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 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 (~1/72 000 donations).

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".

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 (~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 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 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

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 Negative for HBsAb	Definite	Alive
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

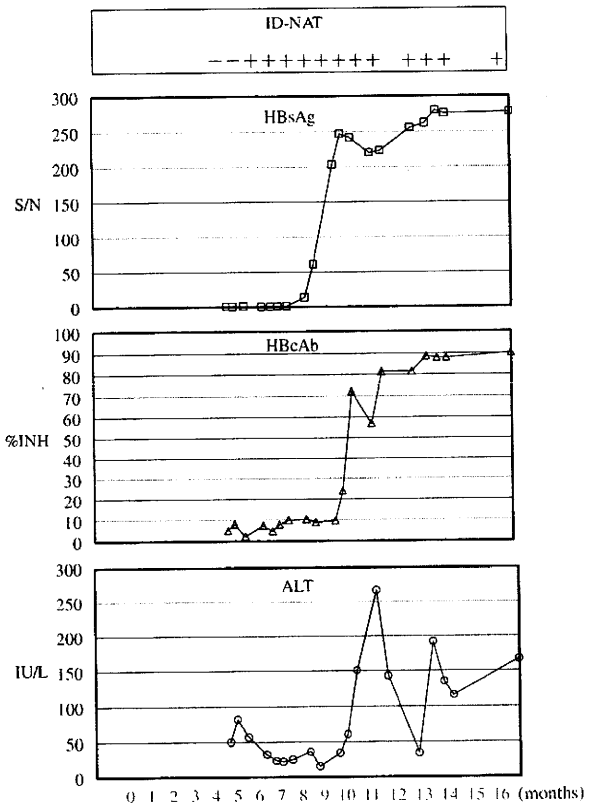

Fig. 1. HBV markers and ALT of R1. R1 was negative for HBsAb during the observation period.

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.

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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 precore 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.

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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 (≥ 80 ng/mg cholesterol) showed significantly ($P < 0.05$) high values for the change of LDL cholesterol concentration (+7.4 ± 3.4%, mean ± SEM, $n = 17$) compared with those with lower 27-hydroxycholesterol levels (–5.3 ± 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]

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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_1C < 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, [$^2H_7$]27-hydroxycholesterol and [$^2H_7$]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,†	*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-

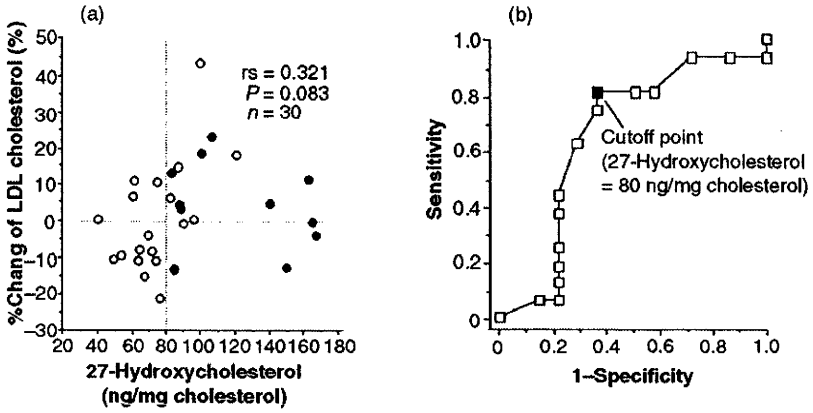

Figure 1 Relationships of baseline serum 27-hydroxycholesterol concentrations with % of change of LDL cholesterol concentrations by cholesterol loading (a), and a receiver operating characteristic (ROC) curve for determining a cutoff point of the 27-hydroxycholesterol concentration that optimally discriminated 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 (≥ 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† (≥ 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, ≥ 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.

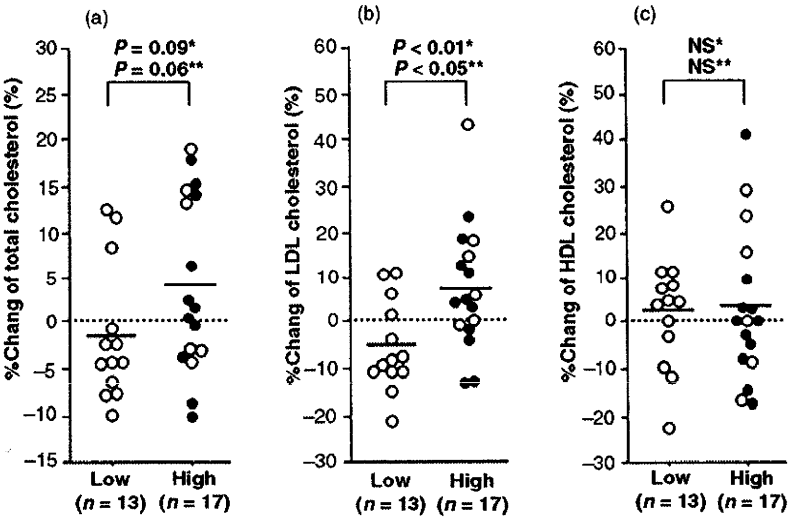

Figure 2 Comparison of the individual responses of serum total cholesterol (a), LDL cholesterol (b), and HDL cholesterol (c) concentrations after cholesterol loading between the subjects with low baseline serum 27-hydroxycholesterol levels (< 80 ng/mg cholesterol) and those with high levels (≥ 80). The percent changes of these plasma sterol concentrations were calculated as (concentration after cholesterol loading – concentration before loading)/concentration before loading x100%. 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

