

遺伝子型	HBs抗原消失率	HBs抗原消失率	HBs抗原消失率	HBV-DNA減少率
B	41% ^{a)}	44% ^{c)}	14% ^{d)}	-3.42% ^{e)}
C	15% ^{a)}	28% ^{c)}	10% ^{d)}	-3.65% ^{e)}
A	37% ^{b)}	47% ^{c)}	—	-3.58% ^{e)}
D	6% ^{b)}	25% ^{c)}	—	-3.68% ^{e)}

P<0.01 (B vs C, A vs D), P=NS (B vs D, C vs D), P=0.01 (A vs D)

表2 各種ウイルス剤における治療効果とHBV遺伝子型との関係

a) : Kaoら(文献9)、b) : Erhardtら(文献11)、c) : Janssenら(文献12)、d) : Chanら(文献17)、e) : Westlandら(文献19)

一方B型急性肝炎におけるわが国の遺伝子型分布については遺伝子型Cが最も多く、68%に認められ、ついで遺伝子型Aが19%に認められている(図1)。先のB型慢性肝炎におけるわが国における遺伝子型分布と比較して急性肝炎では遺伝子型Aの割合が増加しており、特に都市部での性行為ルートによる若年層からの感染が急速に広がっていると考えられている。臨床像との関連については遺伝子型Aは肝炎の程度は軽いが遷延化する傾向にあるとされ、一方では遺伝子型Bjの高い劇症化率が示されている⁴⁾。

HBV 遺伝子型の 亜型分類とその臨床像

HBVのある遺伝子領域において、もとの遺伝子型とは異なる遺伝子型を示すHBV組換え遺伝子型の存在が知られている。遺伝子型Bは日本以外のアジアに広く分布しているプレコア/コア領域が遺伝子型Cに置換された組換え遺伝子型Ba(アジア型)とわが国に特異的に分布する組換えのない遺伝子

型Bj(日本型)に分類されている⁵⁾。さらに遺伝子型BjはBaと比較して早期にHBe抗原陽性からHBe抗体陽性へとセロコンバージョンを起こし、肝炎が沈静化するとされている⁶⁾。また台湾においては遺伝子型Bと若年発症肝臓がんとの関連が報告されているが、わが国では遺伝子型Bはむしろ高齢発症肝臓がんとの関連が認められており、若年発症肝臓がんと組換え遺伝子型Baの関与が推測されている。

遺伝子型Aについては、その地理的分布の違いから東南アジア・アフリカに広く分布するAa(アジア・アフリカ型)と欧米地域に分布するAe(欧米型)に亜型分類がなされている⁷⁾。遺伝子型AaはAeと比較してより若年でHBe抗原の消失が認められかつ肝臓がんとの関連が推測されている。ウイルス学的には遺伝子型Aaは他の遺伝子型では認められないプレコアスタートコドン上流のコザック配列の変異およびプレコア領域のエンカプシデーションシグナル配列のG1862TとG1888A変異を認め、血中からの早期HBe抗原消失に関与していると考えられている。

さらに最近では中央アフリカから新たな亜型の遺伝子型Acも報告され遺伝子型Aの起源についても論じられているようである。

わが国に広く分布する遺伝子型Cについても亜型分類がなされ、主にその地理的分布の違いから香港・東南アジア・中国の南部に分布する遺伝子型C1(南アジア型)とわが国を含む韓国・中国の北部に広く分布する遺伝子型C2(東アジア型)である⁸⁾。最近の報告では遺伝子型C1とC2は同じ遺伝子型Cでありながらも異なる遺伝子変異(V1753とT1653)により、それぞれが肝臓がんに関連しているとされ、また遺伝子型C2はC1と比較してより肝臓がんに関連する遺伝子型であると結論されている。

HBV 遺伝子型と 治療効果

1. インターフェロン治療とHBV遺伝子型

B型慢性肝炎に対するインターフェロン(IFN)治療はわが国では保険上の

制約もあり、投与期間の問題などから必ずしも満足の得られる結果が得られているわけではない。しかし、厚生労働省の肝炎班会議 (B型およびC型肝炎ウイルスの感染者に対する治療の標準化に関する臨床研究) から公表されたB型慢性肝炎の治療ガイドラインに示されているように、35歳以下の若年の慢性肝炎例ではIFNが第一選択となっている。IFN治療効果とHBV遺伝子型との関連については、台湾からの報告では遺伝子型Bの方がCに比べ有意に有効率が高く^{9,10)}、またドイツからの報告では、遺伝子型Dに比べAが有意に高率にセロコンバージョンを起こしたとしており¹¹⁾、いわゆる従来型のIFN治療においては遺伝子型による効果の違いは明らかである (表2)。最近では、わが国においてはまだB型肝炎には認可されていないペグインターフェロン (Peg-IFN) 治療の有効性が海外から多く報告されている。Peg-IFNとHBV遺伝子型との関連についてJanssenらは52週投与後の26週観察後のHBe抗原消失率は遺伝子型BとCではそれぞれ44%と28%で有意差はなかったが、遺伝子型AとDではそれぞれ47%と25%であり、遺伝子型Aにおいて有意に有効性が高かったと報告している (表2)¹²⁾。一方でLauらは48週投与後の24週観察後のHBe抗原消失率は遺伝子型A: 52%、B: 30%、C: 31%、D: 22%で各遺伝子型間に差はなかったとしている¹³⁾。

2. 核酸アナログ治療とHBV遺伝子型

a. ラミブジン

ラミブジンはIFNなどの従来薬剤に比べ強い抗ウイルス効果を発揮する

ことが報告されており、海外でのHBe抗原陽性例に対する52週投与ではHBe抗原消失率が32%、セロコンバージョン率が17%であった¹⁴⁾。しかし、1~2年ぐら経過すると耐性株が出現し抗ウイルス効果は減退する。わが国において熊田らは耐性株の出現については1年目、2年目、3年目、5年目でそれぞれ19.7%、32.2%、43.8%、62.5%と報告している¹⁵⁾。ラミブジン治療効果とHBV遺伝子型との関連については、われわれは複数の施設の協力を得て遺伝子型Ba、BjとC症例において、ケースコントロールスタディを行ったが、治療開始2年後のALT正常化率、HBV DNA陰性化率について、各遺伝子型群間に有意差はみられなかった¹⁶⁾。また、香港からの報告では、遺伝子型BとCの患者でラミブジン治療におけるセロコンバージョン率を検討したが、遺伝子型Bにやや有効率が高いものの、有意差はみられなかったとしているが (表2)¹⁷⁾、一方では遺伝子型Bの方がCより有効率が良好であったとする報告もあり、まだ一定の見解は得られていない。またラミブジン治療による耐性株の出現頻度についても遺伝子型との関係の有無についてもいくつか報告されている。われわれの検討では遺伝子型Cの耐性株の出現頻度は50%であり、Bj群の28%とBa群の13%にくらべ有意に高率であった (図2)¹⁶⁾。

b. アデフォビル

ラミブジンの長期投与に伴う耐性化の問題に関しては、その後わが国で認可されたアデフォビルの併用投与によってラミブジン耐性株も強力に抑制

できるようになっている。アデフォビルの単独投与については、海外では48週投与でHBe抗原陽性例に対するHBV DNA陰性化率は21%、セロコンバージョン率は12%と良好な成績が報告されている¹⁸⁾。また、米国においてアデフォビルの効果を各遺伝子型間で比較したところ、治療効果に差はみられず、人種、地理、HBe抗原の有無などの因子も効果には関連がみられなかったと報告されている (表2)。日本でも認可されたラミブジンとアデフォビルとの併用療法については、ラミブジン耐性患者に対し、アデフォビル単独、あるいはラミブジンとアデフォビルの併用投与で、それぞれ48週後でHBV DNAレベルで-4.0logおよび-3.6logの低下と、47%、53%のALTの正常化率が得られている¹⁹⁾。

c. エンテカビル

最近日本でも認可されたエンテカビルにおける海外での治験成績では、エンテカビル0.5mg、48週間投与でALTの正常化率は68%、セロコンバージョンは21%に認められている²⁰⁾。また24週投与でのHBV DNA陰性化率は84%であり、対照のラミブジンの効果を上回ったと報告されている。一方耐性化については、初回投与例では投与96週で3%にウイルス学的リバウンドがみられたとされており、ラミブジンに比して耐性化出現は低率であると思われる。しかし、ラミブジン耐性化例に対してエンテカビルを投与した場合は、1年で1%、2年で9%のウイルス学的リバウンドが認められている。エンテカビルの治療効果および耐性化出現率とHBV遺伝子型についての関連

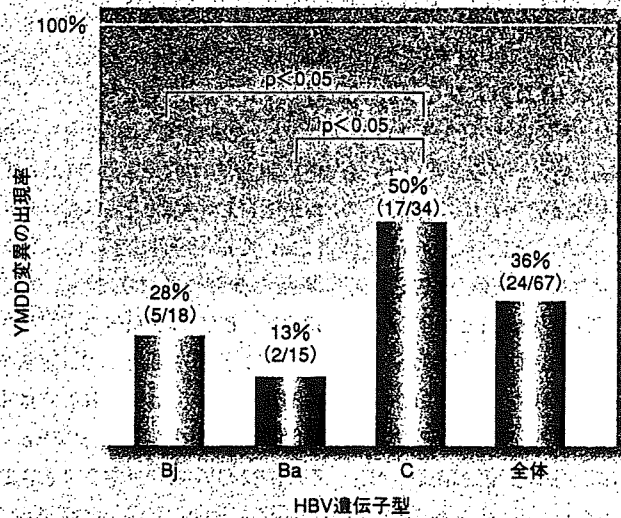


図2 各遺伝子型におけるラミブジン治療2年目のYMDD変異株出現率の比較

は現在までのところ遺伝子型間の明らかな効果の差を示す報告はないが、十分なスタディーが行われておらず今後の検討課題である。

おわりに

HBVの遺伝子型はその地理的分布や臨床像および予後との関連のみでなく、さまざまな抗ウイルス剤に対する

治療効果や耐性化に大きく影響を及ぼしている。HBV遺伝子型の測定はそれぞれの患者にあったより適切な治療法の選択のために有用であると考えられる。

文献

- 1) Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; 46: 329-38.
- 2) Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; 34: 590-4.
- 3) Orito E, Mizokami M, Sakugawa H, et al. A case-control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* 2001; 33: 218-23.
- 4) Sugauchi F, Orito E, Ohno T, et al. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. *Hepato Res* 2006; 36: 107-14.
- 5) Sugauchi F, Orito E, Ichida T, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. *J Virol* 2002; 76: 5985-92.
- 6) Sugauchi F, Orito E, Ichida T, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology* 2003; 124: 925-32.
- 7) Sugauchi F, Kumada H, Acharya SA, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. *J Gen Virol* 2004; 85: 811-20.
- 8) Tanaka Y, Orito E, Yuen MF, et al. Two subtypes (subgenotypes) of hepatitis B virus genotype C: A novel subtyping assay based on restriction fragment length polymorphism. *Hepato Res* 2005; 33: 216-24.
- 9) Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. *J Hepatol* 2000; 33: 998-1002.
- 10) Wai CT, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated

