≪よく見かける感染症 ── 診断・治療の最新事情≫

ウイルス肝炎の現状と撲滅への取り組み

酒 井 明 人　　金 子 周 一

特集　日常診療で診る感染症 ── この疾患を見落とすな

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要旨

- B 型急性肝炎は，慢性化する可能性のある Genotype A が本邦で広がっている．
- 肝炎ウイルス検査を行ったときは，検査を行ったことと，その結果を確実に伝えてほしい．
- B 型，C 型肝炎ウイルスキャリアはともに肝癌ハイリスクグループであることを伝え，自覚症状がなくとも定期的に通院するよう経過観察の重要性を説く．
- 治療はガイドラインが示されているが，肝進展度の判断・治療必要性など専門医と連携をとることが重要であり，国の診療連携のガイドラインにも示されている．

はじめに○

　B 型肝炎ウイルス・C 型肝炎ウイルス感染症は，感染初期の急性肝炎から長い年月を経ての肝硬変・肝癌まで感染者の一生にかかわる問題である．近年ウイルス性肝疾患の診断，治療に関する進歩は目覚ましく，慢性肝炎，肝硬変，そして肝癌に関してのガイドラインも整備されつつある．しかしながら多くのウイルス性慢性肝炎患者は自覚症状に乏しく，検査を受けなければ自分がウイルスキャリアであることがわからない．肝炎にかかわる社会情勢が厳しくなり，国はウイルスキャリアの発掘，医療助成，診療体制の整備，肝炎に関する研究費など多方面にわたるサポートを行っており，2009 年 11 月には肝炎対策基本法が制定され，肝炎撲滅を目指した新たな局面に入っている．

急性ウイルス（B 型，C 型）性肝炎の現状○

　献血事業における核酸増幅検査（NAT）の導入により，輸血後ウイルス性肝炎は激減している．ウインドピリオドの関係で輸血後 B 型肝炎が年間数例報告されているが，C 型肝炎は皆無に等しい．モデル地区を対象とした研究では，輸血をされた 1,755 例中，輸血による感染と判定されたのは B 型肝炎 1 例だけであった[1]．よって現在の B，C 型急性肝炎の大部分の感染ルートは，不潔な注射の回し打ちと性行為であると推測される．近年問題となっているのは，主に 30 代以下の世代を中心として Genotype A の B 型肝炎感染が広がっていることである．これはわが国にほとんどみられ

Table 1. HBV DNA 陽性献血者の Genotype 別
陽性例数（1999〜2006 年）

年齢	Genotype							
	A		B		C		その他	
	男	女	男	女	男	女	男	女
10 代	3		1	5	18	41	1	
20 代	65	1	16	18	109	100	1	4
30 代	38	2	14	4	72	35	3	
40 代	9		12	3	31	11	1	1
50 代	4		15	5	28	10	2	
60 代			7	3	20	4		
計	119	3	65	38	278	201	8	5
	122		103		479		13	

［文献 2）表 4 より引用，改変］

石川県K町調査
K町における肝炎ウイルス検診対象者
（平成14〜19年）
906人中744人受診（82.1％）

肝炎検査を受けたことがあるか？
平成20年度アンケート調査
20歳以上全住民4,543人中
2,552人回答（56.2％）

わからない　302人（11.8％）
受けたことがある　498人（19.5％）
受けたことがない　1,684人（66.0％）
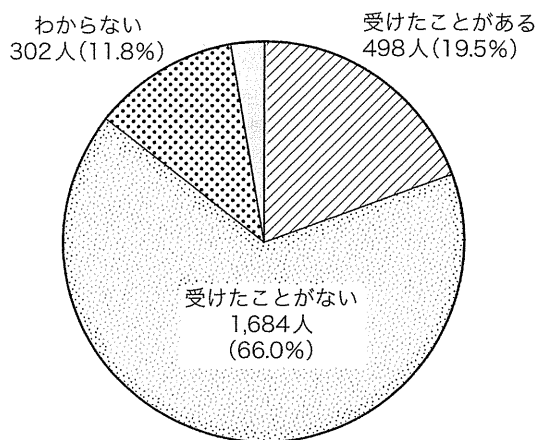
Fig. 1.　石川県 K 町における全住民に対するアンケート調査

なかったヨーロッパタイプといわれ，成人での感染にもかかわらず慢性化する可能性がある．Table 1 に 1999〜2006 年における HBV DNA 陽性献血者の年代，Genotype 別の頻度を示す．20〜30 代の 481 例中，106 例（22.0％）が Genotype A に感染しており，ほとんどが男性である[2]．学会などの報告でもこれらの群は男性同性愛者が多数を占め，HIV 同時感染の率も高いとされている．

　B 型，C 型急性肝炎は，発症前に HBs 抗原，HCV 抗体が陰性であることが確認されていれば診断は容易だが，実際は患者本人も検査されたことがあるかも覚えていないことが多い．班会議報告では，B 型の急性感染の場合は IgM-HBc 抗体が全例陽性であったが，キャリアからの急性増悪例でも 51％ で陽性であった[3]．キャリアからの発症例では，IgM-HBc 抗体価が低値であるが完全な鑑別は困難であり，急性感染例に比べるとキャリアからの発症は重症化することが多い．急性感染と思われてもキャリア例の可能性を否定できない症例には，抗ウイルス療法のタイミングを逃してはならない．ただし事前の問診が大切で，前述の Genotype A が疑われる状況であれば，同意のうえ HIV 検索も行わなければならない．インターフェロンであれば問題ないが，entecavir など核酸アナログ製剤には抗 HIV 作用があり，HIV の耐性

を誘導する恐れがある．C 型急性肝炎の場合は発症前陰性が確認されるか，HCV 抗体陰性 HCV RNA 陽性であるか，HCV 抗体価が低く経過中に上昇を認めれば診断できる．発症後 4ヵ月以降は自然治癒する症例はみられず慢性化に移行すると考えられるので[4]，インターフェロン療法を開始すべきである．

肝炎ウイルス検査の結果を伝えること○

　本邦での悪性新生物死亡数の男性第 4 位，女性第 5 位である肝癌を撲滅するには，潜在肝炎ウイルスキャリアの発見，キャリアの医療機関への受診，そして適切な医療（治療）を行うという 3 つのステップが必要である．肝障害の鑑別，入院時検査，あるいは検診として肝炎ウイルス検査を行うことが多いと考えられるが，陰性であっても患者には「肝炎ウイルス検査」をして「陰性」であったことを伝えてほしい．Fig. 1 に石川県 K 町において全住民対象のアンケート調査を行ったが，同町は肝炎検診受診率が高かったにもかかわらず，「肝炎

Table 2. 慢性 B 型肝炎の治療ガイドライン（2010 年度）（35 歳未満）

HBV-DNA	≧7 log copies/ml	<7 log copies/ml
e 抗原陽性	① IFN 長期投与（24～48 週） ② entecavir*	① IFN 長期投与（24～48 週） ② entecavir
e 抗原陰性 Plt15 万/μl 未満または F2 以上の進行例は ETV	① sequential 療法（ETV＋IFN 連続） ② entecavir	① 経過観察または ETV ② IFN 長期投与（24 週）

治療対象は ALT 31 IU/l 以上で：

e 抗原陽性は HBV DNA　5 log 以上

e 抗原陰性は　　　　　　　4 log 以上

肝硬変は　　　　　　　　　3 log 以上

*ETV を使用し e 抗原が陰性化し HBV DNA が陰性化したものは sequential 療法に切り替えて drug free を目指す．

検査を受けたことがあるか？」の問いに，受けたことがあると答えたのは 20% 未満であった．患者は検査を受けても，検診の一検査として陰性であれば忘れてしまうか，あるいは結果自体をインフォームされていないと考えられる．前述の急性肝炎の鑑別で述べたように，「陰性」結果は重要な情報である．

B 型肝炎ウイルスキャリアへの対応○

B 型肝炎ウイルスキャリアは青年期までは ALT が完全に正常な e 抗原陽性無症候性キャリアの状態であり，肝炎発症までは経過観察のみでよい．事前に画像診断で慢性化の徴候がないかチェックしておく必要がある．生化学的所見，ICG 15 分値までがまったく正常な肝硬変もときに存在する[5]．性のパートナーには HBs 抗原抗体を調べ，両者陰性であればワクチン接種を勧める．日常でみる多くの症例は e 抗体陽性無症候性キャリアであると考えられるが，1 回の診察では真に e 抗体陽性無症候性キャリアであるか，reactivation があるか判断することはできないので，ALT 値，e 抗原，e 抗体，HBV DNA 量を測定し，e 抗体陽性無症候性キャリアと思われてもはじめの 1 年は数回これらの項目をチェックしていく必要がある．決して「問題ない」や「治っている」といってはよくなく，肝癌のハイリスクグループであることを伝え，経過観察の重要性を説かねばならない．

