatively low. Interestingly, the infections remained at a subclinical level in these vaccines, who subsequently resolved the HBV infection, suggesting that gt-A2 vaccination could not prevent non-A2 infection but can inhibit the development of clinical manifestations [28]. Therefore, it is possible that HBV specific antibodies, induced by gt-C vaccines, might be able to protect against clinical hepatitis caused by infection with non-C genotypes, even with lower anti-HBs concentrations. Further investigations are needed to determine clinical effectiveness of gt-C vaccine to induce cross-genotype immune responses.