- with better response to interferon therapy in HBeAg chronic hepatitis than genotype C. *Hepatology* 2002; 36: 1425-30.
- 11) Erhardt A, Reineke U, Blondin D, et al. Mutations of the core promoter and response to interferon treatment in chronic replicative hepatitis B. *Hepatology* 2000; 31: 716-25.
 - 12) Janssen HLA, van Zonneveld M, Senturk H, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B : a randomized trial. *Lancet* 2005; 365: 123-9.
 - 13) Lau G, Piratvisuth T, Luo KX, et al. Peginterferon alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; 352: 2682-95.
 - 14) Dienstag JL, Schiff ER, Wright TL, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; 341: 1256-63.
 - 15) Akuta N, Suzuki F, Kobayashi M, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during longterm treatment. *J Hepatol* 2003; 38: 315-21.
 - 16) Orito E, Fujiwara K, Tanaka Y, et al. A case-control study of response to lamivudine therapy for 2 years in Japanese and Chinese patients chronically infected with hepatitis B virus of genotypes Bj, Ba and C. *Hepatol Res* 2006; 35: 127-34.
 - 17) Chan HLY, Wong ML, Hui AY, et al. Hepatitis B virus genotype has no impact on hepatitis B e antigen seroconversion after lamivudine treatment. *World J Gastroenterol* 2003; 9: 2695-7.
 - 18) Peters MG, Hann Hw H, Martin P, et al. Adefovir dipivoxil alone or in combination with lamivudine in patients with lamivudine-resistant chronic hepatitis B. *Gastroenterology* 2004; 126: 91-101.
 - 19) Westland C, Delaney W 4th, Yang H, et al. Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil 1. *Gastroenterology* 2003; 125: 107-16.
 - 20) Chang TT, Gish RG, de Man R, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; 354: 1001-10.

B型肝炎ウイルス (hepatitis B virus: HBV) 感染症対策：
日本において接種可能である(あった)4種HBワクチンの
HBs抗体誘導能の凝集法 (passive hemagglutination assay) 評価と
国際単位表示法 (chemiluminescent immunoassay) 評価との比較検討

小 方 則 夫*

Comparison of Antibody Responses to Hepatitis B Surface Antigen among
Four Recipient Groups of Hepatitis B Vaccines That Have been Approved in Japan:
Evaluation Using Passive Hemagglutination Assay and Chemiluminescent Immunoassay

Norio OGATA, MD, PhD*

In hepatitis B virus (HBV) infection-preventing programs, serum or plasma levels of antibody to hepatitis B surface antigen (anti-HBs) are important to determine whether individuals are protective or not. We compared anti-HBs responses using passive hemagglutination assay (Mycell) and chemiluminescent immunoassay (Architect) among four recipient groups of HB vaccines, Meinyu, HBY, Bimmugen and Heptavax II, that have been approved in Japan. Overall, in a total of 1,875 vaccinees Mycell results showed recipient groups of Meinyu and HBY acquired higher anti-HBs levels than those of Bimmugen and Heptavax II. Comparison of anti-HBs responses by both Mycell and Architect in recipient groups of Meinyu (n=150), HBY (n=218), Bimmugen (n=260), and Heptavax II (n=47) demonstrated the order of vaccinees' responses, such as geometric mean titers, ratios of acquiring high antibody levels (Mycell titers over 1,024, Architect measurements over 1,000mIU/mL), and ratios of having unsuccessful antibody responses (Mycell titers under 8, Architect measurements under 10mIU/mL), were somewhat different between the two assays. Comparison of Architect measurements at given Mycell titers revealed Bimmugen-recipients showed significantly lower values than HBY- or Heptavax II-recipients. Around critical protective levels, 5 of 22 Bimmugen-recipients with Mycell titers 16 or 32 showed Architect measurements under 10mIU/mL, while 8 of 11 Heptavax II-recipients with Mycell titers below 8 demonstrated Architect measurements over 10mIU/mL. Thus, discrepancies in anti-HBs evaluation between Mycell and Architect seemed to partly depend on administered vaccines. These results indicate anti-HBs concentration should be evaluated carefully so that we could completely prevent HBV infection.

[Rinsho Byori 57 : 954~960, 2009]

Corresponding author: Norio OGATA, MD, PhD, Japan Labour Health and Welfare Organization Tsubame Rosai Hospital, Tsubame 959-1228, Japan. E-mail: ogata-rofuku-go@tsrh.jp

受付 2009 年 6 月 1 日・受理 2009 年 8 月 12 日

*労働者健康福祉機構燕労災病院(〒959-1228 燕市佐渡 633 番地)

本論文は第 55 回日本臨床検査医学会学術集会における座長推薦論文である。

【Key Words】 hepatitis B virus: HBV (B型肝炎ウイルス), antibody to hepatitis B surface antigen: anti-HBs (HBs抗体), hepatitis B vaccine: HB vaccine (B型肝炎ワクチン), passive hemagglutination assay: PHA (受身赤血球凝集反応), chemiluminescent immunoassay: CLIA (蛍光免疫反応)

B型肝炎ウイルス (hepatitis B virus: HBV) 感染症は現在でも大きな臨床領域であり, 世界保健機関 (World Health Organization: WHO) 勧奨¹⁾に基づくユニバーサル・ワクチネーションはその予防に大きな効果をあげている²⁾。

本邦におけるHBV感染予防対策は, 1980年代より開始された母子間感染予防や医療従事者感染予防の対策としてのHBワクチネーションの有効性をうけて, 当時の実施プロトコール³⁾⁴⁾が長年踏襲されてきたが, 2000年代より米国疾病対策センター (Centers for Disease Control and Prevention: CDC) 勧奨プロトコール⁵⁾⁶⁾を採用する施設が増加している。CDC勧奨は多数の臨床疫学的根拠と綿密な医療経済的効果を基盤に作製された秀逸なものであり, 練り上げられた簡潔な記載項目を踏襲することは容易である。しかしながらCDC勧奨を本邦に導入するためには解決すべきいくつかの課題があることが見過ごされている感があり, そのひとつがHBワクチン接種により獲得するHBV感染防御最小HBs抗体濃度の設定とこれを維持するためのHBワクチン追加接種の要・不要論であると考えられる。血清または血漿中感染防御最小HBs抗体濃度は, 日本では受身赤血球凝集反応 (passive hemagglutination assay: PHA) 法による陽性判定 (通常, 凝集価8倍以上) が使用されてきたが³⁾⁴⁾, 米国ではWHO基準品に準拠した国際単位表示法による10mIU/mLが採用されている⁵⁾⁶⁾。後者はradioimmunoassay (RIA) 法を経て現在ではenzyme immunoassay (EIA) 法やchemiluminescent immunoassay (CLIA) 法が主体である。凝集法は国際的に普及しておらず, しかも国際単位表示法との比較検定が実施されていないため国際単位表示法へ換算することも困難である。このように, 両アッセイ法を含め多種のHBs抗体測定キットが普及している現状においては施設間・個人間においてさえHBs抗体評価の共有が困難である。さらに, 筆者らは, 1999年度から2002年度にかけて2種のHBワクチン各接種者が示す血清HBs抗体濃度を複数の測定法を使用して検討した結果, ワクチン種と測定法の組み合わせによる乖離事象を見出し⁷⁾, 上記混乱はさらに大きくなったものと考えている。このた

め筆者らは国内におけるHBs抗体測定法の標準化作業を提唱し⁸⁾, これは現在進行中である⁹⁾¹⁰⁾。

以上の背景に基づき, 本研究では, 国内で汎用されてきた上記2種を含めた4種のHBワクチンのHBs抗体誘導能を, 国産PHA法とWHO基準CLIA法により系統的に比較解析した。結果として見出した事象は, 臨床検査現場・感染対策現場, それぞれにおける実務者の一助となると考えるため報告する。

I. 対象と方法

A. 対象

1999年度から2007年度までの富山医科薬科大学 (現, 富山大学) 医学部 (医学科・看護学科)・薬学部 (薬学科) 学生, ならびに2006年度から2008年度までの燕労災病院新規採用職員を対象とした。

B. 方法

健康診断時に血清HBs抗原・HBs抗体両者陰性, かつ問診によりHBワクチン接種歴がないことを確認し, HBワクチンを標準3回スケジュール, すなわち, 0, 1, 6ヵ月の3回接種を行い, 3回目接種1ヵ月後に血清HBs抗体を測定した。

HBワクチン接種にあたっては, 各HBワクチンのインタビュー・フォームに記載されている効果と副反応等を要約した文書を配布し理解を得た後, 本人と未成年者の場合は保護者からも文書による同意を取得した。

1. HBワクチン種

富山 (医科薬科) 大学学生に対しては, 1999年度・2000年度は酵母由来遺伝子組換えサブタイプ *ayw*・major S蛋白含有ワクチン (エッチビーワイ, 吉富製薬, 2001年度より供給停止), 2001年度・2002年度は酵母由来遺伝子組換えサブタイプ *adr*・major S蛋白含有ワクチン (ビームゲン, 藤沢薬品-アステラス製薬), 2003年度より2007年度まではヒト培養細胞由来遺伝子組換えサブタイプ *adr*・pre-S2+major S蛋白含有ワクチン (明乳, 明治乳業, 2008年度より供給停止) を, それぞれ接種した。燕労災病院職員に対しては2006年度より2008年度まで酵母由来遺伝子組換えサブタイプ *adw*・major S蛋白含有ワクチン (ヘプタバックスII, メルク-万有