Table 3. 慢性 B 型肝炎の治療ガイドライン（2010 年度）（35 歳以上）

HBV-DNA	≧7 log copies/ml	<7 log copies/ml
e 抗原陽性	① entecavir ② sequential 療法 （ETV＋IFN 連続）	① entecavir ② IFN 長期投与 （24～48 週）
e 抗原陰性	entecavir	① entecavir ② IFN 長期投与 （24～48 週）

治療対象は ALT 31 IU/l 以上で：

e 抗原陽性は HBV DNA 5 log 以上

e 抗原陰性は　　　　　　4 log 以上

肝硬変は　　　　　　　　3 log 以上

sequential 療法とは核酸アナログ投与により HBV DNA が感度以下になった症例に対し IFN を 4 週間併用し，その後 IFN 単独で 20 週間投与し薬剤を中止する．

肝炎が持続し，e 抗体陽性に転じず，ウイルス量の確かな低下が得られない症例は，C 型慢性肝炎よりも早期に肝病態が進行するために注意が必要である．年齢，ウイルス量，肝病態進展度に応じてインターフェロンや，抗ウイルス薬療法を決めていく必要があり，必要に応じ専門医の助言を仰ぐのが望ましい．治療に関しては厚生労働省班会議より毎年改定されたガイドラインが示されており（Table 2, 3），本年度より医療費補助があることも患者に伝え，治療の必要性と，とくに核酸アナログ製剤を使用する場合は決して自己中断しないことを守らせないといけない．

Table 4. C 型慢性肝炎の治療ガイドライン（2010 年度）

初回投与	Genotype 1	Genotype 2
高ウイルス量 5.0 log 以上	Peg-IFNα2a/b ribavirin（48〜72 週間） IFNβ＋ribavirin（48〜72 週間）	Peg-IFNα2b ribavirin（24 週間） IFNβ＋ribavirin（24 週間）
低ウイルス量 5.0 log 未満	IFN 単独（24 週間） Peg-IFNα2a（24〜48 週間）	IFN（8〜24 週間） Peg-IFNα2a（24〜48 週間）

C 型肝炎ウイルスキャリアへの対応○

　B 型肝炎同様，自覚症状がなく，たとえ ALT 値が低くとも「問題ない」といってはいけない．肝癌のハイリスクグループであることは B 型肝炎以上である．ALT 値が比較的低く，血小板数が正常でも肝生検をしてみると比較的進行した症例も存在する．当科で肝生検時 ALT 50 IU/l 以下，血小板 17 万/μl 以上であった 44 症例の肝生検では，40% 以上が F2 以上の慢性肝炎であった．B 型肝炎と異なり感染防御に有効なワクチンは存在しないが，夫婦間感染率は年間 0.23%，母子感染は報告により差はあるが約 4% であり，B 型に比べ低率である．

　C 型肝炎は進展度により肝発癌率が異なり，線維化の軽い F1 であれば年間肝発癌率は 0.5% であるが，F2 で 1.5%，F3 で 5%，F4 すなわち肝硬変では年 8% であり，症例に応じて肝画像検査の頻度を考えなければならない．また高齢になるほど慢性肝炎からの肝癌発症頻度が増えるので，注意が必要である．無用のトラブルを避けるためにも定期的・適切な画像診断が重要である．

　C 型慢性肝炎は超高齢者，重度のうつ病，間質性肺炎の合併など一部の症例を除いてはインターフェロン療法が考慮されるべきである．ウイルスの型（セロタイプ），ウイルス量の測定のうえ，基本はガイドライン（Table 4）に準じた治療が望まれる．現在のペグインターフェロン・ribavirin 併用療法であれば，1 型高ウイルス量症例で約 50%，それ以外であれば 80% 以上で著効（ウイルスの消失）が期待される．インターフェロン療法の経験が少ない施設では，専門医に説明を含めた治療導入，および定期的な専門医との併診が望まれる．平成 22 年度よりインターフェロン治療費助成上限額がさらに安くなっており（原則月 1 万円），利用しやすくなっていることも患者に伝えてほしい．

おわりに○

　肝癌撲滅のためには，肝炎ウイルスキャリアへの適切なフォロー・治療が重要である．とくに自覚症状がなく，異常がなければ同じことの繰り返しとなり患者は通院を中断する恐れが高い．一医師のみならず，専門医，行政などが一体となって地域で肝炎患者をケアしていく必要がある.「都道府県における肝炎検査後肝疾患診療体制に関するガイドライン http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou09/03.html」には，地域の医療体制，専門医受診の重要性が，「肝炎対策基本法 http://www.mhlw.go.jp/bunya/kenkou/kekkaku-kansenshou09/hourei_01.html」には，行政，医療機関，そして国民の役割，責務を掲げているので一読されたい．

文　献○

1) 西口修平ほか：輸血に伴うウイルス（HBV, HCV, HIV）感染発生の実態調査. B 型及び C 型肝炎の疫学及び検診を含む肝炎対策に関する研究，厚生労働科学特別研究事業平成 16〜18 年度総合研究報告書, p39-43, 2007
2) 柚木久雄ほか：核酸増幅検査（NAT）により見出された HBV DNA 陽性献血者の特性. B 型及び C 型肝炎の疫学及び検診を含む肝炎対策に関する研究，厚生労働科学特別研究事業平成 16〜18 年度総合研究報告書, p1-4, 2007

3）藤原研司ほか：遅発性（LOHF：late onset hepatic failure）の全国集計（2003 年）．難治性の肝疾患に関する研究，厚生労働科学特別研究事業平成 16 年度研究報告書，p93-107，2005

4）Gerlach JT et al：Acute hepatitis C：high rate of both spontaneous and treatment-induced viral clearance. Gastroenterology **125**：80，2003

5）稲垣　豊ほか：正常肝機能を示す潜在性肝硬変例の臨床病理学的検討．肝臓 **27**：305，1987

INFORMATION

第 22 回 中之島リウマチセミナー

日　時　2010 年 12 月 25 日（土）　13：30～
　　　　　12 月 26 日（日）　9：30～

会　場　大阪国際会議場（特別会議場　12 F）

代表世話人　七川歡次

テーマ　1）抗シトルリン蛋白抗体 2010
　　　　　2）類骨骨腫のラジオ波焼灼術
　　　　　3）ウイルス感染と RA 治療――特に肝炎ウイルス合併症と生物学的製剤
　　　　　4）Behçet 病 2010
　　　　　5）仙腸関節の痛みとその診断
　　　　　6）手首の骨折――橈骨遠位端骨折を中心に
　　　　　その他 24 題

会　費　20,000 円（当日のテキスト，12 月 25 日の懇親会費，12 月 26 日の昼食費用を含みます．また，出席者には講演集「リウマチ病セミナーXXII」を翌年送付いたします．学生および研修医は指導者の証明（1 人）があれば 10,000 円で御参加いただけます．但し，講演集は含まれません）

研修単位　日本リウマチ財団教育研修単位，日本リウマチ学会教育研修会単位

締め切り　2010 年 11 月 25 日

申込方法　Fax または E-mail に住所・氏名・電話・（Fax・E-mail）を御記入のうえ下記事務所までお申込ください．

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ORIGINAL ARTICLE

Infectivity of HBV DNA positive donations identified in look-back studies in Hyogo-Prefecture, Japan

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SUMMARY

Aims/Objectives: To clarify transfusion incidence of hepatitis B virus (HBV) infected blood negative for mini pool-nucleic acid amplification testing (MP-NAT).
Background: Japanese Red Cross (JRC) blood centres screen donated blood to avoid contamination with HBV. However, a low copy number of HBV may be overlooked.
Methods/Materials: In Hyogo-Prefecture, JRC blood centres screened 787 695 donations for HBV from April 2005 to March 2009. Of these, 685 844 were donations from the repeat donors. To detect the donors with HBV, serological tests, MP-NAT and/or individual donation (ID)-NAT were performed. To detect the recipients with transfusion-transmitted HBV infection (TTHBI), serological analysis and/or ID-NAT were performed.
Results: In this study, 265 of the 685 844 repeat donations were serologically and/or MP-NAT positive for

HBV. Their repository samples from the previous donation were examined in a look-back study; 13 of the 265 repository samples proved ID-NAT positive. Twelve recipients were transfused with HBV-infected blood components derived from 10 of the 13 HBV-infected donors. Only 1 of the 12 recipients was identified as TTHBI case. Seven of the 12 recipients escaped from our follow-up study and 4 recipients were negative for HBV during the observation period.
Conclusion: On the basis of the look-back study among the repeat donors in Hyogo-Prefecture, Japan, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations (~1/53 000 donations). However, more than half of the recipients transfused with HBV-infected blood negative for MP-NAT could not be followed up. It is necessary to establish a more cautious follow-up system.

Key words: HBV, ID-NAT, look-back study, MP-NAT, TTHBI.