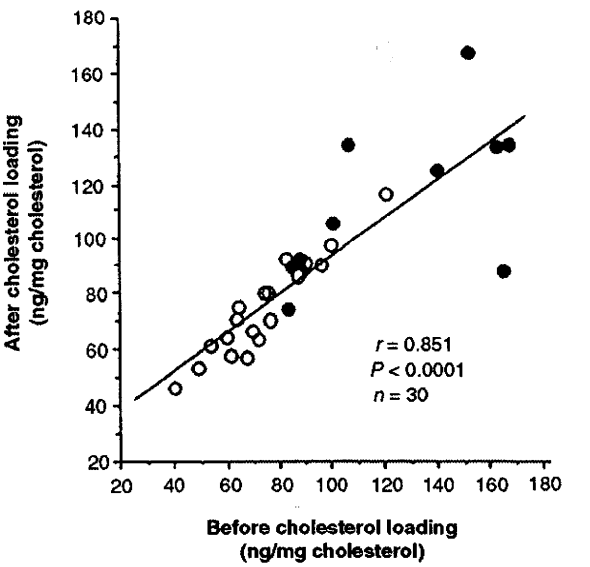

Figure 3 Relationships of serum 27-hydroxycholesterol concentrations before cholesterol loading (baseline concentrations) with those after cholesterol loading. The open circles indicate female subjects (n = 19), and the closed circles represent male subjects (n = 11).

(≥ 80) 27-hydroxycholesterol concentrations than in those with low (< 80) concentrations [+7.1 ± 3.6% ($n = 16$) vs. −4.0 ± 2.6% ($n = 14$); $P < 0.05$, significantly different by both the two-sample Student *t*-test and the Mann–Whitney test].

DISCUSSION

SERUM CONCENTRATIONS OF several marker sterols reflect cholesterol metabolism in the body. First, serum concentrations (relative to cholesterol) of plant sterols, sitosterol and campesterol, are positively correlated with the fractional absorption of dietary cholesterol and negatively correlated with fecal endogenous cholesterol outputs.[27,28] Second, serum concentration (relative to cholesterol) of lathosterol, a cholesterol precursor, reflects whole body cholesterol synthesis[29] or hepatic activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme in the cholesterol biosynthesis.[30] Third, serum concentration (relative to cholesterol) of 7α-hydroxy-4-cholesten-3-one, an intermediate in bile acid synthesis, has been used as a marker for hepatic activity of cholesterol 7α-hydroxylase (CYP7A1),[23] the rate-limiting enzyme in the classic bile acid biosynthetic pathway, and total bile acid synthesis.[31] To explore a biomarker that might predict responsiveness to cholesterol intake, we tried to measure serum baseline concentrations of the above sterols. However, they were not at all correlated with the percent change of LDL cholesterol by cholesterol loading ($r_s = 0.000–0.138$, $P = 1.000–0.466$).

Serum 27-hydroxycholesterol is another candidate for a predictor of cholesterol responsiveness. In fact, although the result did not reach statistical significance, a higher correlation coefficient was obtained between baseline 27-hydroxycholesterol concentrations and the percent change of LDL cholesterol because of cholesterol loading ($r_s = 0.321$, $P = 0.083$). This sterol is synthesized by CYP27A1 that is expressed in many tissues, including liver,[32] intestine,[32] vascular endothelium,[33] macrophages,[34] and atherosclerotic plaque.[35] CYP27A1 seems to protect the human body from cholesterol overload by at least three concurrent but separate mechanisms. The first mechanism operates via a suppression of cholesterol biosynthesis[36] by the inhibition of SREBP2 processing.[37] The second, 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid (immediate metabolite of 27-hydroxycholesterol by the same CYP27A1), are more polar than cholesterol and are transported into the liver and metabolized to bile acids more easily than cholesterol.[38] The third, 27-hydroxycholesterol, is one of the endogenous ligands for LXRα and inhibits the accumulation of cholesterol by activating this nuclear receptor.[39]

Cholesterol homeostasis in mammals is maintained by a balance between absorption from the intestine, *de novo* synthesis in the liver and extrahepatic tissues, and excretion to the bile as cholesterol or bile acids.[40] Our results that subjects with high baseline serum 27-hydroxycholesterol concentrations (≥ 80 ng/mg cholesterol) showed a higher percent change of LDL cholesterol by cholesterol loading suggest that these subjects had positive cholesterol balance in the body and less extra capacity to preserve serum LDL cholesterol concentrations after cholesterol loading. Thus serum 27-hydroxycholesterol concentrations seemed to predict to some extent the responsiveness to dietary cholesterol.

Since serum 27-hydroxycholesterol concentrations were fairly stable and not significantly affected by cholesterol loading (Fig. 3), the concentration appears to be determined by endogenous factors rather than dietary cholesterol. Therefore it may also be true that a restriction of cholesterol results in a more effective reduction of serum LDL cholesterol in subjects with high serum 27-hydroxycholesterol concentrations compared to those with low concentrations. An interesting finding in the present study is that the subjects with low 27-hydroxycholesterol concentrations (< 80 ng/mg cholesterol) were all females, and 65% of the subjects with high 27-hydroxycholesterol concentrations (≥ 80) were males (Table 2). Several studies have suggested that a low cholesterol diet reduces serum total cholesterol and LDL cholesterol concentrations more greatly in males than in females,[41,42] which may be explained in part by our idea that serum 27-hydroxycholesterol concentrations predict the effects of cholesterol restriction on serum LDL cholesterol.