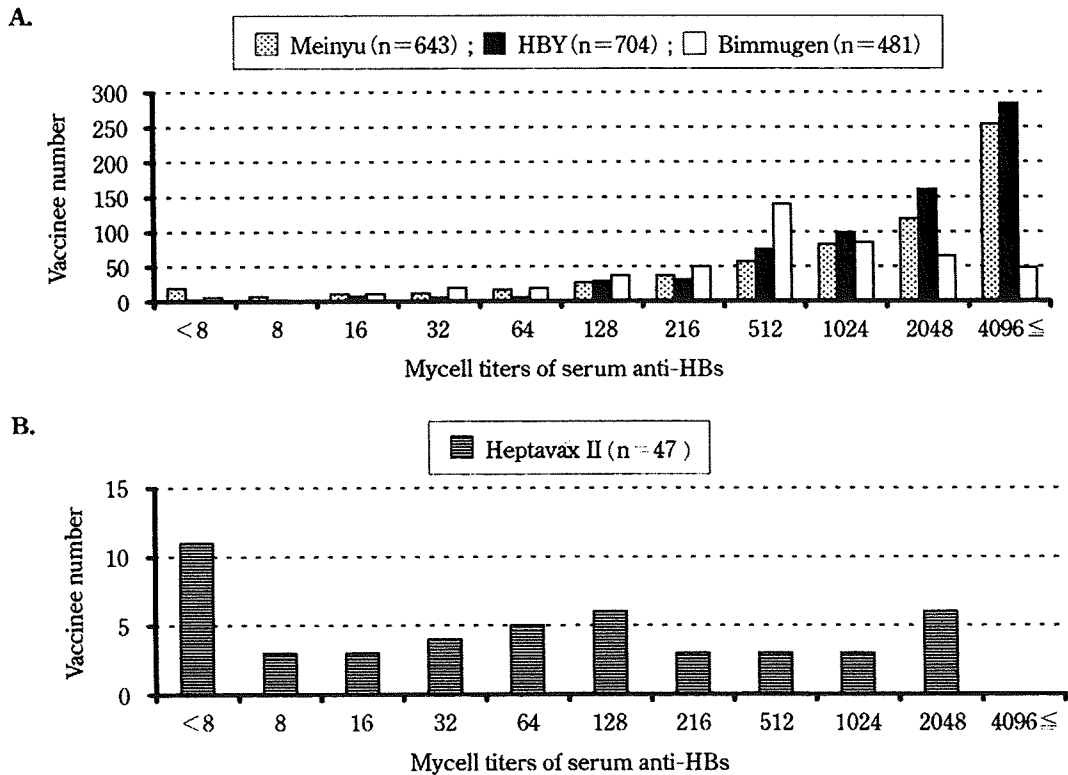


Figure 1 Distribution of number of HB vaccinees at demonstrated Mycell titers of serum anti-HBs assayed one month after completing the vaccination.

Panel A, Recipient groups of Meinyu, HBY and Bimmugen; Panel B, A recipient group of Heptavax II.

製薬)を接種した。

2. HBs 抗体測定法

血清 HBs 抗体は、PHA 法(マイセル II, 特殊免疫研究所)ならびに CLIA 法(アーキテクト・オーサブ, アボットジャパン)を使用して測定した。

3. 統計学的解析

HB ワクチン接種者が示す血清 HBs 抗体幾何平均値(Geometric Mean Titer: GMT)の比較は分散分析と Sheff の F 検定を使用した。人数度数の比較は χ^2 検定または Fisher の直接検定を使用した。

II. 結 果

A. HB ワクチン接種者全員におけるマイセルに

よる血清 HBs 抗体評価の概略

明乳(n=643)・エッチビーワイ(n=704)・ビームゲン(n=481)・ヘプタボックス II(n=47)各接種者、総計 1,875 名のマイセル凝集価別人数分布は、明乳・エッチビーワイ各接種者はともに 4,096 倍以上が最多、ビームゲン接種者は 512 倍が最多であり(Fig. 1A)、ヘプタボックス II 接種者は 8 倍未満が最多であり 8 倍から 2,048 倍まで突出した多人数分

布は認めなかった(Fig. 1B)。

B. HB ワクチン接種者におけるマイセルとアーキ

テクトとによる血清 HBs 抗体評価の比較

明乳(n=150, 男性 81 名・女性 69 名, 年齢 18~31 歳, 中央値 21.1 歳)・エッチビーワイ(n=218, 男性 119 名・女性 99 名, 年齢 18~29 歳・中央値 21.3 歳)・ビームゲン(n=260, 男性 122 名・女性 138 名, 年齢 18~36 歳・中央値 20.9 歳)各接種者の単年度採血順連続検体と、ヘプタボックス II 接種者(n=47, 男性 3 名・女性 44 名, 年齢 21~34 歳, 中央値 22.1 歳)の 3 年度間全検体とを対象に、HBs 抗体をマイセルとアーキテクトにて同時測定した。

1. HBs 抗体平均値

血清 HBs 抗体 GMT は、マイセル評価では、明乳接種者が最も高凝集価を示し、次いでエッチビーワイ・ビームゲン各接種者が同等、ヘプタボックス II 接種者の順であり、アーキテクト評価では、明乳・エッチビーワイ各接種者が同等の高定量値を示し、次いでビームゲン接種者、ヘプタボックス接種者の順であった(Table 1)。

Table 1 Comparison of GMTs of serum anti-HBs levels determined by Mycell and Architect among four recipient groups of HB vaccines

	Meinyu (n=150)	HBV (n=218)	Bimmugen (n=260)	Heptavax II (n=47)	p
Mycell (titer)	960	598	561	64	<0.0001 ¹⁾
Architect (mIU/mL)	1,234.0	1,228.3	372.7	148.8	<0.0001 ²⁾

¹⁾Meinyu vs. HBV, p<0.05; Meinyu vs. Bimmugen, p<0.05; Meinyu vs. Heptavax II, p<0.0001.

HBV vs. Heptavax II, p<0.0001. Bimmugen vs. Heptavax II, p<0.0001.

²⁾Meinyu vs. Bimmugen, p<0.0001; Meinyu vs. Heptavax II, p<0.0001.

HBV vs. Bimmugen, p<0.0001; HBV vs. Heptavax II, p<0.0001. Bimmugen vs. Heptavax II, p<0.05.

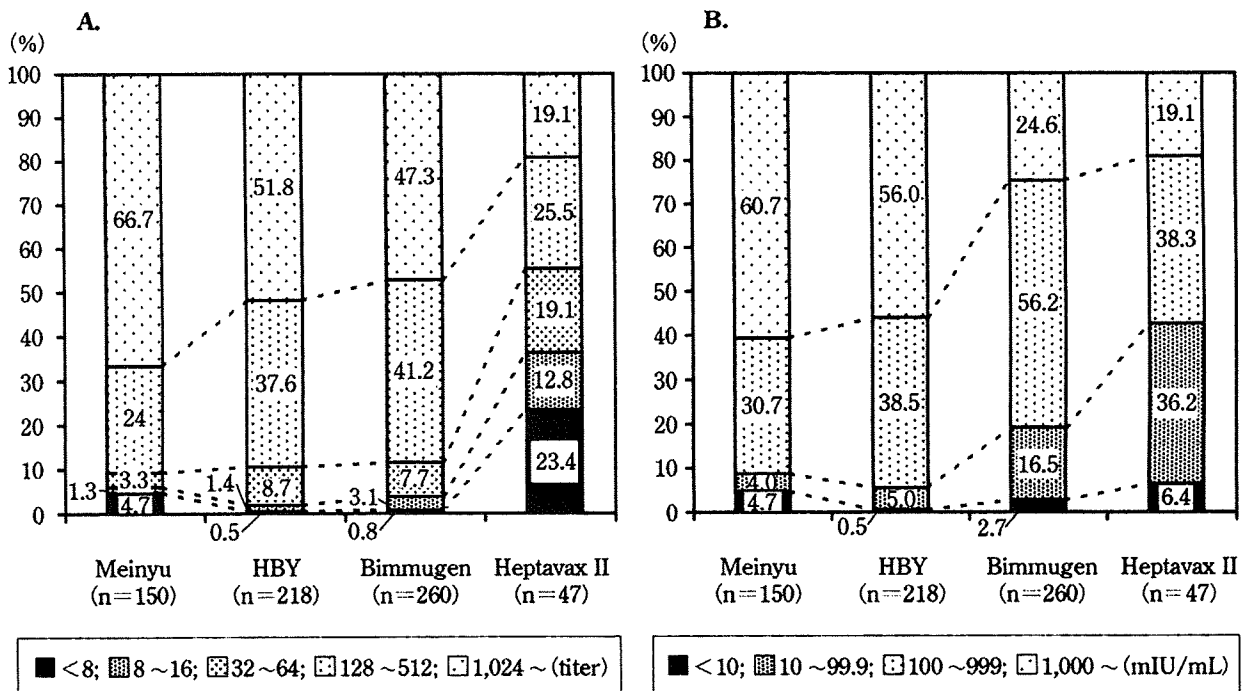


Figure 2 Ratios of number of HB vaccinees stratified by serum anti-HBs levels determined one month after completing the vaccination. Panel A, Mycell titers; Panel B, Architect measurements.

2. HBs 抗体濃度層別の人数比率

初めに HBV 中和抗体保持期間の目安となる HBs 抗体高濃度領域を検討した。マイセル 1,024 倍以上を示した高反応者は、明乳接種者は 100 名 (66.7%)、エッチビーワイ・ビームゲン各接種者は 113 名 (51.8%)・123 名 (47.3%)、ヘプタボックス II 接種者は 9 名 (19.1%) であり (Fig. 2A), 明乳接種者は他の 3 ワクチン接種者に比べ高率で (各, p<0.01, p<0.001, p<0.0001), エッチビーワイ・ビームゲン各接種者はヘプタボックス接種者に比し高率であった (各, p<0.0001, p<0.001)。アーキテクト 1,000mIU/mL 以上を示した高反応者は、明乳・エッチビーワイ各

種者は 91 名 (60.7%)・122 名 (56.0%)、ビームゲン・ヘプタボックス II 各接種者は 64 名 (23.5%)・9 名 (19.1%) であり (Fig. 2B), 明乳・エッチビーワイ各接種者がビームゲン・ヘプタボックス各接種者に比べ高率であった (各, p<0.0001)。

次いで、HBV 感染防御閾値設定の指標となる HBs 抗体低濃度領域を検討した。マイセル 8 倍未満を示した無反応者は、明乳接種者は 7 名 (4.7%)、エッチビーワイ接種者は 1 名 (0.5%)、ビームゲン接種者は 2 名 (0.8%)、ヘプタボックス接種者では 11 名 (23.4%) で (Fig. 2A), エッチビーワイ接種者は明乳・ヘプタボックス II 各接種者に比し低率であり

Table 2 Comparison of GMTs of serum anti-HBs levels measured by Architect at given Mycell titers among four recipient groups of HB vaccines

Mycell (titer)	GMT of Architect measurements (mIU/mL)				
	Meinyu	HBV	Bimmugen	Heptavax II	p
32	86.5	84.3	19.9	77.1	<0.0001 ¹⁾
64	67.3	114.0	48.3	136.2	<0.01 ²⁾
128	139.2	299.0	96.3	325.5	<0.0001 ³⁾
256	228.8	459.7	161.9	542.6	<0.0001 ⁴⁾
512	518.8	763.2	249.1	706.7	<0.0001 ⁵⁾
1,024	1,047.5	2,463.4	658.2	1,104.6	<0.0001 ⁶⁾
2,048	1,949.1	4,269.5	1,625.3	2,821.8	<0.0001 ⁷⁾

¹⁾ HBV vs. Bimmugen, p=0.001; Heptavax II vs. Bimmugen, p<0.01.