For the purpose of the prevention of transfusion-transmitted hepatitis B virus infection (TTHBI), Japanese Red Cross (JRC) blood centres have been screening blood with serological tests for hepatitis B surface antigen (HBsAg) since 1972 and for hepatitis B core antibody (HBcAb) since 1989 (Japanese Red Cross NAT Screening Research Group, 2000). Currently, nucleic acid amplification testing (NAT) methods are widely

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used in Japan as useful diagnostic tools for virus detection.

In 1999, mini pool (MP)-NAT for hepatitis B virus (HBV) was implemented: MP-NAT with 50-member mini pool samples (50-MP-NAT) had been included in the JRC donor screening system since 2000, but that was replaced by 20-MP-NAT in 2004 (Yugi et al., 2006). The screening system including 20-MP-NAT or individual donation-NAT (ID-NAT) has been the standard method in Japan since 2004. Table 1 shows the HBV tests for donor selection that are now conducted in JRC.

However, it may be impossible to exterminate HBV infections, despite the introduction of sensitive NAT. The current NAT system cannot completely eliminate the risk

Table 1. HBV tests for donor selection

Period	Items	Test	Methods	Kit (Company)	Normal range
~July 2008	HBsAg	Serological test for screening	Reverse passive haemagglutination	JRC HBsAg (JRC)	$<2^1$
	HBsAg	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBsAg Dynapack (Abbott)	<2.0 S/N
	HBcAb	Serological test for screening	Haemagglutination inhibition	JRC HBcAb (JRC)	$<2^5$
	HBcAb	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBcAb Dynapack (Abbott)	$<50.0\%$INH
	HBsAb	Serological test for screening	Passive haemagglutination	JRC HBsAb (JRC)	$<2^4$
	HBsAb	Serological test for confirmation	Enzyme immunoassay	AxSYM® HBsAb Dynapack (Abbott)	<200 mIU/mL
	HBV DNA	Mini pool NAT for screening	TaqMan PCR	AMPLINAT MPX (Roche Diagnostics)	<100 copies/mL^1
	HBV DNA	Individual NAT for confirmation	TaqMan PCR	Single NAT HBV (Roche Diagnostics)	<100 copies/mL^1
August 2008~	HBsAg	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBsAg (Fujirebio)	<1.0 C.O.I.
	HBcAb	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBcAb (Fujirebio)	<12.0 C.O.I.
	HBsAb	Serological test for screening and confirmation	Chemiluminescence enzyme immunoassay	Lumipulse® HBsAb (Fujirebio)	<200 mIU/mL
	HBV DNA	Mini pool NAT for screening	TaqMan PCR	Cobas TaqScreen MPX (Roche Diagnostics)	<3.2 IU/mL^1
	HBV DNA	Individual NAT for confirmation	TaqMan PCR	Cobas TaqScreen HBV (Roche Diagnostics)	<2.4 IU/mL^1

^{1}HBV DNA detection limit.
C.O.I, cut off index; S/N, sample rate/index calibrator mean rate.

of TTHBI, because the limited sensitivity of NAT may overlook blood contaminated with extremely low levels of HBV donated by individuals with an early stage HBV infection (window period) or with HBV carrier status (Tadokoro, 2007).

When a new donation of a repeat donor turned out to be positive for HBsAg, HBcAb or NAT for HBV, repository samples of the preceding donation are subjected to a look-back study. In the look-back study, repository tubes from previous donations are re-investigated by ID-NAT. JRC has stored repository tubes of every blood donation for 11 years since 1996 (Satake *et al.*, 2007). A look-back system of JRC may detect donors with acute HBV infection in the window period and occult carrier donors with low HBcAb titers (Satake & Tadokoro, 2008).

To investigate transfusion incidents with HBV-infected blood negative for MP-NAT in the current JRC

screening system, we calculated their incidence based on the cumulative data of the look-back study during the period between 1 April 2005 and 31 March 2009. In addition, to investigate the outcome of the recipients who were transfused with HBV-infected blood, we searched for the transfusion incident victims.

MATERIALS AND METHODS

Subjects studied

In Japan, a total of 20 314 745 donated blood samples were screened for HBV infection during the period 1 April 2005 to 31 March 2009. Among 17 875 065 donations from the repeat donors, 10 380 were serologically positive or MP-NAT positive for HBV.

In Hyogo-Prefecture, a total of 787 695 donated blood samples were screened for HBV infection during the

period between 1 April 2005 and 31 March 2009. Among 685 844 donations from the repeat donors, 265 were serologically positive or MP-NAT positive for HBV.

In addition to the donors identified in the look-back study of the repeat donations, a donor with HBV-infected blood negative for MP-NAT, D1, in Hyogo-Prefecture was identified in the look-back study based on a hospital report.

Serological tests of donation samples for HBV

For the detection of HBV-infected blood, a battery of HBsAg, HBcAb, HBsAb and 20-MP-NAT (or ID-NAT) was used to judge the presence or absence of HBV in the donated blood samples. Donated blood samples had been screened for HBsAg by reverse passive haemagglutination (RPHA), for HBcAb by haemagglutination inhibition (HI) and for antibody to HBsAg (HBsAb) by passive haemagglutination (PHA) in JRC blood centres. Since August 2008, to improve the sensitivity of screening for HBsAg, HBcAb and HBsAb, all RPHA, HI and PHA methods had been replaced by chemiluminescence enzyme immunoassay (CLEIA) method (Table 1). Donated blood samples which were positive for HBV tests or which showed elevated alanine aminotransferase (ALT) ($>$60 IU/L) were excluded (Yoshikawa *et al.*, 2005; Yugi *et al.*, 2005).

MP-NAT and ID-NAT of HBV DNA

The outlines of MP-NAT and ID-NAT in the JRC screening system were reported by Mine *et al.* (2003) and Minegishi *et al.* (2003), respectively. In this study, MP-NAT for screening of HBV DNA was performed with AMPLINAT MPX (Roche Diagnostics, Mannheim, Germany) or Cobas TaqScreen MPX (Roche Diagnostics) according to the manufacturer's instruction. ID-NAT for confirmation was performed with Single NAT HBV (Roche Diagnostics) or Cobas TaqScreen HBV (Roche Diagnostics) according to the manufacturer's instruction. The detection limits using these reagents are shown in Table 1. The genotypes of HBV were determined by the sequencing analysis of Okamoto *et al.* (1990).

Detection of recipients with TTHBI

For the detection of recipients with TTHBI, serological analysis, ID-NAT for HBV and biochemical analysis including ALT were performed.

RESULTS

Look-back studies of the repeat donations

In Japan, a total of 20 314 745 donated blood samples were screened for HBV infection during the period 1

Table 2. Donors[1] in Japan-wide and Hyogo-Prefecture surveys during the period 1 April 2005 to 31 March 2009

	Japan-wide survey[2]	Hyogo-Prefecture survey
Total donors	20 314 745	787 695
First donors	2 439 680	101 851
Repeat donors[3]	17 875 065	685 844
Donors who were subjected to the look-back study	10 380	265
Donors who were ID-NAT positive at the preceding donation	247	13

[1]The donors in this study were the people who came to the JRC blood centres to donate their blood.
[2]Ministry of Health, Labour and Welfare of Japan (2009).
[3]Double counting took place if the same people came to the JRC blood centres to donate their blood more than one time. We call these people "repeat donors".

April 2005 to 31 March 2009. Of the total 20 314 745 donations, 17 875 065 were from the repeat donors. Of the 17 875 065 repeat donors, 10 380 were serologically positive or MP-NAT positive for HBV and subjected to look-back study (Ministry of Health, Labour and Welfare of Japan, 2009). When a new donation of a repeat donor turned out to be positive for serological tests and/or MP-NAT, repository samples of that donor's preceding donation were subjected to look-back study using ID-NAT. The look-back study of 10 380 repeat donors revealed that the previously donated blood samples of 247 repeat donors were ID-NAT positive (Table 2). On the basis of the nationwide look-back study in Japan, donations with HBV-infected blood negative for MP-NAT occurred with frequency of 247 in 17 875 065 donations ($\sim$1/72 000 donations).

In Hyogo-Prefecture, a total of 787 695 donated blood samples were screened for HBV infection during the period between 1 April 2005 and 31 March 2009. Of the 787 695 donations, 685 844 were from the repeat donors and 265 were serologically positive or MP-NAT positive for HBV and subjected to the look-back study. The look-back study of 265 repeat donors revealed that the previously donated blood samples of 13 repeat donors were ID-NAT positive (Table 2). On the basis of the look-back study in Hyogo-Prefecture, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations ($\sim$1/53 000 donations).

