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A monoclonal antibody-based enzyme-linked immunosorbent assay of ursodeoxycholic acid 3-sulfates in human urine

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Abstract

Sulfation of the 3-hydroxy group is assumed to be a major metabolic route of ursodeoxycholic acid (UDCA) which is used for treating various hepatobiliary diseases. We have developed a sensitive enzyme-linked immunosorbent assay (ELISA) for determining the total amount of nonamidated, glycine- and taurine-amidated ursodeoxycholic acid 3-sulfates (UDCA 3-Suls) using a newly established monoclonal antibody. In this study, 12 kinds of antibody-secreting hybridoma clones were generated by a fusion experiment between P3/NS1/1-Ag4-1 myeloma cells and the spleen cells from a BALB/c or an A/J mouse which had been immunized with a conjugate of nonamidated UDCA 3-Sul and bovine serum albumin. One of the monoclonal antibodies, Ba-10 (γ2a, κ), had suitable binding properties for clinical application, which was group-specific to the UDCA 3-Suls, and showed negligible cross-reactivities with various related bile acids including potentially interfering compounds, namely, the unconjugated UDCA, UDCA 7-*N*-acetylglucosaminide, the 3-sulfates of cholic acid, chenodeoxycholic acid and deoxycholic acid. The antibody Ba-10 allowed us to develop a sensitive competitive ELISA system whose measurable range was approximately 2–200 pg per assay. A serial dilution study indicated that the ELISA enables the direct measurement of the UDCA 3-Suls in human urine before and after the administration of exogenous UDCA. The daily urinary excretion rate of UDCA 3-Suls from healthy male volunteers ($n = 5$) was determined to be a mean of 131 ± 61.2 (SD) μg as the nonamidated UDCA 3-Sul equivalent.

1. Introduction

Ursodeoxycholic acid (UDCA) is a minor component of endogenous bile acids which has been clinically used for the treatment of patients with various hepatobiliary diseases. It is well established that the oral administration of UDCA leads to a significant improvement in liver enzymes, and is particularly effective for the treatment of primary biliary cirrhosis (PBC) [1] which is considered to be a result of alterations in the immune function. Recent studies have shown that the UDCA administration reduces extraordinarily expressed major histocompatibility complex class I molecules [2], which may be concerned with the action mechanism of PBC. Furthermore, there has been a renewal of interest in UDCA therapy, because it has been shown to be effective for the treatment of chronic active hepatitis [3] and primary sclerosing cholangitis (PSC) [4]. Moreover, UDCA has been shown to reduce the incidence of colonic tumors in animal models [5], and consequently, is being investigated as a tumor-suppressive agent [6].

The majority of the endogenous bile acid is amidated with glycine or taurine at its carboxy group including the C-24, and in hepatobiliary diseases, the proportion of further conjugated metabolites with sulfuric or glucuronic acid is supposed to significantly

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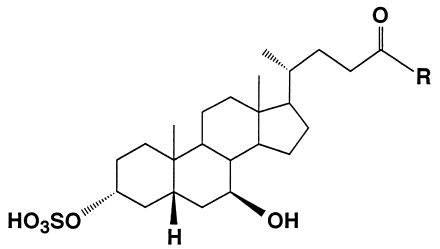

UDCA 3-Sul : R = OH
GUDCA 3-Sul : R = $NHCH_2COOH$
TUDCA 3-Sul : R = $NH(CH_2)_2SO_3H$
Hapten-BSA conjugate : R = NH-BSA

Fig. 1. Structures of 3-Suls of UDCA, GUDCA, TUDCA, and the hapten-BSA conjugate used for immunization.

increase. UDCA sulfates (UDCA Suls[1]) are considered to be a major urinary metabolite of orally administered UDCA both in healthy subjects and in patients with cholestatic and chronic liver disease [7–10], among which the main component is suggested to be UDCA 3-sulfates [11] (UDCA 3-Suls[1]; Fig. 1). It is expected that further studies may reveal that the UDCA 3-Sul levels in biological fluids are available as a useful index for diagnosing a particular disease.