²⁾ HBV vs. Bimmugen, p<0.05; Heptavax II vs. Bimmugen, p<0.05.

³⁾ HBV vs. Bimmugen, p<0.0001; Heptavax II vs. Bimmugen, p<0.0001.

⁴⁾ HBV vs. Bimmugen, p<0.0001; Heptavax II vs. Bimmugen, p<0.05.

⁵⁾ HBV vs. Bimmugen, p<0.0001.

⁶⁾ HBV vs. Bimmugen, p<0.0001; HBV vs. Meinyu, p<0.0001; HBV vs. Heptavax II, p<0.0001; Meinyu vs. Bimmugen, p<0.05; Heptavax II vs. Bimmugen, p<0.05.

⁷⁾ HBV vs. Bimmugen, p<0.0001; HBV vs. Meinyu, p<0.0001; HBV vs. Heptavax II, p<0.0001.

(各, p<0.01, p<0.001), ビームゲン接種者も明乳・ヘプタボックス II 各接種者に比し低率であり(各, p<0.05, p<0.0001), 明乳接種者はヘプタボックス II 接種者に比べ低率であった(p<0.001)。アーキテクト 10mIU/mL 未満を示した無反応者は, 明乳接種者は 6 名(4.7%), エッチビーワイ接種者は 1 名(0.5%), ビームゲン接種者は 7 名(2.7%), ヘプタボックス接種者は 3 名(6.4%)で(Fig. 2B), エッチビーワイ接種者が明乳・ヘプタボックス II 各接種者に比べ低率であり(各, p<0.01, p<0.05), ビームゲン接種者と比べても低率の傾向にあり(p=0.076), 明乳・ビームゲン・ヘプタボックス各接種者間では同等であった。

以上, 2 測定法評価の相違をまとめると, 高反応者比率はビームゲン接種者がマイセル評価に比べアーキテクト評価で低率となり, 結果, ヘプタボックス II 接種者と同等となり, 一方, 無反応者比率はヘプタボックス II 接種者がマイセル評価に比しアーキテクト評価で低率となり, 結果, 明乳・ビームゲン各接種者と同等となった。

C. マイセル凝集価別にみたアーキテクト定量値

同一マイセル凝集価におけるアーキテクト定量値は, エッチビーワイ・ヘプタボックス II 各接種者はビームゲン接種者に比し高値を示し, 明乳接種者は前 2 者よりは低値, 後者よりは高値を示す傾向にあ

った(Table 2)。感染防御閾値においてビームゲン接種者とヘプタボックス II 接種者の測定法間の乖離は深刻で, マイセル評価を基準にするとアーキテクト評価は, ビームゲン接種者では相対的低値を示しマイセル 16 倍・32 倍においてもアーキテクト 10mIU/mL 未満を示す検体が各 8 検体中 2 検体・14 検体中 3 検体存在し, 逆にヘプタボックス II 接種者では相対的高値を示しマイセル 8 倍未満でもアーキテクト 10mIU/mL 以上を示す検体が 11 検体中 7 検体存在した(Fig. 3)。明乳・エッチビーワイ各接種者においては感染防御閾値を示す検体が少数であるため今回の検討には適さなかったが, ワクチン接種完了 1 年後・2 年後の検体を検討した結果, 感染防御閾値におけるマイセル・アーキテクト両測定法間に重大な乖離は認めなかった¹⁰⁾。

III. 考 察

本研究期間中, 各 HB ワクチン接種期間中の製造変更はなく, 各 HBs 抗体測定キットの仕様変更もないため, HBs 抗体測定結果は客観的比較が可能と考える。マイセルとアーキテクトによる同時測定検体が由来する各ワクチン接種者間で, 年齢に差はないが, 性別はヘプタボックス II 接種者で女性が多いため, 他ワクチン接種者に比べ HBs 抗体反応が高い可能性はある^{11)~14)}。

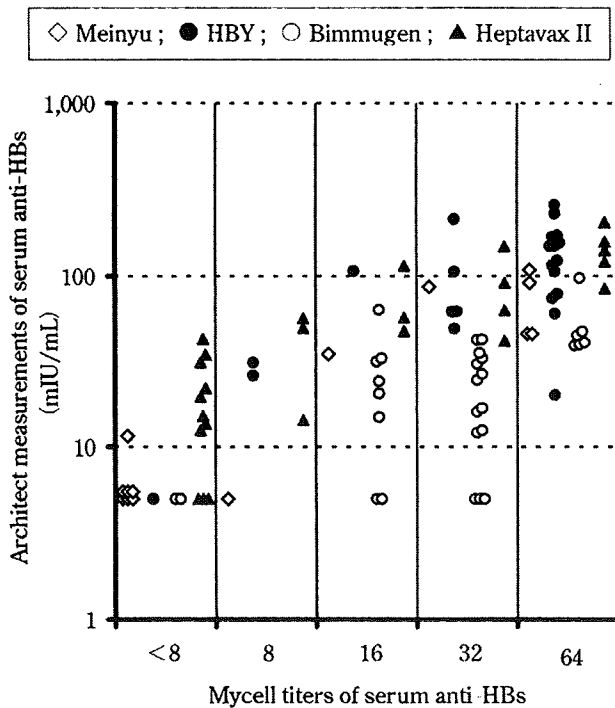


Figure 3 Comparison among four recipient groups of the vaccines of Architect measurements of serum anti-HBs at given Mycell titers determined one month after completing HB vaccination.

各 HB ワクチンの HBs 抗体誘導能に関する本邦第 III 相臨床試験結果は radioimmunoassay (RIA) 法の国際単位換算評価値である。それらによると、血清 HBs 抗体幾何平均値は、明乳¹¹⁾・エッチビーワイ¹²⁾・ビームゲン¹³⁾・ヘプタボックス¹⁴⁾各接種者の順に、587.9mIU/mL・515.1mIU/mL・516.2mIU/mL・100.9mIU/mL である。筆者らの結果は、順位はほぼ同等であるが高値の傾向にあった。血清 HBs 抗体非陽転率は、明乳¹¹⁾・エッチビーワイ¹²⁾・ビームゲン¹³⁾・ヘプタボックス¹⁴⁾各接種者の順に、7.4%・2.2%・3.7%・7.6% であり、筆者らの結果は、順位はやはり同様であるが低率の傾向にあった。これらの理由は、RIA 法と CLIA 法が若干の定量値相違を示す可能性や、今回の対象が 20 歳前後の若年層に集中しているため反応が比較的良好であった可能性が考えられる。

HBV 曝露後対策について、米国 CDC 勧奨では、事故者が HB ワクチン反応者の記録があれば HBs 抗体量が感染防御閾値未満でも免疫記憶が作動するため肝炎発症の危険は少なく¹⁵⁾¹⁶⁾無処置とされる⁵⁾⁶⁾

が、日本⁴⁾¹⁷⁾や他の数国家¹⁸⁾では HBs 抗体量が感染防御閾値未満なら抗 HBs 免疫グロブリン投与や HB ワクチン接種の適応とされる。どちらの立場でも判断の根拠となる HBs 抗体評価は重要である。今回系統的に検討した HB ワクチンの HBs 抗体誘導能について、マイセル評価とアーキテクト評価との間で相違を認めた原因は不明といわざるを得ないが、免疫反応に基づくアッセイ標準化の困難さに加え、ワクチン含有 HBs 蛋白とキット固相 HBs 蛋白のサブタイプの相違やキット構築の相違による乖離である可能性を挙げることができよう。いずれにせよ、同一血清検体における測定法間の乖離、特に感染防御閾値の乖離は認識する必要があると考える。なかでも 2009 年度初頭現在接種可能であるビームゲンとヘプタボックス II 各接種者の血清 HBs 抗体評価の乖離は重度かつ真逆であり、感染防御最小抗体濃度とされるマイセル 8 倍凝集価・アーキテクト 10mIU/mL が一致しない検体が少なからず存在したことは留意する必要があると考える。

HBs 抗体評価は国際的根拠を共有するためにも WHO 基準法に統一すべきであろうが、WHO 基準法でも数種の測定キット間の不一致や偽陽性が報告されている¹⁸⁾¹⁹⁾。このため、米国とは相違して欧州の数国家では HB ワクチン反応基準を安全を期して 100mIU/mL としている¹⁸⁾。HBs 抗体測定法の標準化や抗 HBs 蛋白 α -エピトープ抗体²⁰⁾の実用化が困難な現時点においては、感染防御 HBs 抗体値を個々の施設の実情に応じて安全閾に設定することが方策と考える。

HBs 抗体測定法の標準化作業が完了し、HB ワクチン接種既往者の感染調査結果が集積し、本邦における HB ワクチネーションと HBV 感染予防に関する evidence が確立されることを期待したい。

IV. 結 語

4 種の HB ワクチン、明乳・エッチビーワイ・ビームゲン・ヘプタボックス II の HBs 抗体誘導能を 2 種の HBs 抗体測定法、マイセル・アーキテクトにより比較検討し、各ワクチン性能の相違と、各ワクチン接種者別に両測定法が示す HBs 抗体評価の乖離事象を見出した。HBs 抗体評価の臨床検査現場からの発信は感染対策現場における決定を左右するため、慎重になされるべきものと考えられる。

本研究遂行にあたり多大なご助力を賜りました富山(医科薬科)大学保健管理センター歴代所長、渡邊明治教授・井上博教授・舟田久教授、および同センター職員諸氏、ならびに燕労災病院検査科臨床検査技師諸氏に謝意を表します。

本研究の一部は、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・肝炎ウイルス感染防御を目指したワクチン接種の基盤構築」、独立行政法人労働者健康福祉機構研究費補助金「病院機能向上のための研究活動支援」による。