Transfusion incidents in Hyogo-Prefecture

Table 3 shows the numbers of recipients transfused with HBV-infected blood negative for MP-NAT (or victims of

Table 3. Recipients[1] transfused with HBV-infected blood negative for MP-NAT in Japan-wide and Hyogo-Prefecture surveys during the period 1 April 2005 to 31 March 2009

	Japan-wide survey[2]	Hyogo-Prefecture survey
Recipients transfused with HBV-infected blood negative for MP-NAT	204	12
Recipients who converted to HBV positive	13	1
Recipients who did not convert to HBV positive	58	4
Recipients who escaped from the follow-up study	133	7

[1]The recipients in this study were the people who were transfused with JRC blood products.
[2]Ministry of Health, Labour and Welfare of Japan (2009).

the transfusion incidents) in the Japan-wide and Hyogo-Prefecture surveys during the study period. According to the nationwide survey in Japan, 13 of the 204 recipients (6·3%) who had been transfused with HBV-infected blood negative for 20-MP-NAT were proved to be TTHBI. According to Hyogo-Prefecture survey, only 1 of the 12 recipients (8·3%) who had been transfused with HBV-infected blood negative for 20-MP-NAT was proved to be TTHBI.

HBV markers of the 'look-back study' donors in Hyogo-Prefecture

Table 4 shows the HBV markers at the final donation of 265 repeat donors in Hyogo-Prefecture who were subjected to look-back study. A total of 204 of the 265 'look-back study' donors presented HBsAg negative, NAT negative and HBcAb positive.

Analysis of individual cases of transfusion incidents in Hyogo-Prefecture

Table 5 shows the information of the 14 donors with HBV-infected blood negative for MP-NAT in

Table 4. HBV markers at the final donation of the 'look-back study' donors

Type	HBV markers	Donor numbers
A	[HBsAg (+)]	48
B	[HBsAg (−), NAT (+)]	13
C	[HBsAg (−), NAT (−), HBcAb (+)]	204
	Total	265

Hyogo-Prefecture. All except D1 proved to be ID-NAT positive in the repository sample of the donation prior to the final donation. D1 was accidentally found to be ID-NAT positive in the look-back study started from the hospital report of a patient with TTHBI, R1. The case of D1 was not identified in the look-back study in the repeat donations, because D1 did not donate any more after the index donation. Thus, there was a possibility of higher incidence of TTHBI due to the transfusion with HBV-infected blood negative for MP-NAT than ~1/53 000 donations.

Table 6 shows the clinical data of the 13 recipients transfused with HBV-infected blood negative for MP-NAT in Hyogo-Prefecture. We followed 13 recipients in Hyogo-Prefecture who were transfused with HBV-infected blood negative for MP-NAT. Only one recipient was identified to be suffering from TTHBI. Four of the 12 recipients were negative for HBV during the follow-up period. However, 7 of the 12 recipients did not undergo the post-transfusion HBV examination, because they died soon after the transfusion or did not return for a follow-up.

Case 1: Look-back study based on the hospital report

D1 donated a red blood cell (RBC) component causing TTHBI in a 63-year-old male with myelodysplastic syndrome (MDS), R1. R1 was transfused 28 times and got infected with HBV during chemotherapy. The index RBC component from D1 was ID-NAT positive with low HBV DNA levels, but was not detected by the regular 50-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D1 was in the window period of HBV infection at the index donation.

R1 was negative for HBsAg on 18 February 2002, according to the hospital records. However, he was positive for HBV DNA on 13 November 2002, 175 days after the date of the HBV-positive RBC component transfusion (22 May 2002). The recipient showed to be positive for HBsAg 254 days after the date of the transfusion and positive for HBcAb 315 days after the date of the transfusion (Table 6). R1 showed a transient elevation of ALT just after the negative-to-positive conversion of HBsAg and HBcAb, although HBsAb was still under detection level during his disease course (Fig. 1). R1 died of intracerebral haemorrhage, gastrointestinal haemorrhage and sepsis on 4 January 2004.

To confirm that HBV was transmitted through the blood from D1, HBV strains of the donor and the recipient were determined by the PCR/direct-sequencing method. The sequences in both samples showed genotype

Table 5. Donors involved in HBV-infected blood transfusion incidents

Case	Donor	Recipient	Donation number	Donation date	Index and final donations	HBV markers MP-NAT	ID-NAT	HBsAg	HBcAb
1	D1[1]	R1	3rd	15 Apr 1997		N.D.	N.D.	—	—
			4th	08 May 2002	Index donation	—	+[2]	—	—
2	D2	R2	7th	02 Jun 2003		—	—	—	—
			8th	11 Oct 2004	Index donation	—	+[2]	—	—
			9th	04 May 2005	Final donation	N.D.	—	—	+
3,4	D3	R3, R4	5th	11 Jun 2007		—	—	—	—
			6th	09 Oct 2007	Index donation	—	+[2]	—	—
			7th	20 Jul 2008	Final donation	N.D.	—	—	+
5	D4	R5	7th	18 Jan 2005		—	—	—	—
			8th	16 Mar 2005	Index donation	—	+[2]	—	—
			9th	19 Dec 2008	Final donation	N.D.	—	—	+
6	D5	R6	11th	15 Jun 2008		—	—	—	—
			12th	14 Sep 2008	Index donation	—	+[2]	—	—
			13th	15 Feb 2009	Final donation	+	+	—	—
7	D6	R7	24th	27 Apr 2005		—	—	—	—
			25th	25 Jan 2006	Index donation	—	+[2]	—	—
			26th	04 Mar 2006	Final donation	N.D.	N.D	+	—
8,9	D7	R8, R9	1st	04 Oct 1995		N.D.	N.D.	—	—
			2nd	08 Dec 2004	Index donation	—	+[2]	—	—
			3rd	07 Mar 2006	Final donation	N.D.	N.D	+	+
10	D8	R10	33rd	21 Jun 2006		—	—	—	—
			34th	06 Jul 2006	Index donation	—	+[2]	—	—
			35th	20 Jul 2006	Final donation	+	+	—	—
11	D9	R11	9th	06 Sep 2005		—	—	—	—
			10th	06 Sep 2006	Index donation	—	+[2]	—	—
			11th	27 Feb 2007	Final donation	N.D.	—	—	+
12	D10	R12	5th	04 Dec 2006		—	—	—	—
			6th	01 Feb 2007	Index donation	—	+[2]	—	—
			7th	02 Apr 2007	Final donation	N.D.	N.D	+	+
13	D11	R13	3rd	03 May 2007		—	—	—	—
			4th	04 May 2008	Index donation	—	+[2]	—	+
			5th	24 Feb 2009	Final donation	N.D.	N.D	+	+
	D12[3]	—	18th	14 Jan 2003		—	—	—	—
			19th	05 Oct 2004	Index donation	—	+[2]	—	—
			20th	31 Mar 2005	Final donation	N.D.	—	—	+
	D13[3]	—	8th	16 Mar 2004		—	—	—	—
			9th	25 Mar 2005	Index donation	—	+[2]	—	—
			10th	12 Jan 2006	Final donation	N.D.	—	—	+
	D14[3]	—	19th	10 Jun 2007		—	—	—	—
			20th	21 Oct 2007	Index donation	—	+[2]	—	—
			21st	24 Jul 2008	Final donation	N.D.	—	—	+

N.D., not done.

[1] D1 was identified as HBV carrier based on the hospital reports. No laboratory data of the fifth donation were available, because the fifth donation was not done.

[2] HBV DNA analysis using repository samples.

[3] The components from these donations were not transfused.

C and they were identical except for one nucleotide at nt 533, which was C for the donor and A for the recipient. Thus, R1 was infected by HBV in the RBC component offered by D1.

Case 2: Look-back study of the repeat donations

D2 donated blood nine times. The blood at the final donation (the ninth donation on 4 May 2005) was HBcAb positive, although it was ID-NAT negative. The

Table 6. Recipients involved in HBV-infected blood transfusion incidents

Case	Recipient (age, sex)[1]	Donor	Component	Transfusion date	Examination date (days after transfusion)	Examination result	Diagnosis of TTHBI	Present state
1	R1 (63y, M)[2]	D1	RBC	22 May 2002	13 Nov 2002 (175)	Positive for HBV DNA	Definite	Dead
					31 Jan 2003 (254)	Positive for HBsAg		
					02 Apr 2003 (315)	Positive for HBcAb		
					~29 Oct 2003 (525)	Negative for HBsAb		
2	R2 (63y, M)	D2	RBC	15 Oct 2004	20 May 2005 (217)	Positive for HBsAg, HBcAb and HBV DNA	Definite	Alive
						Negative for HBsAb		
3	R3 (51y, M)	D3	FFP	03 Jun 2008	08 Oct 2008 (127)	Negative for HBsAg, HBcAb, HBsAb and HBV DNA	Uncertain	Alive
4	R4 (87y, F)	D3	RBC	16 Oct 2007	13 Aug 2008 (302)	Negative for HBV DNA	Uncertain	Alive
5	R5 (58y, F)	D4	RBC	22 Mar 2005	20 Jan 2006 (304)	Negative for HBsAg	Uncertain	Dead
					31 Oct 2007 (953)	Negative for HBsAg		
					09 Mar 2008 (1083)	Negative for HBsAg		
6	R6 (68y, M)	D5	RBC	29 Sep 2008	07 Jan 2009 (100)	Negative for HBsAg and HBsAb	Uncertain	Alive
7	R7 (61y, M)	D6	PC	27 Jan 2006	N.D.	N.D.	Uncertain	Dead
8	R8 (67y, F)	D7	FFP	09 Aug 2005	N.D.	N.D.	Uncertain	Dead
9	R9 (82y, F)	D7	RBC	12 Dec 2004	N.D.	N.D.	Uncertain	No available information
10	R10 (75y, M)	D8	PC	08 Jul 2006	N.D.	N.D.	Uncertain	Dead
11	R11 (65y, M)	D9	RBC	14 Sep 2006	N.D.	N.D.	Uncertain	Dead
12	R12 (65y, F)	D10	PC	03 Feb 2007	N.D.	N.D.	Uncertain	Dead
13	R13 (75y, M)	D11	RBC	12 May 2008	N.D.	N.D.	Uncertain	Dead

RBC, red blood cells; FFP, fresh frozen plasma; PC, platelet concentrate; N.D., not done.
[1]Age; age at transfusion of HBV-infected blood.
[2]The index case was found in the hospital reports. Other cases were identified in the look-back study of the repeat donors.

look-back study proved that the repository tube of the index donation (the eighth donation on 11 October 2004) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D2 was in the window period of HBV infection at the index donation.