The importance of serum 27-hydroxycholesterol concentrations and CYP27A1 activity in hepatic and extrahepatic tissues for the response to dietary cholesterol has also been pointed out in experiments using baboons.[43,44] However, the conclusions are completely different from humans. In baboons, baseline serum 27-hydroxycholesterol concentrations were not significantly different between high and low responders, and with a high-cholesterol diet, a significant elevation of 27-hydroxycholesterol concentrations was observed only in the low-responding baboons. Although we excluded subjects with hypocholesterolemia in our study, it may be possible that the treatment of hypocholesterolemic patients with high-cholesterol diets

shows results similar to those of the baboons because basal serum LDL cholesterol concentrations in baboons are very low (less than HDL cholesterol).

In our results, the concentrations of HDL cholesterol were significantly low in subjects with high serum 27-hydroxycholesterol concentrations (Table 2). LXRα upregulates the expression of cholesteryl ester transfer protein (CETP) and CETP transfers cholesteryl ester from HDL to other lipoproteins, so that serum HDL cholesterol levels are reduced. Thus high serum 27-hydroxycholesterol concentrations may reflect the activation of LXRα *in vivo*. A recent report by Higuchi *et al.*[45] suggests that the activation of LXRα is one of the important factors that cause nonalcoholic fatty liver disease (NAFLD) in humans. Further investigations are expected to use serum oxysterol markers for the evaluation of hepatic LXRα activity.

In summary, serum high 27-hydroxycholesterol concentrations were thought to reflect positive cholesterol balance in the body and predict, to some extent, a responsiveness to dietary cholesterol loading. A determination of serum 27-hydroxycholesterol concentrations seems to be useful in predicting tolerance to a high-cholesterol diet and the effects of cholesterol restriction therapies.

REFERENCES

1 The Expert Panel. Repport of the National Cholesterol Education Program Expert Panel on detection, evaluation, and treatment of high blood Cholesterol in adults. *Arch Intern Med* 1988; **148**: 36–69.

2 The Expert Panel. Summary of the second report of the National Cholesterol Education Program (NCEP) Expert Panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel II). *J Am Med Assoc* 1993; **269**: 3015–23.

3 Quintao E, Grundy AM, Ahrens EH Jr. Effects of dietary cholesterol on the regulation of total body cholesterol in man. *J Lipid Res* 1971; **12**: 233–47.

4 Mistry P, Miller NE, Laker M, Hazzard WR, Lewis B. Individual variation in the effects of dietary cholesterol on plasma lipoproteins and cellular cholesterol homeostasis in man: studies of low density lipoprotein receptor activity mononuclear cells. *J Clin Invest* 1981; **67**: 493–502.

5 McNamara DJ, Kolb R, Parker TS *et al.* Heterogeneity of cholesterol homeostasis in man: response to changes in dietary fat quality and cholesterol quantity. *J Clin Invest* 1987; **79**: 1729–39.

6 Katan MB, Beynen AC. Characteristics of human hypo- and hyperresponders to dietary cholesterol. *Am J Epidemiol* 1987; **125**: 387–99.

7 Schaefer EJ, Lamon-Fava S, Ausman LM *et al.* Individual variability in lipoprotein cholesterol response to National Cholesterol Education Program Step 2 diets. *Am J Clin Nutr* 1997; **65**: 823–30.

8 Homma Y, Kobayashi T, Yamaguchi H, Ozawa H, Homma K, Ishiwata K. Apolipoprotein-E phenotype and basal activity of low-density lipoprotein receptor are independent of changes in plasma lipoprotein subfractions after cholesterol ingestion in Japanese subjects. *Nutrition* 2001; **17**: 310–14.

9 Kesäniemi YA, Ehnholm C, Miettinen TA. Intestinal cholesterol absorption efficiency in man is related to apoprotein E phenotype. *J Clin Invest* 1987; **80**: 578–81.

10 Davignon J, Gregg RE, Sing CF Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis* 1988; **8**: 1–21.

11 Miettinen TA. Impact of apo E phenotype on the regulation of cholesterol metabolism. *Ann Med* 1991; **23**: 181–6.

12 Gylling H, Miettinen TA. Cholesterol absorption and synthesis related to low density lipoprotein metabolism during varying cholesterol intake in men with different apoE phenotypes. *J Lipid Res* 1992; **33**: 1361–71.

13 Miettinen HE, Gylling H, Vanhanen H. Serum cholesterol response to dietary cholesterol and apoprotein phenotype. *Lancet* 1988; **2**: 1261.

14 Tikkanen MJ, Huttunen JK, Ehnholm C, Pietinen P. Apolipoprotein E4 homozygosity predisposes to serum cholesterol elevation during high fat diet. *Arteriosclerosis* 1990; **10**: 285–8.

15 Miettinen TA, Gylling H, Vanhanen H, Ollus A. Cholesterol absorption, elimination, and synthesis related to LDL kinetics during varying fat intake in men with different apoprotein E phenotypes. *Arterioscler Thromb* 1992; **12**: 1044–52.

16 Jones PJ, Main BF, Frohlich JJ. Response of cholesterol synthesis to cholesterol feeding in men with different apolipoprotein E genotypes. *Metabolism* 1993; **42**: 1065–71.

17 Clifton PM, Kestin M, Abbey M, Drysdale M, Nestel PJ. Relationship between sensitivity to dietary fat and dietary cholesterol. *Arteriosclerosis* 1990; **10**: 394–401.

18 Savolainen MJ, Rantala M, Kervinen K *et al.* Magnitude of dietary effects on plasma cholesterol concentration: role of sex and apolipoprotein E phenotype. *Atherosclerosis* 1991; **86**: 145–52.