On the other hand, another point of interest in these sulfated UDCA metabolites has arisen, because UDCA Suls have recently been shown to reduce lipid secretion and have greater choleretic activity than UDCA itself. These results suggest that the sulfated metabolites may be more useful for treating hepatobiliary diseases. Furthermore, it is expected that the sulfate conjugation helps the site-specific delivery of UDCA to the colon when one attempts its application to colon tumors (see above), thus protecting the molecule from bacterial degradation and inhibiting its intestinal absorption [6].

A simple, sensitive, specific, and robust assay system of the major metabolite, UDCA 3-Suls, will be helpful not only for monitoring these metabolites as a novel diagnostic marker, but also for evaluating the usefulness of UDCA Suls as a therapeutic agent. The enzyme-linked immunosorbent assay (ELISA) based on a monoclonal antibody with high affinity and specificity is desirable for this purpose. This paper deals with the production and characterization of a monoclonal antibody which group–specifically recognizes UDCA 3-Suls and its application to an ELISA system which enables the direct measurement of the total amount of these sulfates in human urine.

[1] The abbreviations UDCA (3-) Suls and UDCA (7-) NAGs are used as generic terms for the (3-) sulfates and (7-) *N*-acetylglucosaminides of nonamidated, glycine- and taurine-amidated UDCAs, respectively.

2. Experimental

2.1. Materials

UDCA was supplied from Tokyo Tanabe (Tokyo). The other unconjugated bile acids were purchased from Nacalai Tesque (Kyoto). The glycine- and taurine-amidated bile acids as well as the conjugated bile acids with glucuronic acid, sulfuric acid or *N*-acetylglucosamine (NAG) were those previously prepared in our laboratory [12–15]. The mouse monoclonal antibody isotyping kit and horse radish peroxidase (HRP) (EC 1.11.1.7; grade I-C, 263 units per mg protein) were obtained from Amersham (Tokyo) and Toyobo (Osaka), respectively. Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) were purchased from DIFCO (Detroit, MI). AffiniPure rabbit anti-mouse IgG + IgM antibody (the second antibodies in the following ELISA) was purchased from Jackson ImmunoResearch (West Grove, PA). 96-Well EIA/RIA plates (No. 3590), cluster dishes and flasks for cell culture were purchased from Costar (Cambridge, MA). RPMI 1640 medium and fetal bovine serum were obtained from GIBCO (Grand Island, NY). Polyethylene glycol (PEG) 4000 and ORIGEN Hybridoma Cloning Factor (HCF) were purchased from Merck (Darmstadt) and IGEN (Rockville, MD), respectively. Other reagents and solvents were of analytical grade.

2.2. Mediums

Hybridoma medium, RPMI 1640 medium supplemented with 10 mM HEPES buffer (pH 7.3), 0.1 mM kanamycin sulfate, 10% (v/v) fetal bovine serum, 50 μM 2-mercaptoethanol, 2 mM L-glutamine and 1 mM sodium pyruvate; HAT medium, a hybridoma medium supplemented with 0.1 mM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine; HT medium, the same as HAT medium but does not contain aminopterin.

2.3. Buffers

Buffer A, 0.05 M NaH_2PO_4–Na_2HPO_4 (pH 7.3); buffer B, buffer A containing 0.1% gelatin and 0.9% NaCl; buffer C, buffer A containing 0.9% NaCl; buffer D, buffer C containing 0.05% (v/v) Tween 20.

2.4. Cell

The P3/NS1/1-Ag4-1 myeloma cell [16] was donated

by the Health Science Research Resources Bank (Osaka).