R2 was a 63-year-old male with severe anaemia who had been transfused with the index RBC component derived from D2 on 15 October 2004. According to the hospital record, his HBsAg was negative on 13 October 2004 and his HBV DNA was positive 217 days after the date of transfusion (Table 6).

To confirm that HBV was transmitted through the blood from D2, HBV strains of the donor and the recipient were determined by the PCR/direct-sequencing method. The sequences in both samples showed genotype C and they were identical except for one nucleotide at nt 519, which was R for the donor and A for the

recipient. Thus, R2 was infected by HBV in the RBC component offered by D2.

Case 3: Look-back study of the repeat donations

D3 donated blood seven times. The blood at the final donation (the seventh donation on 20 July 2008) was HBcAb positive, although it showed ID-NAT negative. The look-back study proved that the repository tube of the index donation (the sixth donation on 9 October 2007) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D3 was in the window period of HBV infection at the index donation.

R3 was a 51-year-old male with infectious endocarditis and he had been transfused with the index fresh frozen plasma (FFP) component derived from D3 on 3 June 2008. According to the record in the hospital, his
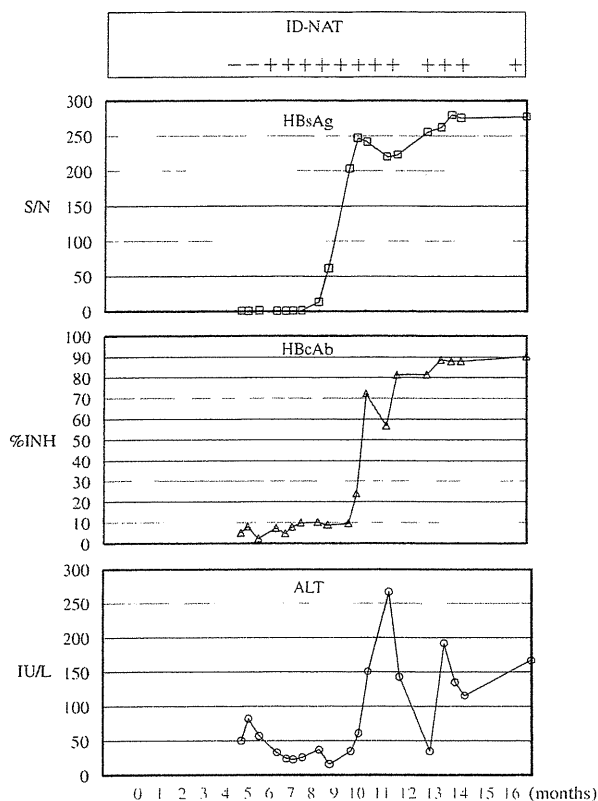
Fig. 1. HBV markers and ALT of R1. R1 was negative for HBsAb during the observation period.

HBsAg, HBsAb, HBcAb and HBV DNA were negative after 127 days from the date of transfusion (Table 6).

Case 4: Look-back study of the repeat donations

R4 was an 87-year-old female with bone fracture and she had been transfused with the index RBC component donated from D3 on 16 October 2007. According to the record in the hospital, her HBV DNA was negative after 302 days from the date of transfusion (Table 6).

Case 5: Look-back study of the repeat donations

D4 donated blood nine times. The blood at the final donation (the ninth donation on 19 December 2008) was HBcAb positive, although it showed ID-NAT negative. The look-back study proved that the repository tube of the index donation (the eighth donation on 16 March 2005) was ID-NAT positive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D4 was in the window period of HBV infection at the index donation.

R5 was a 58-year-old female with aplastic anaemia and she had been transfused with the index RBC component derived from D4 on 22 March 2005. According to the record in the hospital, her HBsAg was negative after 4 months from the date of transfusion, after 304, 953 and 1083 days (Table 6).

Case 6: Look-back study of the repeat donations

D5 donated blood 13 times. The blood at the final donation (the 13 donation on 15 February 2009) was 20-MP-NAT positive and it also showed ID-NAT positive. The look-back study proved that the repository tube of the index donation (the 12th donation on 14 September 2008) was ID-NATpositive, but HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that D5 was in the window period of HBV infection at the index donation.

R6 was a 68-year-old male with chronic renal failure and he had been transfused with the index RBC component derived from D5 on 29 September 2008. According to the record in the hospital, his HBsAg and HBsAb were negative after 100 days from the date of transfusion (Table 6).

Cases 7–13: Look-back study of the repeat donations

The index donations of D6 in Case 7, D7 in Cases 8 and 9, D8 in Case 10, D9 in Case 11 and D10 in Case 12 were ID-NAT positive, but their HBV infection was not detected by the regular 20-MP-NAT, HBsAg and HBcAb screening tests (Table 5). On the basis of these findings, it seemed that they were in the window period of HBV infection at the index donation.

The examination data of the recipients, R7 in Case 7, R8 in Case 8, R9 in Case 9, R10 in Case 10, R11 in Case 11, R12 in Case 12 and R13 in Case 13, were not available in this study.

The index blood components derived from D12, D13 and D14 were not transfused.

DISCUSSION

New HBV infection in the repeat donors

We identified the repeat donors who 'seroconverted' to either HBV DNA in MP-NAT, HBsAg or HBcAb in the look-back study during the period 1 April 2005 to 31 March 2009. If these were all true seroconversions, the incidence of new HBV infection among the repeat donors in Japan and Hyogo-Prefecture would be 1 : 1700 and 1 : 2600 in 4 years, i.e. 1 : 425 and 1 : 650 in a year. The value may be helpful to estimate the incidence

of new HBV infection in Japan and Hyogo-Prefecture, although it may not be the true incidence of new HBV infection.

Frequency of transfusion incidents with HBV-infected blood negative for MP-NAT

We calculated the transfusion incident frequency of HBV-infected blood negative for MP-NAT as 1/53 000–72 000 donations in the current JRC system, based on the look-back study data of the repeat donations. However, it should be noted that the frequency is only a minimum estimate, because donations from first time donors were not be evaluated in the look-back study among the repeat donors.

In addition, even ID-NAT does not always detect a donor with a low copy number of HBV. Some donors with intermittent ID-NAT detectable viremia may not be detected in any HBV screening system. Inaba *et al.* (2006) reported a case of an occult HBV carrier who did not constantly show ID-NAT positive results. The case of Inaba *et al.* suggested that it is necessary to repeat ID-NAT even after the repository tube is once found to be ID-NAT negative.

Incidence of TTHBI due to transfusion with HBV-infected blood negative for MP-NAT

The exact TTHBI incidence in the current JRC system could not be estimated. Two factors hampered estimation of the exact TTHBI incidence: (i) more than half of the recipients who were transfused with HBV-infected blood escaped from the follow-up study and (ii) recipients may become HBV positive after the observation period is over.

In Japan, only 'ID-NAT at 3 months after transfusion' is recommended by the Guideline of Ministry of Health, Labour and Welfare of Japan, because it is thought that recipients will turn out ID-NAT positive within 3 months after HBV-infected blood is transfused (Schreiber *et al.*, 1996; Comanor & Holland, 2006). However, in a recipient, R1, in our study, the period length of the ID-NAT negative window period was more than 161 days. Only 'ID-NAT investigation at 3 months after transfusion' may not be enough to prove the absence of HBV transmission in some recipients.