本論文は第55回日本臨床検査医学会学術集会推薦演題である。

文 献

- 1) World Health Organization. WHO expanded programme on immunization. Global Advisory Group-Part I. *Wkly Epidemiol Rec* 1992; 67: 11-6.
- 2) Zanetti AR, Damme PV, Shouval D. The global impact of vaccination against hepatitis B. *Vaccine* 2008; 26: 6266-73.
- 3) 吉澤浩司, 飯野四郎. B型肝炎. ウイルス肝炎 診断/予防/治療—ウイルス肝炎対策の指針—(第2版). 東京: 文光堂; 2002. p.13-36.
- 4) 袖山 建, 小林正和. 医療機関内におけるHBV感染経路と予防対策—リスクマネージメントの立場から—. *日本臨牀* 2004; 62(増刊: ウイルス性肝炎 下巻): 203-9.
- 5) Centers for Disease Control and Prevention. Prevention and control of infection with hepatitis viruses in correctional settings. *MMWR Recomm Rep* 2003; 52(RR-1): 1-36.
- 6) Centers for Disease Control and Prevention. Updated U.S. public health service guidelines for the management of occupational exposures to HBV, HCV, and HIV and recommendations for postexposure prophylaxis. *MMWR Recomm Rep* 2001; 50(RR-11): 1-42.
- 7) 小方則夫, 島木貴久子, 桑守美千代, 他. ウイルス肝炎対策: 医療系学生のB型肝炎ワクチン接種状況, および獲得HBs抗体評価の問題. *CAMPUS HEALTH* 2003; 40: 252-3.
- 8) 小方則夫. 検査の標準化: 国際基準共有に向けたB型肝炎ウイルス感染防御最小HBs抗体評価標準化の必要性—本邦にて汎用されるHBs抗体測定法の特性乖離—. *臨床病理* 2006; 54: 960-5.
- 9) 水落利明, 小高千加子, 山口一成. 国内で販売されている抗HBs抗体定量用体外診断医薬品の評価: 国内標準品を用いた検討. *臨床検査* 2008; 52: 111-5.
- 10) 小方則夫. B型肝炎ウイルス感染防御HBs抗体に関する研究. 研究代表者 水落利明 厚生労働科学研究費補助金肝炎等克服緊急対策事業「肝炎ウイルス感染防御を目指したワクチン接種の基盤構築」平成20年度研究報告書. 2009. p.29-38.
- 11) 谷内 昭, 菅 充夫, 宮崎 保, 他. 沈降B型肝炎ワクチン(ヒト培養細胞由来, MC-HB)の第III相臨床試験成績. *基礎と臨床* 1992; 26: 891-901.
- 12) 市田文弘. 組換えB型肝炎ワクチン(GB-0892)の臨床第III相試験成績. *基礎と臨床* 1988; 22: 3109-23.
- 13) 矢野右人. 遺伝子組換え酵母由来B型肝炎ワクチン第III相試験成績. *基礎と臨床* 1987; 21: 2681-90.
- 14) 飯野四郎, 黒木哲夫, 鈴木 宏, 他. 組換え沈降B型肝炎ワクチン(酵母由来, HBX-R)の第三相試験成績. *薬理と治療* 1987; 15: 2403-15.
- 15) West DJ, Calandra GB. Vaccine induced immunologic memory for hepatitis B surface antigen: implication for policy on booster vaccination. *Vaccine* 1996; 14: 1019-27.
- 16) European Consensus Group on Hepatitis B Immunity. Are booster immunization needed for lifelong hepatitis B immunity? *Lancet* 2000; 355: 561-5.
- 17) Yoshida T, Saito I. Hepatitis B booster vaccination for healthcare workers. *Lancet* 2000; 355: 1464.
- 18) Huzly D, Schenk T, Jilig W, et al. Comparison of nine commercially available assays for quantification of antibody response to hepatitis B surface antigen. *J Clin Microbiol* 2008; 46: 1298-306.
- 19) Heijitink RA, Schneeberger PM, Postma B, et al. Anti-HBs levels after hepatitis B immunization depend on test reagents: routinely determined 10 and 100 IU/L seroprotection levels unreliable. *Vaccine* 2002; 20: 2899-905.
- 20) Ogata N, Ostberg L, Ehrlich PH, et al. Markedly prolonged incubation period of hepatitis B in a chimpanzee passively immunized with a human monoclonal antibody to the α determinant of hepatitis B surface antigen. *Proc Natl Acad Sci U S A* 1993; 90: 3014-8.

Original Article

Case–control study for the identification of virological factors associated with fulminant hepatitis B

Atsunori Kusakabe,^{1,2} Yasuhito Tanaka,¹ Satoshi Mochida,³ Nobuaki Nakayama,³ Kazuaki Inoue,⁴ Michio Sata,⁵ Norio Isoda,⁶ Jong-Hon Kang,⁷ Yasukiyo Sumino,⁸ Hiroshi Yatsuhashi,⁹ Yasuhiro Takikawa,¹⁰ Shuichi Kaneko,¹¹ Gotaro Yamada,¹² Yoshiyasu Karino,¹³ Eiji Tanaka,¹⁴ Junji Kato,¹⁵ Isao Sakaida,¹⁶ Namiki Izumi,¹⁷ Fuminaka Sugauchi,² Shunsuke Nojiri,² Takashi Joh,² Yuzo Miyakawa¹⁸ and Masashi Mizokami^{1,19}

¹Departments of Clinical Molecular Informative Medicine and ²Gastroenterology and Metabolism, Nagoya City University Graduate School of Medical Sciences, Nagoya, ³Division of Gastroenterology and Hepatology, Internal Medicine, Saitama Medical University, Saitama, ⁴Showa University Fujigaoka Hospital, Yokohama, ⁵Kurume University School of Medicine, Kurume, ⁶Jichi Medical University, Tochigi, ⁷Center for Gastroenterology, Teinekeijinkai Hospital, Sapporo, ⁸Toho University Omori Medical Center, Tokyo, ⁹National Hospital Organization Nagasaki Medical Center, Nagasaki, ¹⁰Iwate Medical University, Morioka, ¹¹Department of Signal Transduction, Cancer Research Institute, Kanazawa University, Kanazawa, ¹²Kawasaki Hospital, Okayama, ¹³Sapporo-Kosei General Hospital, Sapporo, ¹⁴Shinsyu University Graduate School of Medicine, Matsumoto, ¹⁵Sapporo Medical University Hospital, Sapporo, ¹⁶Yamaguchi University Hospital, Ube, ¹⁷Musashino Red Cross Hospital, Musashino, ¹⁸Miyakawa Memorial Research Foundation, Tokyo, and ¹⁹Research Center for Hepatitis and Immunology, International Medical Center of Japan, Kohnodai Hospital, Ichikawa, Japan

Background: Host and viral factors can promote the development of fulminant hepatitis B (FHB), but there have been no case–control studies for figuring out virological parameters that can distinguish FHB.

Methods: In a case–control study, virological factors associated with the development of FHB were sought in 50 patients with FH developed by transient hepatitis B virus (HBV) infection (FH-T) and 50 with acute self-limited hepatitis B (AHB) who were matched for sex and age. In addition, 12 patients with FH developed by acute exacerbation (AE) of asymptomatic HBV carrier (ASC) (FH-C) were also compared with 12 patients without FH by AE of chronic hepatitis B (AE-C).

Results: Higher HBV DNA levels, subgenotype B1/Bj, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent ($P < 0.05$), while hepatitis B e-antigen was less frequent in the FH-T patients than AHB. In multivariate analysis, G1896A mutation (odds ratio [OR],

13.53; 95% confidence interval [CI], 2.75–66.64), serum HBV DNA more than 5.23 log copies/mL (OR, 5.14; 95% CI, 1.10–24.15) and total bilirubin more than 10.35 mg/mL (OR, 7.81; 95% CI, 1.77–34.51) were independently associated with a fulminant outcome by transient HBV infection. On the other hand, in comparison with the patients between FH-C and AE-C groups, there was no significant difference of virological factors associated with the development of FHB.

Conclusion: A number of virological factors have been defined that may distinguish FH-T from AHB in a case–control study. The pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

Key words: acute exacerbation of asymptomatic hepatitis B virus carrier, fulminant hepatitis, genotypes, transient hepatitis B virus infection

Correspondence: Dr Yasuhito Tanaka, Department of Clinical Molecular Informative Medicine, Nagoya, City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Email: ytanaka@med.nagoya-cu.ac.jp
Received 22 August 2008; revision 26 January 2009; accepted 24 February 2009.

INTRODUCTION

IN JAPAN, 634 patients with fulminant hepatitis (FH) were registered from 1998–2003. Of them, 41.8% were infected with hepatitis B virus (HBV) that is the most frequent cause of FH there.¹ HBV is classified into eight genotypes (A–H) based on a sequence divergence of more than 8% in the entire genome of approximately

3200 nucleotides.^{2–5} They have distinct geographical distributions and are associated with the severity of liver disease.^{6,7} Furthermore, subgenotypes have been reported for HBV/A, B and C, and they are named A1/Aa (Asian/African type) and A2/Ae (European type),⁸ B1/Bj (Japanese type) and B2/Ba (Asian type),⁹ and C1/Cs (Southeast Asian type) and C2/Ce (East Asian type).^{10,11} HBV genotypes/subgenotypes and mutations in the pre-core region and the core promoter can influence the viral replication and expression of hepatitis B e-antigen (HBeAg).^{6,12}

Acute HBV infection in adulthood resolves in the most cases by far, but can induce FH or go on to become chronic in some. It has been reported that host and viral factors may influence the development of fulminant hepatitis B (FHB), but the pathogenesis of FHB remains unclear. As for virological factors associated with FHB, mutations in the core promoter (A1762T/G1764A)¹³ and the pre-core region (G1896A)^{14–16} have been reported in association with the development of FHB in Asia and the Middle East. Additional mutations, including T1753V, T1754V and A2339G in the core gene are implicated, also.^{17,18} In regard of HBV genotypes, subgenotype B1/Bj is highly associated with the development of FHB in Japan.¹⁵ In contrast, an association of HBV genotypes with the fulminant outcome has not been reproduced in patients from the USA and Europe.^{19–22} Such a discrepancy would be attributed, at least in part, to distinct geographical distributions of HBV genotypes/subgenotypes over the world.

The original definition by Trey *et al.*²³ about fulminant hepatic failure is widely used all over the world. On the other hand, in Japan, the diagnosis of FH was contingent on a slight modification of Trey's original definition by the Inuyama Symposium (Aichi, Japan in 1981). Furthermore, the Intractable Liver Diseases Study Group of Japan modified the criteria for the etiology of FH and late-onset hepatic failure in 2002. According to the criteria of the Intractable Liver Diseases Study Group of Japan, there are two clinical entities of FHB that are induced, respectively, by transient HBV infection and acute exacerbation (AE) of an asymptomatic HBV carrier (ASC).¹

Recently, FH developing in ASC who undergo AE is increasing in Japan.¹ In patients with hematological malignancy, in particular, rituximab and/or glucocorticoid, can reactivate HBV for the development of FHB.²⁴ The outcome is poor for FHB precipitating in ASC who undergo acute exacerbation,¹ but it has been difficult to identify it by clinical examinations.

As there have been no case-control studies for figuring out virological parameters that can distinguish FHB,

a case-control study was conducted on the patients with FH by transient HBV infection and acute self-limited hepatitis B (AHB) in this study, for the identification of virological factors that influence a fulminant outcome. In addition, the patients with FH by AE of ASC, which is assumed as a different clinical condition from transient HBV infection, were also compared with the patients without FH by AE of chronic hepatitis B (CHB) in a case-control study.

METHODS

Patients

DURING 9 YEARS from 1998 to 2006, in twenty-six hospitals all over Japan, sera were obtained from the 50 FH patients by transient HBV infection (the FH-T group) and the 50 patients with AHB (the AHB group) who were controlled for age and sex. As the elder patients with FHB were enrolled in this study (mean age, 42.8 years), the mean age of AHB patients became relatively high (42.9 years, Table 1). Furthermore, the 12 FH patients developed by AE of ASC (the FH-C group) were also compared with the 12 patients without FH by AE of CHB who were matched by age and sex (the AE-C group).