19 Boerwinkle E, Brown SA, Rohrbach K, Gotto AM Jr, Patsch W. Role of apolipoprotein E and B gene variation in determining response of lipid, lipoprotein, and apolipoprotein levels to increased dietary cholesterol. *Am J Hum Genet* 1991; **49**: 1145–54.

20 Sakai J, Duncan EA, Rawson RB, Hua X, Brown MS, Goldstein JL. Sterol-regulated release of SREBP-2 from cell membranes requires two sequential cleavages, one within a transmembrane segment. *Cell* 1996; **85**: 1037–46.

21 Lu TT, Repa JJ, Mangelsdorf DJ. Orphan nuclear receptors as eLiXiRs and FiXeRs of sterol metabolism. *J Biol Chem* 2001; **276**: 37735–8.

22 Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. *Anal Biochem* 1995; 225: 73–80.
23 Honda A, Yoshida T, Xu G *et al.* Significance of plasma 7α-hydroxy-4-cholesten-3-one and 27-hydroxycholesterol concentrations as markers for hepatic bile acid synthesis in cholesterol-fed rabbits. *Metabolism* 2004; 53: 42–8.
24 Havel RJ, Eder HA, Bragdon JH. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J Clin Invest* 1955; 34: 1345–53.
25 Kataoka S, Paidi M, Howard BV. Simplified isoelectricfocusing/immunoblotting determination of apoprotein E phenotype. *Clin Chem* 1994; 40: 11–13.
26 Ranganathan S, Hattori H, Kashyap ML. A rapid flow cytometric assay for low-density lipoprotein receptors in human peripheral blood mononuclear cells. *J Lab Clin Med* 1995; 125: 479–86.
27 Tilvis RS, Miettinen TA. Serum plant sterols and their relation to cholesterol absorption. *Am J Clin Nutr* 1986; 43: 92–7.
28 Miettinen TA, Tilvis RS, Kesäniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. *Am J Epidemiol* 1990; 131: 20–31.
29 Kempen HJ, Glatz JF, Gevers Leuven JA, van der Voort HA, Katan MB. Serum lathosterol concentration is an indicator of whole-body cholesterol synthesis in humans. *J Lipi Res* 1988; 29: 1149–55.
30 Björkhem I, Miettinen T, Reihnér E, Ewerth S, Angelin B, Einarsson K. Correlation between serum levels of some cholesterol precursors and activity of HMG-CoA reductase in human liver. *J Lipid Res* 1987; 28: 1137–43.
31 Sauter G, Berr F, Beuers U, Fischer S, Paumgartner G. Serum concentrations of 7α-hydroxy-4-cholester-3-one reflect bile acid synthesis in humans. *Hepatology* 1996; 24: 123–6.
32 Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW. Cloning, structure, and expression of the mitochondrial cytochrome P-450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. *J Biol Chem* 1989; 264: 8222–9.
33 Reiss AB, Martin KO, Javitt NB, Martin DW, Grossi EA, Galloway AC. Sterol 27-hydroxylase: high levels of activity in vascular endothelium. *J Lipid Res* 1994; 35: 1026–30.
34 Björkhem I, Andersson O, Diczfalusy U *et al.* Atherosclerosis and sterol 27-hydroxylase: evidence for a role of this enzyme in elimination of cholesterol from human macrophages. *Proc Natl Acad Sci USA* 1994; 91: 8592–6.
35 Crisby M, Nilsson J, Kostulas V, Björkhem I, Diczfalusy U. Localization of sterol 27-hydroxylase immuno-reactivity in human atherosclerotic plaques. *Biochim Biophys Acta* 1997; 1344: 278–85.
36 Axelson M, Larsson O. Low density lipoprotein (LDL) cholesterol is converted to 27-hydroxycholesterol in human fibroblasts. *J Biol Chem* 1995; 270: 15102–10.
37 Janowski BA, Shan B, Russell DW. The hypocholesterolemic agent LY295427 reverses suppression of sterol regulatory element-binding protein processing mediated by oxysterols. *J Biol Chem* 2001; 276: 45408–16.
38 Babiker A, Andersson O, Lund E *et al.* Elimination of cholesterol in macrophages and endothelial cells by the sterol 27-hydroxylase mechanism. *J Biol Chem* 1997; 272: 26253–61.
39 Fu X, Menke JG, Chen Y *et al.* 27-hydroxycholesterol is an endogenous ligand for liver X receptor in cholesterol-loaded cells. *J Biol Chem* 2001; 276: 38378–87.
40 Everson GT. Bile acid metabolism and its role in human cholesterol balance. *Semin Liver Dis* 1992; 12: 420–8.
41 Barnard RJ. Effects of life-style modification on serum lipids. *Arch Intern Med* 1991; 151: 1389–94.
42 Li Z, Otvos JD, Lamon-Fava S *et al.* Men and women differ in lipoprotein response to dietary saturated fat and cholesterol restriction. *J Nutr* 2003; 133: 3428–33.
43 Hasan SQ, Kushwaha RS. Differences in 27-hydroxycholesterol concentrations in plasma and liver of baboons with high and low responses to dietary cholesterol and fat. *Biochim Biophys Acta* 1993; 1182: 299–302.
44 Chen L-D, Kushwaha RS, Rice KS, Carey KD, McGill HC Jr. Effect of dietary lipids on hepatic and extrahepatic sterol 27-hydroxylase activity in high- and low-responding baboons. *Metabolism* 1998; 47: 731–8.
45 Higuchi N, Kato M, Shundo Y *et al.* Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. *Hepatol Res* 2008; [Epub ahead of print].