Our ideas are supported by the report of Satake *et al.* (2007). They reported the difficulty of following the recipients who had been transfused with HBV-infected blood. In those days they screened for HBV with 50-MP-NAT. According to the nationwide data during the period between 2000 and 2004, 12 of the 181 recipients who had been transfused with HBV-infected blood (6·6%) were cases of TTHBI (Satake *et al.*, 2007). Fifty-one of

the 181 recipients showed no evidence of HBV infection after index transfusion during their observation period. However, there is no information on whether these 51 recipients stayed in HBV-free status after the observation period. In addition, a total of 104 patients died without leaving any test results regarding HBV infection. This result also suggests that it is difficult to know the exact TTHBI incidence.

Prolonged window period due to long incubation before ID-NAT positive

In this study, we reported a recipient with MDS, R1, who showed no liver dysfunction, no elevation of serologic markers of HBV infection and no detection of HBV DNA until 175 days after transfusion of ID-NAT positive blood. The delayed appearance of HBV may be explained by two factors: (i) a very low copy number of HBV in the transfused blood and (ii) the immunosuppressive condition of the recipient.

Regarding the longer window period in the immune suppressed patients, it is difficult to explain. Immunosuppressive effect may shorten the window period and facilitate viral replication. However, Wendel *et al.* (2008) suggested a possibility that some free virus may have persisted in the liver, escaping the immune system until the level of immunodeficiency was such that viral replication could take place. They also reported two cases of immunodeficient recipients who were transfused with blood components from a single unit containing a very low level of HBV. One of these recipients had acute lymphoblastic leukaemia (ALL) and developed an acute HBV infection 13 months after transfusion despite carrying vaccine-induced HBsAb. The other recipient, who had MDS and received platelets from the same donation while receiving major chemotherapy, remained uninfected.

Unusual circumstances, such as chemotherapy or immunosuppression, can considerably modify the variables classically defining the early stages of a viral infection. When transfused with HBV-infected blood, immunosuppressive recipients need long-term examination, even if HBV-negative data are obtained at 3 months after transfusion (Hollinger & Dodd, 2009).

Then, a question arises how long it is necessary to observe the recipients after transfusion. It is not easy to answer this question. According to the report of Yuki *et al.* (2003), 14 patients who were recalled at a median of 4·2 years (range: 1·8–9·5 years) after the onset of acute hepatitis B all showed clinical and serologic recovery with circulating HBsAg clearance. However, PCR analysis showed that 3 of these 14 patients had low levels of circulating HBV DNA for up to 4·6–8·9 years after the onset. Although Yuki *et al.* did not show the exact

immunological state of their patients, a much longer observation period may be required than had ever been considered. Regarding the observation period after blood transfusion, we can say that it should be longer than 6 months, based on our experience with R1.

Conclusion

In the current Japanese look-back study, we can find the transfusion cases due to HBV-infected blood that could not be detected by serological tests or MP-NAT. On the basis of the look-back study among the repeat donors in Hyogo-Prefecture, Japan, donations with HBV-infected blood negative for MP-NAT occurred with a frequency of 13 in 685 844 donations (~1/53 000 donations). However, more than half of the recipients transfused with

HBV-infected blood negative for MP-NAT could not be followed up. It is therefore necessary to establish a more cautious follow-up system.

ACKNOWLEDGMENTS

REFERENCES

Comanor, L. & Holland, P. (2006) Hepatitis B virus blood screening: unfinished agendas. *Vox Sanguinis*, **91**, 1–12.

Hollinger, F.B. & Dodd, R.Y. (2009) Hepatitis B virus traceback and lookback: factors to consider. *Transfusion*, **49**, 176–184.

Inaba, S., Ito, A., Miyata, Y. *et al.* (2006) Individual nucleic amplification technology does not prevent all hepatitis B virus transmission by blood transfusion. *Transfusion*, **46**, 2028–2029.

Japanese Red Cross NAT Screening Research Group. (2000) Nationwide nucleic acid amplification testing of hepatitis B virus, hepatitis C virus and human immunodeficiency virus type 1 for blood transfusion and follow-up study of nucleic acid amplification positive donors. *Japanese Journal of Infectious Diseases*, **53**, 116–123.

Mine, H., Emura, H., Miyamoto, M. *et al.* (2003) High throughput screening of 16 million serologically negative blood donors for hepatitis B virus, hepatitis C virus and human immunodeficiency virus type-1 by nucleic acid amplification testing with specific and sensitive multiplex reagent in Japan. *Journal of Virological Methods*, **112**, 145–151.

Minegishi, K., Yoshikawa, A., Kishimoto, S., Yugi, H., Yokoya, N., Sakurada, M., Kiyokawa, H., Nishioka, K. & the Japanese Red Cross NAT Screening Research Group. (2003) Superiority of minipool nucleic acid amplification technology for hepatitis B

virus over chemiluminescence immunoassay for hepatitis B surface antigen screening. *Vox Sanguinis*, **84**, 287–291.

Ministry of Health, Labour and Welfare of Japan. (2009) *Report of the Blood Program Steering Committee, Blood Program division Pharmaceutical Affairs Food Sanitation Council.* URL http://www.mhlw. go.jp/shingi/2009/07/dl/s0728-12_060.pdf (Accessed 28/7/09).

Okamoto, H., Yotsumoto, S., Akahane, Y. *et al.* (1990) Hepatitis B viruses with pre-core region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *Journal of Virology*, **64**, 1298–1303.

Satake, M. (2007) Japanese repositories. *Transfusion*, **47**, 1105.

Satake, M. & Tadokoro, K. (2008) Transfusion-transmitted hepatitis verified from haemovigilance and look-back study. *ISBT Science Series*, **3**, 107–110.

Satake, M., Taira, R., Yugi, H., Hino, S., Kanemitsu, K., Ikeda, H. & Tadokoro, K. (2007) Infectivity of blood components with low hepatitis B virus DNA levels identified in a lookback program. *Transfusion*, **47**, 1197–1205.

Schreiber, G.B., Busch, M.P., Kleinman, S.H. & Korelitz, J.J. (1996) The risk of transfusion-transmitted viral infections. *The New England Journal of Medicine*, **334**, 1685–1690.

Tadokoro, K. (2007) The new Japanese blood law: its impact on blood safety and usage. *Developmental Biology*, **127**, 161–168.

Wendel, S., Levi, J.E., Biagini, S., Candotti, D. & Allain, J.P. (2008) A probable case of hepatitis B virus transfusion transmission revealed after a 13-month-long window period. *Transfusion*, **48**, 1602–1608.

Yoshikawa, A., Gotanda, Y., Itabashi, M., Minegishi, K., Kanemitsu, K., Nishioka, K. & the Japanese Red Cross NAT Screening Research Group. (2005) Hepatitis B NAT virus-positive blood donors in the early and late stages of HBV infection: analyses of the window period and kinetics of HBV DNA. *Vox Sanguinis*, **88**, 77–86.

Yugi, H., Hino, S., Satake, M. & Tadokoro, K. (2005) Implementation of donor screening for infectious agents transmitted by blood by nucleic acid technology in Japan. *Vox Sanguinis*, **89**, 265.

Yugi, H., Mizui, M., Tanaka, J. & Yoshizawa, H. (2006) Hepatitis B virus (HBV) screening strategy to ensure the safety of blood for transfusion through a combination of immunological testing and nucleic acid amplification testing – Japanese experience. *Journal of Clinical Virology*, **36**, S56–S64.

Yuki, N., Nagaoka, T., Yamashiro, M. *et al.* (2003) Long-term histologic and virologic outcomes of acute self-limited hepatitis B. *Hepatology*, **37**, 1172–1179.

Original Article

Serum concentration of 27-hydroxycholesterol predicts the effects of high-cholesterol diet on plasma LDL cholesterol level

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Aim: The effect of dietary cholesterol on plasma cholesterol concentrations varies widely among individuals. Recent studies suggest that the synthesis of oxysterols is up-regulated when tissue cholesterol is saturated. The present study was undertaken to test the hypothesis that a serum high concentration of 27-hydroxycholesterol, one of the oxysterols, reflects positive cholesterol balance in the body and predicts intolerance to a high-cholesterol diet.

Methods: In 30 subjects, 750 mg/day of cholesterol was added for 4 weeks to the ordinary diet. Blood samples were collected at the start and finish of the supplementation. Serum sterol and oxysterol concentrations were measured by high-resolution GC-MS.

Results: A receiver operating characteristic curve was drawn and the cutoff point (80 ng/mg cholesterol) was chosen to maximize sensitivity (81.3%) and specificity (64.3%) for predicting a positive change of LDL cholesterol concentration

after cholesterol loading. Subjects with higher serum 27-hydroxycholesterol concentrations ($\geq$ 80 ng/mg cholesterol) showed significantly ($P < 0.05$) high values for the change of LDL cholesterol concentration ($+7.4 \pm 3.4\%$, mean $\pm$ SEM, $n = 17$) compared with those with lower 27-hydroxycholesterol levels ($-5.3 \pm 2.7\%$, $n = 13$).

Conclusions: In subjects with high serum 27-hydroxycholesterol concentrations were unable to adapt to a high-cholesterol diet. The concentration of serum 27-hydroxycholesterol appears to reflect cholesterol saturation in the body and predicts to some extent a responsiveness to dietary cholesterol.