All the serum samples tested for this study were collected at hospitalization. All 124 patients had hepatitis B surface antigen (HBsAg) in serum. Infection with hepatitis A virus and hepatitis C virus, as well as alcoholic hepatitis, were excluded in them.

The diagnosis of acute hepatitis B was based on sudden manifestation of clinical symptoms of hepatitis and detection of high-titered immunoglobulin (Ig)M anti-hepatitis B core (HBC). Patients with initial high-titered anti-HBC (>90% inhibition by a 1:200 diluted serum) were excluded. The diagnosis of FH was contingent on a slight modification by Inuyama Symposium (Aichi, Japan in 1981) of the original definition by Trey *et al.*:²³ (i) coma of grade II or higher; and (ii) a prothrombin time less than 40% developing within 8 weeks after the onset of hepatitis. To exclude AE of ASC in FH-T and AHB groups, we confirmed the negativity of HBsAg before onset of FHB or AHB and no family histories of hepatitis were found among all the patients. Furthermore, serum HBsAg in all patients with FH-T or AHB became naturally seronegative within 24 weeks. AE of ASC or CHB was defined as the elevation of alanine aminotransferase (ALT >300 IU/L) or total bilirubin (T.bil >3.0 mg/dL).²⁵ All 24 patients with AE of ASC or CHB could be confirmed positive for serum HBsAg before the onset of acute liver injury.

Table 1 Baseline characteristics between fulminant hepatitis B patients by transient infection (FH-T) and acute self-limited hepatitis B (AHB) patients

Features	FH-T (n = 50)	AHB (n = 50)	Differences P-value
Age (years)	42.8 ± 16.1	42.9 ± 14.6	Matched
Men	25 (50%)	25 (50%)	Matched
ALT (IU/L)	3788 ± 2856	2170 ± 1350	<0.001
AST (IU/L)	3131 ± 3673	1676 ± 1851	<0.05
Total bilirubin (mg/dL)	14.8 ± 8.6	9.5 ± 9.8	<0.01
Prothrombin time (%)	16.9 ± 11.2	72.8 ± 26.0	<0.001
HBeAg positive	15 (30%)	28 (56%)	<0.01
Core protein (log U/mL)	3.21 ± 1.28	3.01 ± 1.00	NS
HBcrAg (log U/mL)	5.30 ± 1.32	5.95 ± 1.13	<0.01
HBV DNA (log copies/mL)	5.97 ± 1.87	4.98 ± 1.17	<0.005
Deceased	19 (38%)	0 (0%)	<0.001

AHB, acute self-limited hepatitis B; ALT, alanine aminotransferase; AST, aspartate aminotransferase; FH-T, fulminant hepatitis B by transient HBV infection; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

Serological markers of HBV infection

Hepatitis B surface antigen, HBeAg and the corresponding antibody (anti-HBe) were determined by enzyme immunoassay (EIA) (AxSYM; Abbott Japan, Tokyo, Japan) or chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio, Tokyo, Japan). Anti-HBc of IgM and IgG classes were determined by radioimmunoassay (Abbott Japan). Core protein constituting the viral nucleocapsid and HBV core-related antigen (HBcrAg), both of which correlate with HBV DNA in serum, were measured by CLEIA as described elsewhere.^{26,27}

Quantification of serum HBV DNA

Hepatitis B virus DNA sequences spanning the S gene were amplified by real-time detection polymerase chain reaction (RTD-PCR) in accordance with the previously described protocol²⁸ with a slight modification;⁸ it has a detection limit of 100 copies/mL.

Sequencing and molecular evolutionary analysis of HBV

Nucleic acids were extracted from serum samples (100 µL) using the QIAamp DNA extraction kit (Qiagen, Hilden, Germany) and subjected to PCR for amplifying genomic areas bearing enhancer II/core promoter/pre-core/core regions [nt 1628–2364], as described previously.²⁹ The target of PCR covered several mutations which were associated with FHB. Amplicons were sequenced directly with use of the ABI Prism Big Dye ver. 3.0 kit in the ABI 3100 DNA automated

sequencer (Applied Biosystems, Foster City, CA, USA). All sequences were analyzed in both forward and backward directions.

Hepatitis B virus genotypes were determined by molecular evolutionary analysis. Reference HBV sequences were retrieved from the DDBJ/EMBL/GenBank database and aligned by CLUSTAL X, then genetic distances were estimated with the 6-parameter method in the Hepatitis Virus Database (<http://s2as02.genes.nig.ac.jp/>).³⁰ Based on obtained distances, phylogenetic trees were constructed by the neighbor-joining (NJ) method with the mid-point rooting option. To confirm the reliability of the phylogenetic trees, bootstrap resampling tests were performed 1000 times.

Statistical analysis

Statistical differences were evaluated by the Mann-Whitney *U*-test, Fisher's exact probability test and χ^2 -test, where appropriate. Differences were considered to be statistically significant at $P < 0.05$. Multivariate analyses with logistic regression were utilized to sort out independent risk factors for FHB. STATA Software ver. 8.0 was employed for all analyses.

RESULTS

Baseline characteristics of the patients with FHB by transient HBV infection and AHB

TABLE 1 COMPARES baseline clinical characteristics of the 50 FH-T patients and the 50 AHB who

were matched for age and sex. The peak ALT, AST and T.bil levels were significantly higher (3788 ± 2856 vs 2170 ± 1350 IU/L, $P < 0.001$; 3131 ± 3673 vs 1676 ± 1851 IU/L, $P < 0.05$; and 14.8 ± 8.6 vs 9.5 ± 9.8 mg/dL, $P < 0.01$, respectively), while HBeAg was less frequent (30% vs 56%, $P < 0.01$) in the FH-T patients than AHB. The level of HBcrAg was significantly lower (5.30 ± 1.32 vs 5.95 ± 1.13 log U/mL, $P < 0.01$), while HBV DNA loads were higher (5.97 ± 1.87 vs 4.98 ± 1.17 log copies/mL, $P < 0.005$), in the FH-T patients than AHB. The level of core protein in sera tended to be higher in the FH-T patients than AHB (3.21 ± 1.28 vs 3.01 ± 1.00 log U/mL). Death occurred more often in the FH-T patients than AHB (38% vs 0%, $P < 0.001$).

HBV Genotypes and enhancer II/core promoter/pre-core/core Mutations in Patients with FHB by transient HBV infection and AHB

Figure 1(a) compares the distribution of HBV genotypes/subgenotypes between the FH-T and the AHB patients. The subgenotype C2/Ce was most prevalent in both patients with FH-T and AHB (66% and 62%, respectively), whereas B1/Bj was more frequent in the FH-T patients than AHB (22% vs 6%, $P < 0.05$). Likewise, mutations in enhancer II/core promoter/pre-core/core regions are compared between the FH-T and AHB patients in Figure 1(b). A1762T/G1764A, G1896A, G1899A and A2339G mutation were more frequent in the FH-T patients than AHB (48% vs 16%, $P < 0.001$; 62% vs 6%, $P < 0.001$; 24% vs 4%, $P < 0.001$; and 8% vs 0%, $P < 0.05$, respectively).

Figure 2(a) compares various mutations between the 11 FH-T patients and the three AHB patients who were infected with B1/Bj. Only G1896A was significantly more frequent (73% vs 0%, $P < 0.05$), while the lack of any mutations was less common (0% vs 33%, $P < 0.05$) in the FH-T patients than AHB. In comparison with the 33 FH-T patients and the 31 AHB patients who were infected with C2/Ce (Fig. 2b), A1762T/G1764A (70% vs 19%, $P < 0.001$), G1896A (61% vs 6%, $P < 0.001$) and the combination of all three mutations (A1762T/G1764A and G1896A) (45% vs 6%, $P < 0.001$) were significantly more frequent, while the lack of any mutations was less common (9% vs 70%, $P < 0.001$) in the FH-T patients than AHB. Interestingly, all the AHB patients with both G1896A and A1762T/G1764A mutations suffered acute severe hepatitis B that was defined by prothrombin time less than 40% but without coma of grade II or higher.

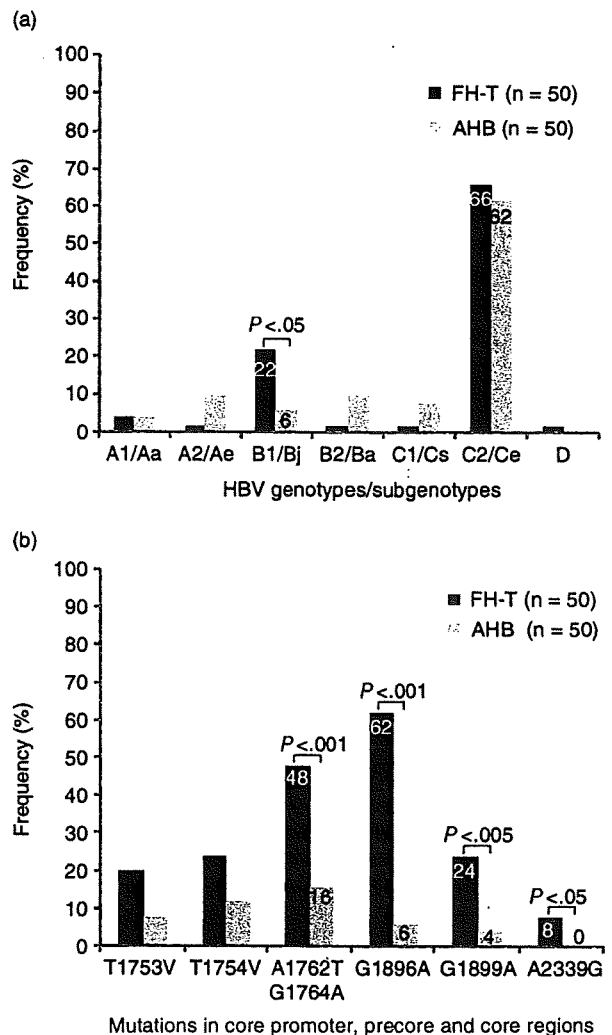


Figure 1 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 50 transient hepatitis B virus infection (FH-T) and the 50 acute self-limited hepatitis B (AHB) patients.