methods

Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS[S]

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Abstract **We describe a highly sensitive and specific method for the quantification of key regulatory oxysterols in biological samples. This method is based upon a stable isotope dilution technique by liquid chromatography-tandem mass spectrometry (LC-MS/MS). After alkaline hydrolysis of human serum (5 μl) or rat liver microsomes (1 mg protein), oxysterols were extracted, derivatized into picolinyl esters, and analyzed by LC-MS/MS using the electrospray ionization mode. The detection limits of the picolinyl esters of 4β-hydroxycholesterol, 7α-hydroxycholesterol, 22R-hydroxycholesterol, 24S-hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol were 2–10 fg (5–25 amol) on-column (signal-to-noise ratio = 3). Reproducibilities and recoveries of these oxysterols were validated according to one-way layout and polynomial equation, respectively. The variances between sample preparations and between measurements by this method were calculated to be 1.8% to 12.7% and 2.9% to 11.9%, respectively. The recovery experiments were performed using rat liver microsomes spiked with 0.05 ng to 12 ng of oxysterols, and recoveries of the oxysterols ranged from 86.7% to 107.3%, with a mean recovery of 100.6%. This method provides reproducible and reliable results for the quantification of oxysterols in small amounts of biological samples.—** Honda, A., K. Yamashita, T. Hara, T. Ikegami, T. Miyazaki, M. Shirai, G. Xu, M. Numazawa, and Y. Matsuzaki. **Highly sensitive quantification of key regulatory oxysterols in biological samples by LC-ESI-MS/MS.** ***J. Lipid Res.*** **2009.** 50: **350–357.**

Supplementary key words liquid chromatography-tandem mass spectrometry • electrospray ionization • 24S,25-epoxycholesterol • 4β-hydroxycholesterol • 7α-hydroxycholesterol • 22R-hydroxycholesterol • 24S-hydroxycholesterol • 25-hydroxycholesterol • 27-hydroxycholesterol

Biological samples contain a large number of oxysterols (1), and most of them are formed from cholesterol by enzymatic oxidation (2–6) (**Fig. 1**) or autoxidation (7). By contrast, the oxysterol 24S,25-epoxycholesterol is not derived from cholesterol but is produced de novo from acetyl-CoA via a shunt in the mevalonate pathway (8).

These oxysterols are important molecules for preserving lipid homeostasis in the body. 7α-Hydroxycholesterol is a product of CYP7A1, which is the rate-limiting enzyme in the classic bile acid biosynthetic pathway. 27-Hydroxycholesterol, 24S-hydroxycholesterol, 4β-hydroxycholesterol, 22R-hydroxycholesterol, and 24S,25-epoxycholesterol are effective endogenous ligands of the nuclear receptors liver X receptor α (LXRα) and LXRβ (9–11). In addition, 27-hydroxycholesterol (12), 25-hydroxycholesterol (13), and 24S,25-epoxycholesterol (14) are known to downregulate the cholesterol biosynthetic pathway, presumably by blocking the processing of the sterol-regulatory element binding protein.

GC-MS has historically been used for the analyses of oxysterols in serum and tissues (1, 15) because the sensitivity and specificity of conventional GC with flame ionization detector is not sufficient to quantify oxysterols in biological samples. However, GC-MS is still not an ideal method, especially for the analysis of 24S,25-epoxycholesterol, because this epoxycholesterol does not survive the temperature required for GC analysis (16). Another approach to quantifying oxysterols in biological samples was HPLC with ultraviolet (UV) detection after derivatization to the Δ^4-3-ketones (16–19). This method made it possible to detect

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Abbreviations: CTX, cerebrotendinous xanthomathosis; ESI, electrospray ionization; LC-APCI-MS, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry; LC-MS/MS, liquid chromatography-tandem mass spectrometry; LXRα, liver X receptor α; SRM, selected reaction monitoring; TMS, trimethylsilyl.

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[S]The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of three tables.