Key words: high-cholesterol diet, 27-hydroxycholesterol, hypercholesterolemia, LDL cholesterol, liver X receptor, oxysterol

INTRODUCTION

IN GENERAL, THE intake of dietary cholesterol is believed to increase plasma LDL cholesterol concentrations. Therefore, diets restricted in cholesterol have been recommended for the prevention and treatment of hypercholesterolemia.[1,2] However, the response of plasma cholesterol to dietary cholesterol varies among

the population.[3–8] A group of people considered high responders showed significant increases in plasma LDL cholesterol after cholesterol consumption. In contrast, individuals considered low responders showed stable or even decreased LDL cholesterol in spite of high intakes of dietary cholesterol. These facts suggest that the restriction of dietary cholesterol is effective only in high-responding people. Therefore great efforts have been made to explore the mechanism of the individual variability and to predict the responsiveness in each subject before dietary intervention.

The most popular approach is to investigate apolipoprotein (apo) E phenotypes. Subjects with apoE4 phenotype displayed higher plasma cholesterol levels, increased cholesterol absorption, and lower cholesterol synthesis than people with apoE2 phenotype.[9–11]

However, the effects of apoE phenotype on the response of plasma cholesterol levels to increased dietary cholesterol are still controversial, that is associated[12–15] or not associated[8,16–19] with the responsiveness.

Another approach is to measure the LDL receptor function. Mistry *et al.* reported a negative correlation between the change in plasma cholesterol concentration and LDL receptor activity in peripheral mononuclear leucocytes.[4] In contrast, Homma *et al.* demonstrated that the capacity of the LDL receptor did not explain the variability in the change of plasma cholesterol concentration induced by cholesterol loading.[8]

The aim of the present study was to discover a new biomarker that predicts responsiveness to a high-cholesterol diet. Recent biochemical studies unveiled the regulation of cholesterol metabolism at the molecular level. Cholesterol biosynthesis is down-regulated by oxysterols, intermediates in bile acid biosynthesis, through the modulation of a transcription factor, sterol regulatory element-binding protein (SREBP).[20] The elimination of sterols from the intestine and the liver are also stimulated by oxysterols through the activation of another transcription factor, liver X receptor α (LXRα).[21] Thus oxysterols appear to be messenger molecules that represent positive cholesterol balance in the body. Our results suggested that the baseline serum concentrations of 27-hydroxycholesterol, one of the most abundant oxysterol in human serum,[22] predicted to some extent a responsiveness to dietary cholesterol.

METHODS

Subjects

THIRTY JAPANESE SUBJECTS (11 males and 19 females; aged 29–84 years; BMI 18–28 kg/m^2) were studied, including healthy volunteers and patients with hypercholesterolemia. Patients with hypertension (> 140/90 mmHg, n = 14), well-controlled non-insulin-dependent diabetes mellitus (fasting plasma glucose < 126 mg/dL and hemoglobin A$_1$C < 7.0%, n = 3), stable angina pectoris (n = 2), old myocardial infarction (n = 1), and old cerebral infarction (n = 2) were included. Patients with hypocholesterolemia or familial hyperlipoproteinemia were excluded from this study. Informed consent was obtained from all subjects, and the study procedures were in accordance with the ethical standards of the Helsinki Declaration.

Experimental design

A daily dose of 750 mg of cholesterol was added to the ordinary diet for 4 weeks as freeze-dried egg yolk. The

subjects were requested not to change their dietary and drinking habits or their exercise patterns. During this study, all subjects were on a free-living Japanese diet that contains 250–350 mg/day cholesterol as estimated from daily food diaries. Patients who received antihyperlipidemic agents were excluded, and the treatment for complications except for hyperlipidemia was continued unaltered during the study period.

At the start and end of supplemental cholesterol feeding, blood samples were collected in the morning before breakfast after an overnight fasting, and serum was stored at −20°C until analyzed.

Chemicals

Sitosterol and campesterol were purchased from Sigma (MO, USA). Lathosterol and 5α-cholestane were obtained from Steraloids (NH, USA). 27-Hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one, [^{2}H$_7$]27-hydroxycholesterol and [^{2}H$_7$]7α-hydroxy-4-cholesten-3-one were prepared as described previously.[23]

Measurement of serum cholesterol concentration

Subfractions of serum lipoproteins were obtained by sequential ultracentrifugation.[24] The concentrations of total cholesterol in serum and the lipoprotein subfractions were measured by a Hitachi autoanalyzer (Hitachi, Japan).

Determination of apoE phenotype

ApoE phenotyping was performed by an isoelectric focusing immunoblotting method by Kataoka *et al.*[25]

Assay of LDL receptor activity in lymphocytes

LDL receptor activity was evaluated by the use of peripheral lymphocytes under the method of Ranganathan *et al.*[26] Briefly, mononuclear cells collected by the Ficoll precipitation method were cultured in lipoprotein-deficient medium for 72 h. Nonadherent mononuclear cells (lymphocytes) were collected and incubated with fluorescent LDL at 37°C for 2 h. Fluorescence of the washed lymphocytes was measured with a FACScan flow cytometer (Becton-Dickinson, NJ, USA). The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).

Determination of serum sterol concentrations

Serum levels of sitosterol, campesterol and lathosterol were measured by gas chromatography-mass spectrom-

Table 1 Correlations between baseline serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading ($n = 30$)

Serum marker sterols	Change of LDL cholesterol (%)	
	r_s†	P-value
Sitosterol (ng/mg cholesterol)	0.000	1.000
Campesterol (ng/mg cholesterol)	0.092	0.631
Lathosterol (ng/mg cholesterol)	0.138	0.466
27-Hydroxycholesterol (ng/mg cholesterol)	0.321	0.083
7α-hydroxy-4-cholesten-3-one (pg/mg cholesterol)	0.037	0.847

†Nonparametric Spearman's rank-order correlation coefficient.

etry (GC-MS). 5α-Cholestane (2 μg) was added to 50 μl of serum as an internal standard, and alkaline hydrolysis was carried out in 1 mL of 1 N ethanolic KOH at 60°C for 1 h. After an addition of 0.5 mL of distilled water, the sterols were extracted twice with 2 mL of n-hexane, and the extract was evaporated to dryness under nitrogen. The extracted sterols were converted into trimethylsilyl (TMS) ethers with 100 μL of TMSI-H (GL Sciences, Japan) for 15 min at 55°C. GC-MS with selected-ion monitoring was performed with a JMS-SX102 instrument equipped with a data processing XMS-system (JEOL, Japan). The accelerating voltage was 10 kV, the ionization energy was 70 eV, the trap current was 300 μA, and the mass spectral resolution was about 10 000. An Ultra Performance capillary column (25 m x 0.32 mm i.d.) coated with methylsilicone (Agilent Technologies, CA, USA) was used at a flow rate of helium carrier gas of 1.0 mL/min. The column oven was programmed to change from 100°C to 260°C at 30°C/min, after a 1-min delay from the start time. The multiple ion detector was focused on *m/z* 357.3521 for 5α-cholestane and sitosterol, *m/z* 343.3364 for campesterol, and *m/z* 458.3943 for lathosterol.

Serum 27-hydroxycholesterol and 7α-hydroxy-4-cholesten-3-one levels were quantified as described previously.[23]

Statistical analysis

Data are expressed as the mean ± SEM. The statistical significance between the results in the different groups was evaluated by a parametric two-sample t-test and a nonparametric Mann-Whitney test. The change of values after cholesterol supplementation was evaluated by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test. The correlations were tested by calculating Pearson's correlation coefficient, *r*, or a nonparametric Spearman's rank-order correlation coefficient, *r_s*. Independence was evaluated by Fisher's exact

probability test for a 2 × 2 contingency table and by the χ^2-test for a 3 × 2 contingency table. In all the statistical tests, significance was accepted at the level of $P < 0.05$.

RESULTS

Search for a new biomarker that predicts responsiveness to a high-cholesterol diet

THE CORRELATIONS BETWEEN the serum sterol concentrations and percent change of LDL cholesterol levels by cholesterol loading are summarized in Table 1. Although no statistically significant correlation was observed, a relatively low P-value was obtained for the relationship of 27-hydroxycholesterol concentrations with the percent changes of LDL cholesterol.

Figure 1a depicts the relationship and Figure 1b represents a receiver operating characteristic (ROC) curve to determine a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes. The cutoff point was chosen to maximize sensitivity and specificity, and it was 80 ng/mg cholesterol; the sensitivity and specificity for predicting a positive change of LDL cholesterol concentration because of cholesterol loading were 81.3% and 64.3%, respectively.