Factors independently associated with the development of FHB by transient HBV infection

The following independent factors, promoting the development of FHB, were evaluated by multivariate analysis: ALT, AST, T.bil, HBeAg, HBV DNA, core protein, HBcrAg, genotypes/subgenotypes (B1/Bj or not) and mutations (T1753V, T1754V, A1762T/G1764A, G1896A, G1899A and A2339G). T.bil more than 10.35 mg/dL (OR, 7.81 [95% CI, 1.77-34.51], $P = 0.0067$), G1896A mutation (OR, 13.53 [95% CI,

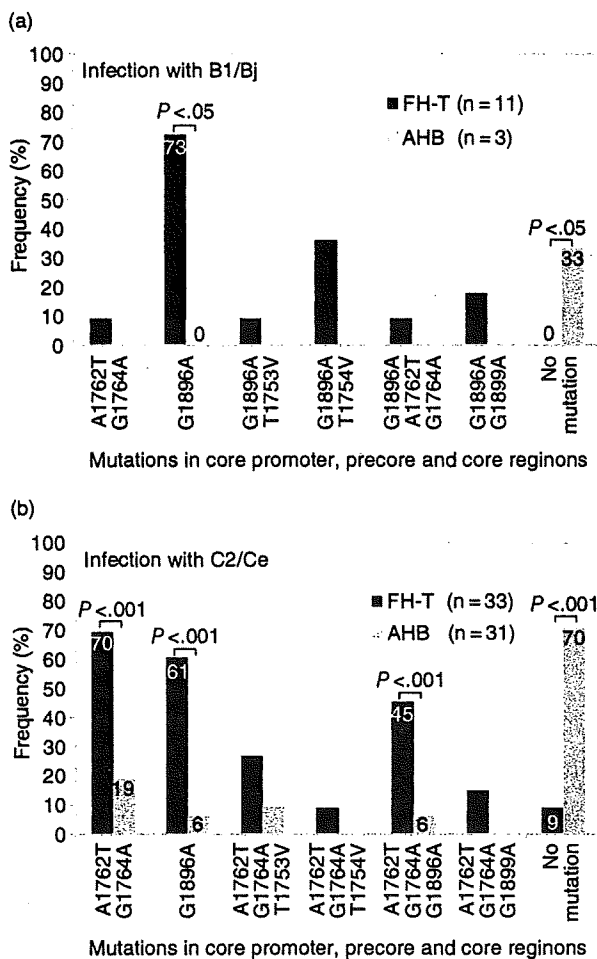


Figure 2 Frequencies of core promoter, pre-core and core mutations compared between the transient hepatitis B virus infection (FH-T) and the acute self-limited hepatitis B (AHB) patients who were infected with HBV of subgenotype B1/Bj (a) or C2/Ce (b).

2.75–66.64], $P = 0.0014$) and serum HBV DNA more than 5.23 log copies/mL (OR, 5.14 [95% CI, 1.10–24.15], $P = 0.0379$) were independent risk factors for the development of FHB by transient HBV infection (Table 2). Other mutations (T1753V, T1754V, A1762T/G1764A, G1899A and A2339G) were not significantly associated with the development of FHB by transient HBV infection, however.

Baseline clinical characteristics for distinguishing between the patients with FHB by AE of ASC (FH-C) and those without FHB by AE of CHB (AE-C)

Table 3 compares baseline clinical characteristics between the 12 FH-C patients and the 12 AE-C patients who were matched for age and sex. The levels of T.bil were significantly higher in the FH-C patients (15.0 ± 7.3 vs 7.3 ± 8.8 mg/dL, $P < 0.05$), but the peak ALT and AST levels tended to be slightly higher in the FH-C patients than AE-C (887 ± 681 vs 641 ± 620 IU/L and 701 ± 451 vs 601 ± 753 IU/L, respectively). There were also no significant differences in levels of sera HBV DNA, core protein and HBcrAg between these two groups (7.44 ± 1.51 vs 6.60 ± 1.10 log copies/mL, 5.04 ± 1.45 vs 5.07 ± 1.07 log U/mL, and 6.35 ± 1.70 vs 6.29 ± 1.95 log U/mL, respectively).

HBV genotypes and enhancer II/core promoter/pre-core/core mutations between the patients with FH-C and those with AE-C

There were no significant differences in the frequencies of any HBV genotypes between the 12 FH-C patients and the 12 AE-C patients (Fig. 3a). In addition, there were also no significant differences in the frequencies

Table 2 Multivariate analysis for factors independently associated with fulminant hepatitis by transient HBV infection

Factors	Odds ratio	95% confidence interval	P-value
Total bilirubin (mg/dL)†			
<10.35	1		
≥10.35	7.81	1.77–34.51	0.0067
G1896A mutation			
Absent	1		
Present	13.53	2.75–66.64	0.0014
HBV DNA (log copies/mL)†			
<5.23	1		
≥5.23	5.14	1.10–24.15	0.0379

†Median values. HBV, hepatitis B virus.

Table 3 Baseline characteristics between patients with FH by AE of ASC (FH-C) and those without FH by AE of CHB (AE-C)

Features	FH-C (n = 12)	AE-C (n = 12)	Differences P-value
Age (years)	51.7 ± 14.7	49.9 ± 5.6	Matched
Male	10 (83%)	9 (75%)	Matched
ALT (IU/L)	887 ± 681	641 ± 620	NS
AST (IU/L)	701 ± 451	601 ± 753	NS
Total bilirubin (mg/dL)	15.0 ± 7.3	7.3 ± 8.8	<0.05
Prothrombin time (%)	25.8 ± 6.6	48.4 ± 21.5	<0.005
HBeAg positive	4 (33%)	3 (25%)	NS
Core protein (log U/mL)	5.04 ± 1.45	5.07 ± 1.07	NS
HBcrAg (log U/mL)	6.35 ± 1.70	6.29 ± 1.95	NS
HBV DNA (log copies/mL)	7.44 ± 1.51	6.60 ± 1.10	NS

AE, acute exacerbation; ALT, alanine aminotransferase; ASC, asymptomatic HBV carrier; AST, aspartate aminotransferase; CHB, chronic hepatitis B; HBcrAg, hepatitis B core related antigen; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; NS, not significant.

of any specific mutations between these two groups (Fig. 3b).

DISCUSSION

THE MAGNITUDE OF liver injuries depends on the replication level of HBV and cytotoxic immune responses of the host raised against viral epitopes in general.³¹ Various viral factors have been proposed that promote the development of FHB, represented by pre-core (G1896A) and core promoter (A1762T/G1764A) mutations.^{13–16} Impact of virological factors on the development of FHB has remained controversial, however, especially because these mutations are rarely detected in the patients from the USA and France.^{19–21} It has been argued that the development of FHB is not promoted by these mutations and is dependent on host factors including the human leukocyte antigen (HLA) environment.²²

The expression of HBeAg is terminated by G1896A mutation in the pre-core region at the translation level,³² and downregulated by the A1762T/G1764A double mutation at the transcription level.^{33,34} Lamberts *et al.* are the first to implicate a negative influence of HBeAg on the replication of HBV.³⁵ Should HBeAg suppress the replication of HBV, presumably by inhibiting the encapsidation of pre-genome,³⁵ the lack or decrease of HBeAg would enhance the reproduction of HBV. Furthermore, HBeAg acts as a tolerogen to T cells recognizing epitopes on core protein, thereby, obviating immune injury of hepatocytes.^{36,37} In the absence or decrease of HBeAg, therefore, hosts would mount vigorous cytotoxic T-cell responses to core epitopes excessively

presented on hepatocytes, and develop severe liver injuries culminating in FHB.³⁸

There is a possibility that influence of viral factors such as HBV mutants with a HBeAg-negative phenotype, on the induction of FHB, may have been confounded by host factors and created disagreement. Therefore, the sheer influence of virological factors on FHB would need to be evaluated in case-control studies, as has been attempted to sort out the influence of HBV genotypes on development of cirrhosis and hepatocellular carcinoma.⁸ These backgrounds have instigated us to identify virological factors accelerating the severity of liver disease in the 50 FHB patients by transient HBV infection and the 50 AHB patients who were of the same ethnicity and matched for age as well as sex.

In this case controlled study, A1762T/G1764A, G1896A, G1899A and A2339G mutation were significantly more frequent in the patients with FH-T than AHB, providing further corroboration of previous studies;^{13–16} these mutations could enhance viral replication. Interestingly, our recent study using an *in vitro* replication model, showed that A2339G mutation in the core region enhanced viral replication and the effect of A2339G mutation may be associated with inhibition of the cleavage of the core protein by a furin-like protease, resulting in the high expression of the complete core protein.¹⁸ Such enhanced HBV would induce significant immune response, resulting in development of FHB.

In multivariate analysis, higher levels of serum HBV DNA and G1896A mutation were independent virological risk factors for the development of FHB by transient

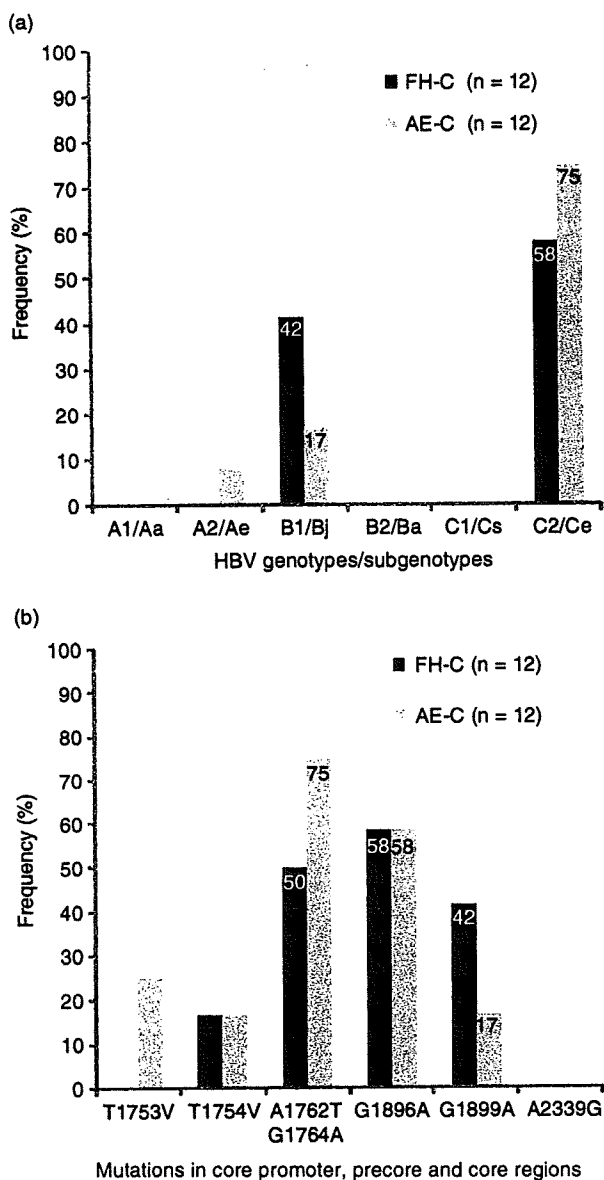


Figure 3 Genotypes/subgenotypes (a) and mutations in core promoter, pre-core and core regions (b) between the 12 transient hepatitis B virus infection (FH-T) and the 12 acute self-limited hepatitis B (AHB) patients.