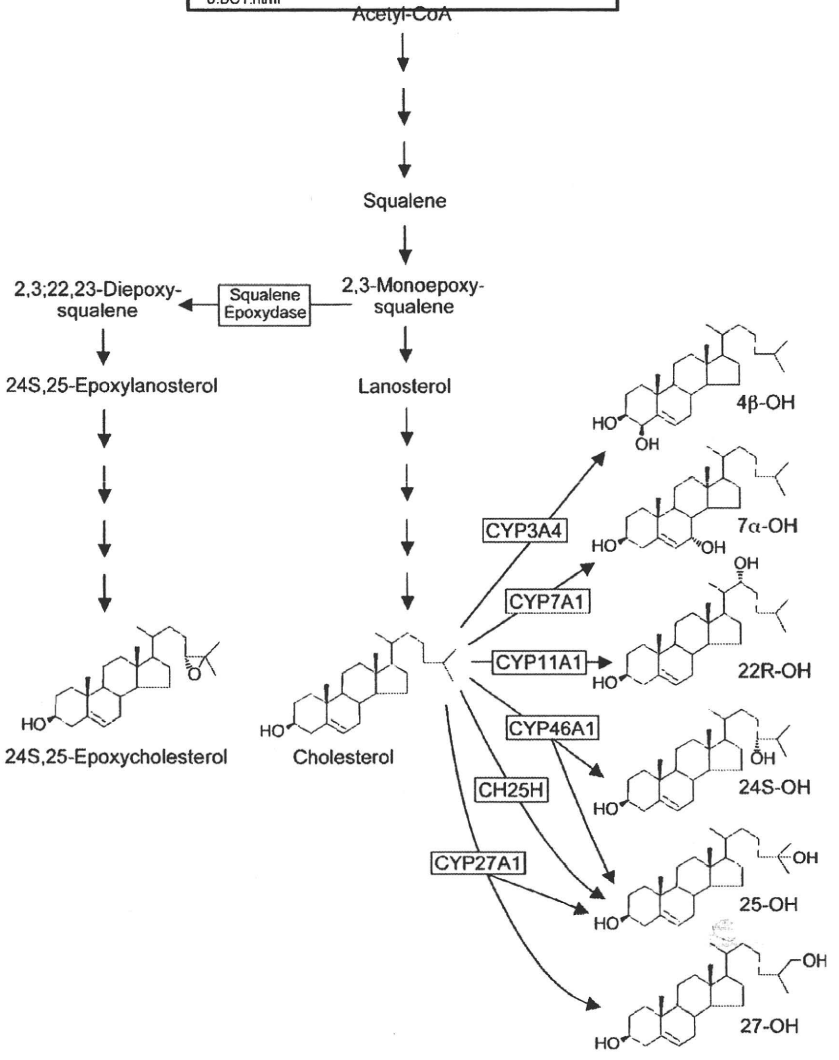

Fig. 1. Biosynthetic pathways for key regulatory oxysterols. Hydroxycholesterols are synthesized from cholesterol, whereas 24S,25-epoxycholesterol is derived from a shunt in the cholesterol biosynthetic pathway. CH25H, cholesterol 25-hydroxylase; 4β-OH, 4β-hydroxycholesterol; 7α-OH, 7α-hydroxycholesterol; 22R-OH, 22R-hydroxycholesterol; 24S-OH, 24S-hydroxycholesterol; 25-OH, 25-hydroxycholesterol; and 27-OH, 27-hydroxycholesterol.

the 24S,25-epoxycholesterol derivative as an intact form, but the lower limit of detection for the Δ^4-3-ketones of oxysterols was about 2 ng on-column (16), which was not sufficient for quantification of the oxysterols in a small amount of biological sample.

Recently, liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS) was introduced as a sensitive, specific, and rapid method for the quantification of oxysterols (20, 21). In addition, LC-tandem mass spectrometry (LC-MS/MS) using electrospray ionization (ESI) has also been applied to the analysis of oxysterols (22). In general, ESI is not the best ionization method for neutral steroids because of its poor ionization efficiency. However, our recent study demonstrated that the derivatization of monohydroxysterols into picolinyl esters markedly enhanced the ionization efficiency in the ESI process, and the method was much more sensitive than the assay of native monohydroxysterols by LC-APCI-MS/MS (23). In this study, we have applied our derivatization method to dihydroxy- and epoxysterols. In each case, singly charged ions were observed as the base peaks in the positive ESI mass spectra and amol levels of these oxysterols were detectable.

MATERIALS AND METHODS

Chemicals

4β-Hydroxycholesterol (cholest-5-en-3β,4β-diol), 7α-hydroxycholesterol (cholest-5-en-3β,7α-diol), 22R-hydroxycholesterol (cholest-5-en-3β,22R-diol), 24S-hydroxycholesterol (cholest-5-en-3β,24S-diol), 25-hydroxycholesterol (cholest-5-en-3β,25-diol),

and 24S,25-epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-ol) were purchased from Steraloids (Wilton, NH). [25,26,26,26,27,27,27-$^2H_7$]4β-hydroxycholesterol, [26,26,26,27,27,27-$^2H_6$]24-hydroxycholesterol, [27,27,27-$^2H_3$]25-hydroxycholesterol, and [26,26,26,27,27,27-$^2H_6$]24,25-epoxycholesterol were obtained from Avanti Polar Lipids (Alabaster, AL). 27-Hydroxycholesterol [(25R)-cholest-5-en-3β,26-diol], [25,26,26,26,27,27,27-$^2H_7$]27-hydroxycholesterol, and [25,26,26,26,27,27,27-$^2H_7$]7α-hydroxycholesterol were prepared as described previously (24).

Picolinic acid and 2-methyl-6-nitrobenzoic anhydride were purchased from Tokyo Kasei Kogyo (Tokyo, Japan), and 4-dimethylaminopyridine and triethylamine were obtained from Wako Pure Chemical Industries (Osaka, Japan). Additional reagents and solvents were of analytical grade.

Sample collection

Blood samples were collected from healthy human volunteers and from a patient with cerebrotendinous xanthomatosis (CTX). After coagulation and centrifugation at 1,500 *g* for 10 min, serum samples were stored at −20°C until analysis. Informed consent was obtained from all subjects, and the experimental procedures were conducted in accordance with the ethical standards of the Helsinki Declaration. Rat liver microsomes were prepared in our previous study (25) and had been stored at −70°C until they were used in the present experiments.