Characteristics of subjects with high serum 27-hydroxycholesterol concentrations

The baseline characteristics of subjects with low (< 80 ng/mg cholesterol) and high (≥ 80) serum 27-hydroxycholesterol concentrations were compared in Table 2. The subjects with low 27-hydroxycholesterol concentrations were all females, whereas 65% of those with high 27-hydroxycholesterol concentrations were males. The concentrations of HDL cholesterol were significantly low in subjects with high (≥ 80 ng/mg choles-
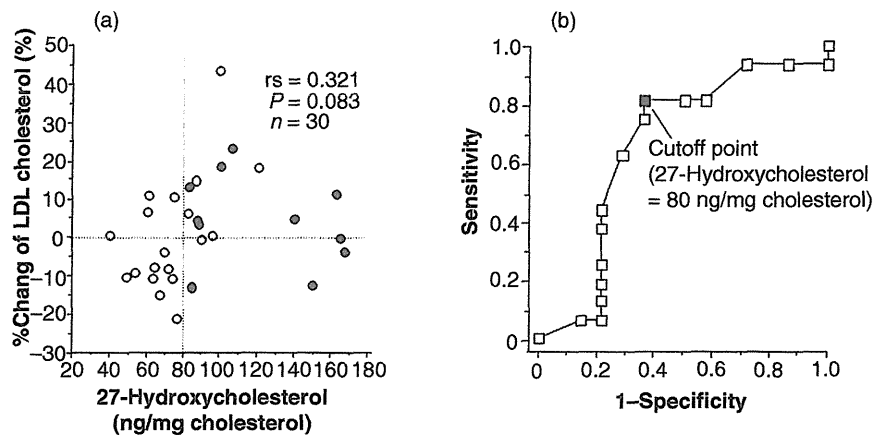
Figure 1 Relationships of baseline serum 27-hydroxycholesterol concentrations with % of change of LDL cholesterol concentrations by cholesterol loading (a), and a receiver operating characteristic (ROC) curve for determining a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated the subjects with positive changes of serum LDL cholesterol by cholesterol loading from those with negative changes (b). The percent change of LDL cholesterol concentration was calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading x100%. The open circles indicate female subjects ($n = 19$), and the closed circles represent male subjects ($n = 11$).

terol) serum 27-hydroxycholesterol concentrations. The other baseline data, including LDL receptor activity and apoE phenotype, were not significantly different between the two groups.

Figure 2 compares individual responses of serum lipid concentrations after cholesterol loading between the subjects with low (< 80 ng/mg cholesterol) and high

($\geq$ 80) baseline serum 27-hydroxycholesterol concentrations. The percent change of LDL cholesterol was significantly higher in subjects with high baseline 27-hydroxycholesterol concentrations than in those with low concentrations (Fig. 2b). The percent change of total cholesterol also tended to be high in subjects with high baseline 27-hydroxycholesterol concentra-

Table 2 Baseline characteristics of subjects with high serum 27-hydroxycholesterol versus low serum 27-hydroxycholesterol concentrations

	27-Hydroxycholesterol concentration (ng/mg cholesterol)		
	Low† (< 80)	High† ($\geq$ 80)	*P*-value‡
n (male/female)	0/13	11/6	< 0.0005
Age (years)	63.6 ± 4.5§	62.5 ± 2.7	0.63
BMI (kg/m²)	22.3 ± 0.7	23.1 ± 0.6	0.43
Total cholesterol (mg/dL)	246 ± 14	229 ± 11	0.34
LDL cholesterol (mg/dL)	164 ± 13	143 ± 9	0.16
HDL cholesterol (mg/dl)	66 ± 4	52 ± 4	< 0.05
LDL receptor activity (%)¶	112 ± 5	106 ± 5	0.36
ApoE phenotype (E2/E3/E4)††	1/8/4	0/13/4	0.43

†Each subject was assigned to one of the two groups by serum 27-hydroxycholesterol concentration; Low, < 80 ng/mg cholesterol; High, $\geq$ 80 ng/mg cholesterol.
‡The *P*-value for gender was calculated by Fisher's exact probability test and that for apoE phenotype by the χ^2-test for independence. The other *P*-values were calculated by the nonparametric Mann–Whitney test. §All such values are mean ± SEM.
¶The activities in normolipidemic volunteers were measured with every assay to provide an internal control value (100%).
††E2, E2/2 + E3/2; E3, E3/3; E4, E4/3 + E4/4.

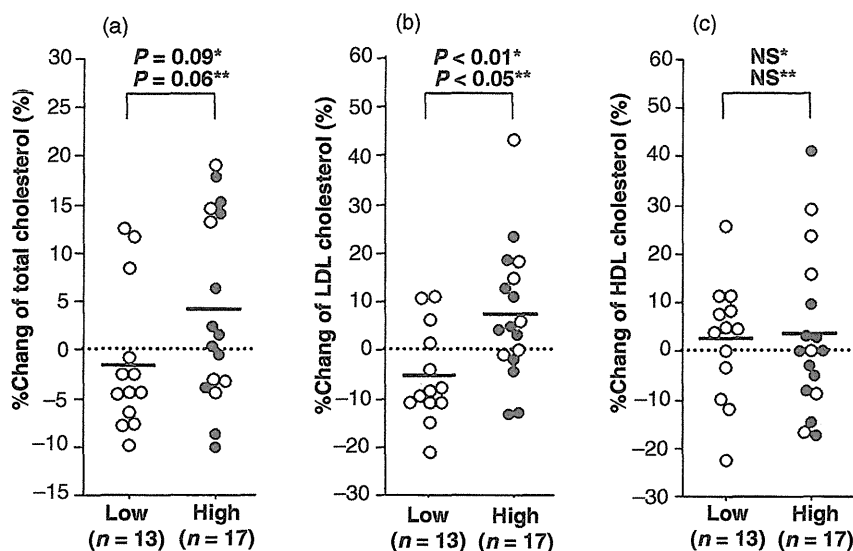© 2008 The Japan Society of Hepatology

Figure 2 Comparison of the individual responses of serum total cholesterol (a), LDL cholesterol (b), and HDL cholesterol (c) concentrations after cholesterol loading between the subjects with low baseline serum 27-hydroxycholesterol levels (< 80 ng/mg cholesterol) and those with high levels (≥ 80). The percent changes of these plasma sterol concentrations were calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading ×100%. The mean value for each group is indicated by a horizontal line. The open circles indicate female subjects (n = 19), and the closed circles represent male subjects (n = 11). *Analyzed by a parametric two-sample t-test. **Analyzed by a nonparametric Mann–Whitney test.

tions, but the difference was not statistically significant (Fig. 2a). In contrast, the percent change of HDL cholesterol was not significantly different between subjects with low baseline 27-hydroxycholesterol concentrations and those with high concentrations (Fig. 2c).

Effects of cholesterol loading on serum 27-hydroxycholesterol concentrations

As shown in Figure 3, strong positive correlations were observed between baseline 27-hydroxycholesterol concentrations and the concentrations after cholesterol loading ($r = 0.851$, $P < 0.0001$; $r_s = 0.911$, $P < 0.0001$). Furthermore, serum 27-hydroxycholesterol concentrations before and after cholesterol loading were compared by a parametric paired t-test and a nonparametric Wilcoxon signed-ranks test (92.2 ± 6.4 vs. 88.2 ± 5.2 ng/mg cholesterol), and no significant change was observed. Therefore similar results were obtained even if 27-hydroxycholesterol concentrations after cholesterol loading were used as a predictor instead of baseline 27-hydroxycholesterol concentrations. When the same 80 ng/mg cholesterol was used as a cut-off value for 27-hydroxycholesterol concentration after cholesterol loading, the percent change of LDL cholesterol was significantly higher in subjects with high
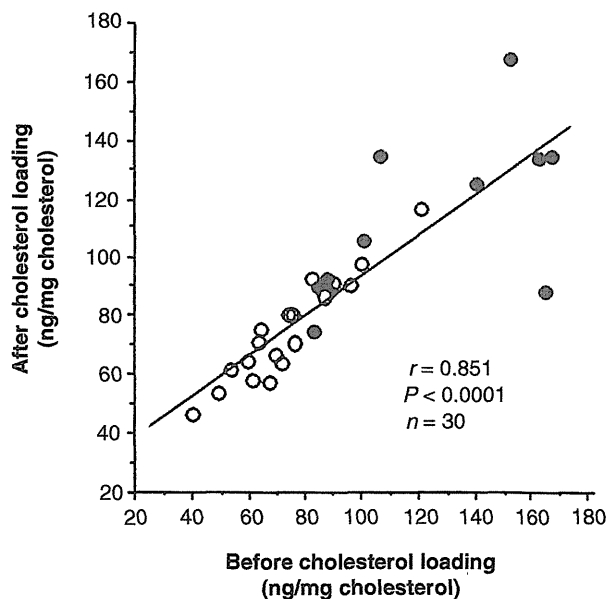

Figure 3 Relationships of serum 27-hydroxycholesterol concentrations before cholesterol loading (baseline concentrations) with those after cholesterol loading. The open circles indicate female subjects (n = 19), and the closed circles represent male subjects (n = 11).