2.5. Immunization

The hapten UDCA 3-Sul was conjugated with bovine serum albumin via its *N*-succinimidyl ester to give an immunogenic conjugate [17], and its hapten/carrier molar ratio was determined by the titration of residual free amino groups on the conjugate using trinitrobenzenesulfonic acid [18]. Female BALB/c and A/J mice (five heads each; 7–8 weeks of age) purchased from Japan SLC (Hamamatsu) were immunized with the conjugate at approximately 3-week intervals. The conjugate (60 μg) was subcutaneously injected with an emulsion (0.2 ml) of FCA (primary immunization) or FIA (booster immunizations) and sterile saline (1:1, v/v), in the foot pads and multiple sites on the back. Seven days after the fifth booster injection, blood was collected from the retrobulbar plexus and the binding ability of the serum antibodies to UDCA 3-Sul was determined by the ELISA procedure (see below). The conjugate (60 μg) in sterile saline (0.2 ml) was then intraperitoneally injected into a selected mouse based on the binding ability, and spleen cells were prepared 3 days later.

2.6. Monoclonal antibody production

The immune spleen cells (approximately, 1×10^8 cells) and one-fifth the number of myeloma cells were fused with 40% PEG 4000 in a sterile phosphate-buffered saline containing 10% (v/v) DMSO and 0.001% poly-L-arginine·HCl solution (1 ml). The fused cells were suspended in HAT medium supplemented with 10% (v/v) HCF, and cultured in 96-well cluster dishes (100 μl/well) overnight under 5% CO_2–95% air at 37°C. After further culture for approximately 2 weeks in HAT medium, the hybridoma supernatants were submitted to the screening by the ELISA described below. The antibody-secreting hybridomas were expanded in HT medium, and then cloned by limiting dilution in the hybridoma medium supplemented with 10% (v/v) HCF. Cloned hybridomas were grown in the medium until confluence was reached, whose supernatant was used for characterization of the monoclonal antibody contained in it.

2.7. Preparation of enzyme-labeled antigen

An HRP-labeled antigen was prepared according to the reported method [19]. Briefly, a solution of HRP (2.0 mg) in buffer A (400 μl) was added to UDCA 3-Sul *N*-succinimidyl ester (synthesized according to the reported method [20]; 2 mol eq to the enzyme) in dioxane (400 μl), and the mixture was stirred at 4°C for 4 h. The solution was then dialyzed against buffer A at 4°C for 2 days, and the resulting solution was adjusted to contain 400 μg/ml of the labeled antigen in buffer B, which was stored at 4°C until use.

2.8. ELISA for screening hybridomas, characterization of monoclonal antibodies, and measurement of human urine specimens

A solution of the second antibody diluted at 1:400 with buffer A (100 μl) was distributed in each well of the EIA/RIA plates, which were left overnight at 4°C. After washing three times with buffer C, the wells were blocked with a 5% skimmed milk solution in buffer C (200 μl) at 37°C for 1 h. The wells were washed three times with buffer D to which suitably diluted hybridoma supernatants or monoclonal antibody solutions diluted with buffer B (100 μl) were then added. After incubation at room temperature for 1 h, the solutions were aspirated off and the wells were washed three times with buffer D. The enzyme-labeled antigen (4 ng) dissolved in buffer B (100 μl) and standard bile acid solutions (or human urine specimens) diluted with 50% ethanol (50 μl) were then added, mixed and incubated as described above. After washing in the same manner, a citrate–phosphate buffer (pH 5.0) containing 0.04% *o*-phenylenediamine·2HCl and 0.018% H_2O_2 was distributed (100 μl) and the plates were incubated at room temperature for 30 min. This enzymic reaction was terminated by the addition of 1 M H_2SO_4 (100 μl), and the absorbance at 492 nm was measured using a MPR A4i microplate reader (Tosoh, Tokyo).