HBV infection (Table 2). In particular, G1896A mutation was the most important factor associated with the development of FHB. Host responses, represented by T.bil, contributed to the development of FHB as well.

As for HBV genotypes, B1/Bj alone was significantly more frequent in the FH-T patients in univariate analy-

sis. In the patients infected with B1/Bj, G1896A was more frequent in those with FH-T than AHB. In *in vitro* replication analysis, Ozasa *et al.*¹⁵ observed extremely high expressions of intra- and extracellular HBV DNA in culture transfected with an HBV clone of B1/Bj genotype having the G1896A mutation; a high replication would be induced by this pre-core mutation for the induction of FHB. Our clinical results stand in support of this *in vitro* analysis. Taken altogether, chances for developing severe acute or FH would be high in the patients with acute hepatitis who are infected with HBV/B1 having the pre-core mutation. By contrast, in patients infected with C2/Ce, G1896A or A1762T/G1764A, or both was much more frequent in the FH-T patients than AHB. Of note, the co-occurrence of G1896A and A1762T/G1764A mutations was invariably accompanied by either FHB or acute severe hepatitis B in this study. Hence, these pre-core and core-promoter mutations might have additive or synergetic effects for exacerbating hepatitis, when they emerge in the patients infected with C2/Ce. Such high-risk patients deserve special care and surveillance for signs and symptoms of fulminant or severe acute hepatitis B.

In the present study, serum levels of HBV DNA were significantly higher in the patients with FH-T than AHB. High serum levels of HBV DNA have been reported in patients with FHB;³⁹ they are followed by rapid decrease as the sequel of virus elimination operated by vigorous immune responses. Because of rapid and extensive elimination of HBV by the host immune system, HBV DNA in serum, in general, has decreased to low levels in patients with FHB at the presentation.⁴⁰ HBV DNA levels may be subject to the time that has elapsed from the onset of hepatitis to its measurement.³⁹ Also, serum levels of core protein (the product of the C gene) closely correlate with serum HBV DNA levels in patients with hepatitis B,²⁷ and they were compared between the FH-T patients and AHB. The core protein was determined by the newly developed CLEIA method; it is much easier and less expensive than the determination of HBV DNA. The level of core protein has turned out to be marginally higher in the FH-T patients than AHB (Table 1), and therefore might not contribute to an early diagnosis of FHB by transient infection.

Fulminant hepatitis B by AE of ASC is assumed as a different clinical condition from FHB by transient HBV infection. In this study, as there was no case-control study on virological factors associated with FHB for the patients with AE of ASC, we also attempted to identify virological factors associated with the development of FHB in the 12 FH-C and the 12 AE-C patients who were

matched for age as well as sex. Disappointingly, no differences of virological factors such as HBV genotypes and pre-core mutations, which were strongly associated with the development of FHB by transient infection, were found between the FH-C and AE-C patients (Fig. 3a,b). Furthermore, there were also no significant differences about HBeAg-positive rate and the levels of serum HBV DNA or core protein (Table 3), suggesting that several host factors may play a more important role in the development of FHB in ASC instead of virological factors. In this case-control study, however, there seems to be some problems: a small number of patients, different duration of HBV infection, different clinical stage (ASC or CHB) at the onset of AE, and HBV quasispecies complexity. Further investigations are needed to identify factors associated with FHB precipitating in asymptomatic HBV carriers.

In conclusion, virological factors associated with enhancement of viral replication seemed to be important for the development of FHB in the patients by transient HBV infection. But no virological factors were identified for differentiation of the FH-C patients from the AE-C patients. Hence, the pathogenic mechanism of FHB between transient HBV infection and AE of ASC would be different.

ACKNOWLEDGMENTS

WE WOULD LIKE to thank Dr S. Baba, Showa University Hospital, Dr Y. Koga, Kurume University School of Medicine and the other doctors for collecting serum samples in this study. We would also thank Dr N. Maki, Advanced Life Science Institute (Saitama, Japan) for measuring core protein in serum. This study was supported in part by a Grant-in-Aid from the Ministry of Health, Labor and Welfare of Japan, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology.

REFERENCES

- 1 Fujiwara K, Mochida S, Matsui A, Nakayama N, Nagoshi S, Toda G. Fulminant hepatitis and late onset hepatic failure in Japan. *Hepatol Res* 2008; 38: 646–57.
- 2 Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO. Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. *J Gen Virol* 1992; 73 (Pt 5): 1201–8.
- 3 Okamoto H, Tsuda F, Sakugawa H *et al.* Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988; 69: 2575–83.
- 4 Stuyver L, De Gendt S, Van Geyt C *et al.* A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; 81 (Pt 1): 67–74.
- 5 Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; 83 (Pt 8): 2059–73.
- 6 Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; 46: 329–38.
- 7 Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002; 35: 1274–6.
- 8 Tanaka Y, Hasegawa I, Kato T *et al.* A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. *Hepatology* 2004; 40: 747–55.
- 9 Sugauchi F, Orito E, Ichida T *et al.* Hepatitis B virus of genotype B with or without recombination with genotype C over the pre-core region plus the core gene. *J Virol* 2002; 76: 5985–92.
- 10 Huy TT, Ushijima H, Quang VX *et al.* Genotype C of hepatitis B virus can be classified into at least two subgroups. *J Gen Virol* 2004; 85 (Pt 2): 283–92.
- 11 Tanaka Y, Orito E, Yuen MF *et al.* Two subtypes (subgenotypes) of hepatitis B virus genotype C: a novel subtyping assay based on restriction fragment length polymorphism. *Hepatol Res* 2005; 33: 216–24.
- 12 Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus—large-scale analysis using a new genotyping method. *J Infect Dis* 1997; 175: 1285–93.
- 13 Sato S, Suzuki K, Akahane Y *et al.* Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med* 1995; 122: 241–8.
- 14 Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the pre-core region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991; 324: 1699–704.
- 15 Ozasa A, Tanaka Y, Orito E *et al.* Influence of genotypes and pre-core mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006; 44: 326–34.
- 16 Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991; 324: 1705–9.
- 17 Imamura T, Yokosuka O, Kurihara T *et al.* Distribution of hepatitis B viral genotypes and mutations in the core promoter and pre-core regions in acute forms of liver disease in patients from Chiba, Japan. *Gut* 2003; 52: 1630–7.
- 18 Sugiyama M, Tanaka Y, Kurbanov F, Nakayama N, Mochida S, Mizokami M. Influences on hepatitis B virus replication by a naturally occurring mutation in the core gene. *Virology* 2007; 365: 285–91.

- 19 Laskus T, Persing DH, Nowicki MJ, Mosley JW, Rakela J. Nucleotide sequence analysis of the pre-core region in patients with fulminant hepatitis B in the United States. *Gastroenterology* 1993; 105: 1173–8.
- 20 Liang TJ, Hasegawa K, Munoz SJ *et al.* Hepatitis B virus pre-core mutation and fulminant hepatitis in the United States. A polymerase chain reaction-based assay for the detection of specific mutation. *J Clin Invest* 1994; 93: 550–5.
- 21 Feray C, Gigou M, Samuel D, Bernuau J, Bismuth H, Brechot C. Low prevalence of pre-core mutations in hepatitis B virus DNA in fulminant hepatitis type B in France. *J Hepatol* 1993; 18: 119–22.
- 22 Karayiannis P, Alexopoulou A, Hadziyannis S *et al.* Fulminant hepatitis associated with hepatitis B virus e antigen-negative infection: importance of host factors. *Hepatology* 1995; 22: 1628–34.
- 23 Trey C, Lipworth L, Chalmers TC *et al.* Fulminant hepatic failure. Presumable contribution to halothane. *N Engl J Med* 1968; 279: 798–801.
- 24 Ng HJ, Lim LC. Fulminant hepatitis B virus reactivation with concomitant listeriosis after fludarabine and rituximab therapy: case report. *Ann Hematol* 2001; 80: 549–52.
- 25 Fujiwara K, Mochida S, Matsui A. [Prospective study for the efficiency of lamivudine for the patients with acute exacerbation of HBV carrier.] *Annual Report of Intractable Liver Disease Study Group of Japan, the Ministry of Health, Welfare and Labor* 2004. (In Japanese.)
- 26 Kimura T, Rokuhara A, Sakamoto Y *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; 40: 439–45.
- 27 Kimura T, Rokuhara A, Matsumoto A *et al.* New enzyme immunoassay for detection of hepatitis B virus core antigen (HBcAg) and relation between levels of HBcAg and HBV DNA. *J Clin Microbiol* 2003; 41: 1901–6.
- 28 Abe A, Inoue K, Tanaka T *et al.* Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J Clin Microbiol* 1999; 37: 2899–903.
- 29 Sugauchi F, Mizokami M, Orito E *et al.* A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. *J Gen Virol* 2001; 82 (Pt 4): 883–92.
- 30 Shin IT, Tanaka Y, Tatenno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. *Hepatol Res* 2008; 38: 234–43.
- 31 Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol* 1995; 13: 29–60.
- 32 Carman WF, Jacyna MR, Hadziyannis S *et al.* Mutation preventing formation of hepatitis B e antigen in patients with chronic hepatitis B infection. *Lancet* 1989; 2 (8663): 588–91.
- 33 Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on pre-core gene expression and viral replication. *J Virol* 1996; 70: 5845–51.
- 34 Okamoto H, Tsuda F, Akahane Y *et al.* Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994; 68: 8102–10.
- 35 Lamberts C, Nassal M, Velhagen I, Zentgraf H, Schroder CH. Precore-mediated inhibition of hepatitis B virus progeny DNA synthesis. *J Virol* 1993; 67: 3756–62.
- 36 Chen MT, Billaud JN, Sallberg M *et al.* A function of the hepatitis B virus pre-core protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA* 2004; 101: 14913–8.
- 37 Chen M, Sallberg M, Hughes J *et al.* Immune tolerance split between hepatitis B virus pre-core and core proteins. *J Virol* 2005; 79: 3016–27.
- 38 Bocharov G, Ludewig B, Bertoletti A *et al.* Underwhelming the immune response: effect of slow virus growth on CD8+T-lymphocyte responses. *J Virol* 2004; 78: 2247–54.
- 39 Sainokami S, Abe K, Sato A *et al.* Initial load of hepatitis B virus (HBV), its changing profile, and pre-core/core promoter mutations correlate with the severity and outcome of acute HBV infection. *J Gastroenterol* 2007; 42: 241–9.
- 40 Tassopoulos NC, Papaevangelou GJ, Roumeliotou-Karayannis A, Ticehurst JR, Feinstone SM, Purcell RH. Search for hepatitis B virus DNA in sera from patients with acute type B or non-A, non-B hepatitis. *J Hepatol* 1986; 2: 410–8.