Sample preparation

[$^2H_7$]4β-hydroxycholesterol (5 ng), [$^2H_7$]7α-hydroxycholesterol (10 ng) [$^2H_6$]24-hydroxycholesterol (5 ng), [$^2H_3$]25-hydroxycholesterol (1 ng), [$^2H_7$]27-hydroxycholesterol (10 ng), and [$^2H_6$]24,25-epoxycholesterol (1 ng) as internal standards and 5 μg of butylated hydroxytoluene were added to serum (5 μl) or microsomes (1 mg protein), and saponification was carried out in 0.5 ml of 1 N ethanolic KOH at 37°C for 1 h. After the addition of 0.25 ml of distilled water, sterols were extracted twice with 1 ml of *n*-hexane, and the extract was evaporated to dryness under a stream of nitrogen. Derivatization to the picolinyl ester was performed according to our previous method (23) with minor modifications. The reagent mixture for derivatization consisted of 2-methyl-6-nitrobenzoic anhydride (100 mg), 4-dimethylaminopyridine (30 mg), picolinic acid (80 mg), pyridine (1.5 ml), and triethylamine (200 μl). The freshly prepared reagent mixture (170 μl) was added to the sterol extract, and the reaction mixture was incubated at 80°C for 60 min. After the addition of 1 ml of *n*-hexane, the mixture was vortexed for 30 s and centrifuged at 700 *g* for 3 min. The clear supernatant was collected and evaporated at 80°C under nitrogen. The residue was redissolved in 50 μl of acetonitrile, and an aliquot (1 μl) was injected into the following LC-MS/MS system.

LC-MS/MS analysis

The LC-MS/MS system consisted of a TSQ Quantum Ultra quadrupole mass spectrometer (Thermo Fisher Scientific, San Jose, CA) equipped with an H-ESI probe and a Nanospace SI-2 HPLC system (Shiseido, Tokyo, Japan). Chromatographic separation was performed using a Hypersil GOLD column (150 × 2.1 mm, 3 μm, Thermo Electron) at 40°C, and the following gradient system was used at a flow rate of 300 μl/min: initially, the mobile phase was composed of acetonitrile-methanol-water (40:40:20, v/v/v) containing 0.1% acetic acid; then it was programmed in a linear manner to acetonitrile-methanol-water (45:45:10, v/v/v) containing 0.1% acetic acid over 20 min. The final mobile phase was kept constant for an additional 20 min.

The general LC-MS/MS conditions were as follows: spray voltage, 1,000 V; vaporizer temperature, 350°C; sheath gas (nitrogen) pressure, 85 psi; auxiliary gas (nitrogen) flow, 60 arbitrary units; ion transfer capillary temperature, 350°C; collision gas (argon) pressure, 1.5 mTorr; and ion polarity, positive. Selected reaction monitoring (SRM) was conducted using the characteristic precursor-to-product ion transition under optimized collision energy, as listed in **Table 1**.

Statistics

Data are reported as the mean ± SD. Reproducibility was analyzed by one-way layout (JMP software; SAS Institute Inc., Cary, NC). Recovery was analyzed using a polynomial equation (26). Linearity of the calibration curves was analyzed by simple linear regression. Regression analysis was also used to calculate the estimated amount ±95% confidence limit in the recovery study. For all analyses, significance was accepted at the level of $P < 0.05$.

RESULTS

Selection of monitoring ions for SRM

Seven oxysterols were converted into the corresponding picolinyl ester derivatives and positive ESI-MS, MS/MS,

TABLE 1. Positive ESI-MS, MS/MS, SRM, and HPLC data of the picolinoyl ester derivative of each oxysterol

Oxysterols (Derivatives)	MS Data $[M+Na]^+$ (Relative Intensity)	MS/MS Data[a] (Collision Energy at Maximum Intensity)	SRM Data[b] Collision Energy	SRM Data[b] Precursor to Product	SRM Data[b] S/N[c]	HPLC Data[b] (RRT[d])
	m/z (%)	*m/z* (*V*)	*V*	*m/z*		
4β-Hydroxycholesterol (cholest-5-en-3β,4β-dipicolinates)	635 (100)	146 (22) 512 (20)	22	635 → 146	200	0.77
7α-Hydroxycholesterol (cholest-5-en-3β,7α-dipicolinates)	635 (100)	146 (15) —[e]	15	635 → 146	200	0.62
22R-Hydroxycholesterol (cholest-5-en-3β,22R-dipicolinates)	635 (100)	146 (26) 512 (22)	22	635 → 512	40	0.45
24S-Hydroxycholesterol (cholest-5-en-3β,24S-dipicolinates)	635 (100)	512 (22) 146 (31)	22	635 → 512	80	0.48
25-Hydroxycholesterol (cholest-5-en-3β,25-dipicolinates)	635 (100)	512 (19) 146 (28)	22	635 → 512	40	0.51
27-Hydroxycholesterol (cholest-5-en-3β,27-dipicolinates)	635 (100)	512 (12) 146 (33)	22	635 → 512	80	0.56
24S,25-Epoxycholesterol (cholest-5-en-24S,25-epoxy-3β-picolinate)	528 (100)	146 (20) —[e]	20	528 → 146	80	0.41

ESI, electrospray ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RRT, relative retention time; S/N, signal-to-noise ratio; SRM, selected reaction monitoring.

[a] $[M+Na]^+$ was used as a precursor ion for each MS/MS analysis. Major product ions were arranged in the order of abundance from left to right.

[b] The same HPLC column and flow rate described in Materials and Methods were employed.

[c] S/Ns were determined by injecting 100 fg of each derivative.

[d] RRTs are expressed relative to the retention time of cholesterol 3β-picolinate.

[e] Intense ion (>5% of base peak) was not observed.