3. Results

3.1. Cell fusion and monoclonal antibody production

To establish a simple assay method for monitoring the sulfation of UDCA, it is advantageous to measure the total amount of the 3-sulfates of UDCA, irrespective of the amidation status at the carboxyl group on the side chain. For this reason, a hapten-carrier conjugate in which nonamidated UDCA 3-Sul molecules were directly linked with bovine serum albumin via their own carboxyl group (hapten/carrier molar ratio 10) was used as an immunogen (Fig. 1). Although BALB/c mice are the commonest spleen donor in the cell fusion, we also immunized A/J mice with the same schedule and the titer of the anti-UDCA 3-Sul antibody in the serum was compared using ELISA after the fifth booster immunization; this was intended to enlarge the opportunity for generating a desirable monoclonal antibody (discussed in our previous paper [20]). No significant difference was observed in the titer

of these strains, thus we compared the inhibition of bound enzymic activity due to the addition of the nonamidated UDCA 3-Sul to assume average affinity of the serum antibodies (data not shown). An animal giving the largest inhibition value was selected from the BALB/c and A/J groups (''BALB/c-4'' and ''A/J-2'' mice) as a spleen donor, and two fusion experiments were performed using the P3/NS1/1-Ag4-1 myeloma cells. About 10 days after the fusion, hybridomas were observed in over 95% of the microwells. The screening by the ELISA, examining binding ability to the enzyme-labeled hapten, demonstrated that 30 (derived from the BALB/c spleen cells) and 47 (derived from the A/J spleen cells) kinds of hybridoma microcultures in which an antibody against UDCA 3-Suls was obviously secreted. Hybridomas were further selected based on the titer of their culture supernatant and the inhibition experiment. Cloning of these hybridomas by limiting dilution resulted in 12 kinds of antibody-secreting hybridoma clones (seven kinds from the BALB/c-fusion, and five kinds from the A/J-fusion) each derived from different microwells in the initial cluster dishes (Table 1). The monoclonal antibodies used below were those secreted in the culture supernatants of these cloned hybridomes.

3.2. Characterization of monoclonal antibodies

Isotypes of the heavy and light chains of each monoclonal antibody were determined as shown in Table 1. It is noteworthy that the antibodies Ba-8, Ba-30, Ba-40 and Ba-44, derived from the BALB/c mouse, possessed a λ-chain which is rare among the mouse immunoglobulins. The binding characteristics of these antibodies were investigated by the competitive ELISA system described above. The optimum dilution of each antibody (hybridoma supernatant) in this ELISA was arbitrarily determined as the dilution rate which binds the enzymic activity corresponding to 0.6–0.8 absorption unit by a 1 h enzymic reaction.

Table 1
Immunochemical and binding properties of monoclonal antibodies

Antibody	Titer	Midpoint (pg/assay)	Isotype
Aj-5	1:1500	18	γ2a, κ
Aj-31	1:900	17	γ2a, κ
Aj-61	1:600	11	γ2b, κ
Aj-64	1:300	80	γ2b, κ
Aj-68	1:1500	30	γ2a, κ
Ba-8	1:2000	15	γ1, λ
Ba-10	1:1000	17	γ2a, κ
Ba-13	1:500	38	γ2a, κ
Ba-30	1:50	40	γ1, λ
Ba-33	1:1000	25	γ2a, κ
Ba-40	1:2000	16	γ2a, λ
Ba-44	1:1000	15	γ1, λ