SRM, and HPLC data were obtained for each of them (Table 1). All picolinyl ester derivatives exhibited $[M+Na]^+$ ions as the base peaks. The fragmentation pattern of the base peak ion of each derivative was examined under various levels of collision energy, and [M+Na−picolinic acid $(C_6H_5NO_2)]^+$ (m/z = 512) or [picolinic acid $(C_6H_5NO_2)$ + $Na]^+$ (m/z = 146) ions were observed as the most-abundant product ions, so that they were selected as monitoring ions for authentic oxysterols by SRM. The monitoring ions and optimal collision energies for deuterated internal standards were m/z 642 → 146 (22 V) for 3β,4β-dipicolinates of [$^2H_7$] 4β-hydroxycholesterol, m/z 642 → 146 (15 V) for 3β,7α-dipicolinates of [$^2H_7$]7α-hydroxycholesterol, m/z 641 → 518 (22 V) for 3β,24-dipicolinates of [$^2H_6$]24-hydroxycholesterol, m/z 638 → 515 (22 V) for 3β,25-dipicolinates of [$^2H_3$]25-hydroxycholesterol, m/z 642 → 519 (22 V) for 3β,27-dipicolinates of [$^2H_7$]27-hydroxycholesterol, and m/z 534 → 146 (20 V) for 3β-picolinate of [$^2H_6$]24,25-epoxycholesterol.

Sensitivity of the present method

To determine the sensitivity of our SRM method, the standard mixture solution of the seven oxysterol derivatives was diluted and injected into the LC-MS/MS system. The limit of detection (signal-to-noise ratio of 3) of each steroid was 2 fg (5 amol) on-column for 4β-hydroxycholesterol and 7α-hydroxycholesterol, 5 fg (12.5 amol) on-column for 24S-hydroxycholesterol, 27-hydroxycholesterol, and 24S,25-epoxycholesterol, and 10 fg (25 amol) on-column for 22R-hydroxycholesterol and 25-hydroxycholesterol.

Calibration curves

A calibration plot was established for each oxysterol. Different amounts of authentic oxysterol were mixed with deuterated internal standard, derivatized to the picolinyl ester, and quantified as described in the Materials and Methods. The weight ratio of each oxysterol, relative to the corresponding deuterated internal standard, was plotted on the abscissa, and the peak area ratio of the picolinyl ester of the authentic oxysterol to the deuterated variant measured by SRM was plotted on the ordinate. Because deuterium-labeled 22R-hydroxycholesterol was not available, [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol. The linearity of the standard curves, as determined by simple linear regression, was excellent, as shown in **Table 2.**

Representative SRM

The separation of various authentic oxysterol picolinates by SRM is shown in **Fig. 2A.** All oxysterol picolinates tested were successfully separated. 7β-Hydroxycholesterol, an autoxidation product of cholesterol, gave a peak just before 7α-hydroxycholesterol (not shown in the figure), and the retention times (relative to cholesterol) of these oxysterols (as picolinates) were 0.61 and 0.62, respectively. Figure 2B–D shows typical SRM chromatograms obtained from 1 mg of protein from rat liver microsomes (Fig. 2B) and 5 μl of sera from a control subject (Fig. 2C) and a CTX patient (Fig. 2D). In rat liver microsomes, a significant amount of 24S,25-epoxycholesterol was detected, whereas only a trace amount of 24S-hydroxycholesterol was observed. In contrast, human serum contained a very low concentration of 24S,25-epoxycholesterol, but a significant amount of 24S-hydroxycholesterol was present. When serum oxysterol profiles were compared between controls and CTX, markedly reduced serum 25- and 27-hydroxycholesterol concentrations were observed.

Precision and accuracy of the present method

The following studies were performed to determine the precision and accuracy of the present method using rat liver microsomes. Reproducibility was investigated by analyzing four samples in triplicate by LC-MS/MS (**Table 3**). The results were analyzed by a one-way layout, in which the analytical errors were divided into two sources: sample preparation and SRM measurement. The variances were not considered to be attributable to the sample preparation, because the errors during sample preparation were not significantly larger than those between the measurements (see supplementary Tables I, II). The inter-assay coefficients of variation for the between- and within-sample variations were 1.8% to 12.7% and 2.9% to 11.9%, respectively.

For the recovery experiment, known amounts of oxysterols (a, 2a, 3a; a = 0.05–4.0 ng) were spiked into 1 mg of rat liver microsomal protein (n = 2). After alkaline hydrolysis and derivatization, LC-MS/MS was carried out in triplicate for each sample. The recoveries of the known spiked amounts of the oxysterols ranged from 86.7% to 107.3%, with a mean of 100.6% (**Table 4**). In addition, the amounts of each endogenous oxysterol found in 1 mg of unspiked microsomal protein were within the 95% confidence limit for the estimated amount of each

TABLE 2. Linearities of calibration plots for each oxysterol

Oxysterol	Range (n)	Linear Regression Equation[a]	Correlation Coefficient (r)
	ng		
4β-Hydroxycholesterol	0.05 – 10 (7)	$Y = 0.436X - 0.009$	0.999
7α-Hydroxycholesterol	0.1 – 20 (7)	$Y = 1.075X - 0.011$	1.000
22R-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.084X - 0.000$	0.993
24S-Hydroxycholesterol	0.05 – 5 (6)	$Y = 0.615X - 0.010$	0.996
25-Hydroxycholesterol	0.01 – 1 (6)	$Y = 0.935X - 0.007$	1.000
27-Hydroxycholesterol	0.1 – 10 (6)	$Y = 1.400X - 0.020$	0.998
24S,25-Epoxycholesterol	0.01 – 2 (7)	$Y = 0.444X - 0.004$	0.998

[a] X is the weight ratio of each oxysterol to the corresponding deuterated internal standard, and Y is the peak area ratio calculated as the peak area of the oxysterol-picolinate (s) divided by that of deuterated oxysterol-picolinate(s) (internal standard). [$^2H_6$]24-hydroxycholesterol was used as an internal standard for 22R-hydroxycholesterol.