All the antibodies afforded a dose-response curve for the nonamidated UDCA 3-Sul with a practical sensitivity, as shown by the midpoint value (<100 pg), which is the amount of antigen required to inhibit the bound enzymic activity by half. Antibodies Aj-5, Aj-31, Aj-61 (derived from A/J mouse spleen cells), Ba-8, Ba-10, Ba-40 and Ba-44 (from BALB/c mouse) provided extremely sensitive dose-response curves whose midpoint was less than 20 pg per assay. The cross-reactivities with four bile acids were then determined by the 50% displacement method [21] to evaluate the applicability of these antibodies to biological specimens (Table 2). As we initially expected, almost all the antibodies exhibited very large cross-reactivities with 3-sulfates of glycine-amidated UDCA (GUDCA; $>50\%$ except for Aj-61, Ba-30 and Ba-44) and taurine-amidated UDCA (TUDCA 3-Sul; $>50\%$ except for Ba-8 and Ba-40); this is a desirable feature for the group-specific determination of the three 3-sulfated UDCAs. The antibodies Aj-31 and Aj-68 exhibited several fold higher reactivities to TUDCA 3-Sul than the nonamidated 3-sulfate. Cross-reactivity with chenodeoxycholic acid 3-sulfate (CDCA 3-Sul) is one of the crucial factors determining whether an antibody is practical or not, because the urinary concentration of this sulfate has been reported to be extremely high among the various bile acid metabolites [7,9]. Although the five antibodies derived from the A/J mouse exhibited significantly high cross-reactivities with CDCA 3-Sul (37–360%), the seven antibodies from the BALB/c mouse well discriminated this metabolite, among which the antibodies Ba-10 and Ba-13 showed negligibly low cross-reactivities ($<0.1\%$). This difference between the hybridomas from the A/J-2 mouse and those from the BALB/c-4 mouse was compatible with the nature of the antibodies in the serum which had been collected from these mice before the fusion experiment; the cross-reactivities with CDCA 3-Sul were 150% (A/J) and 0.1% (BALB/c), respectively. The BALB/c-derived monoclonal antibodies also showed lower cross-reactivities ($<0.2\%$) with unconjugated UDCA than the A/J-antibodies (maximum, 5.6%).

These results demonstrated that Ba-10 is the most promising antibody among the 12 monoclonal antibodies generated here, because it afforded an excellent sensitivity in the ELISA system, satisfactory group-specificity recognizing the 3-sulfates of GUDCA and TUDCA as well as the nonamidated sulfate, and essentially no cross-reactivity with CDCA 3-Sul and UDCA. Thus, the cross-reactivities of the antibody Ba-10 with five additional bile acid conjugates were tested. UDCA 3-glucuronide and UDCA 7-NAG, both of which are contained in human urine specimens

Table 2
Percent cross-reaction of monoclonal antibodies[a,b]

Antibody	UDCA 3-Sul	CDCA 3-Sul	GUDCA 3-Sul	TUDCA 3-Sul	UDCA
Aj-5	100	360	70	200	< 0.02
Aj-31	100	68	100	430	2.1
Aj-61	100	37	34	210	5.6
Aj-64	100	80	67	270	2.7
Aj-68	100	75	160	750	4.3
Ba-8	100	0.02	100	40	< 0.06
Ba-10	100	< 0.03	170	50	< 0.01
Ba-13	100	< 0.06	250	71	< 0.03
Ba-30	100	2.1	43	120	< 0.17
Ba-33	100	0.50	160	70	< 0.03
Ba-40	100	0.20	200	42	< 0.02
Ba-44	100	0.60	21	150	< 0.01

[a] Calculated by 50% displacement method [21].
[b] Cross-reactivities of the antibody Ba-10 with five additional compounds are described in the text.

and thus may interfere with the assay of the 3-sulfates, were well discriminated (< 0.03 and < 0.08%, respectively). Negligibly low cross-reactivities were found with UDCA 7-Sul (0.04%), deoxycholic acid (DCA) 3-Sul (0.02%) and cholic acid (CA) 3-Sul (< 0.08%), which indicate the antibody strictly recognizes not only the position of the sulfuric acid moiety but also modifications on the steroid nuclei.

The antibody Ba-10 afforded highly sensitive dose-response curves for the 3-Suls of GUDCA and TUDCA as well as the nonamidated UDCA, which are similar to one another (Fig. 2). The practical measurable range on these standard curves was approximately 2–200 pg per assay, thus obviously more sensitive than the reported immunoassays of the bile acid conjugates [22].

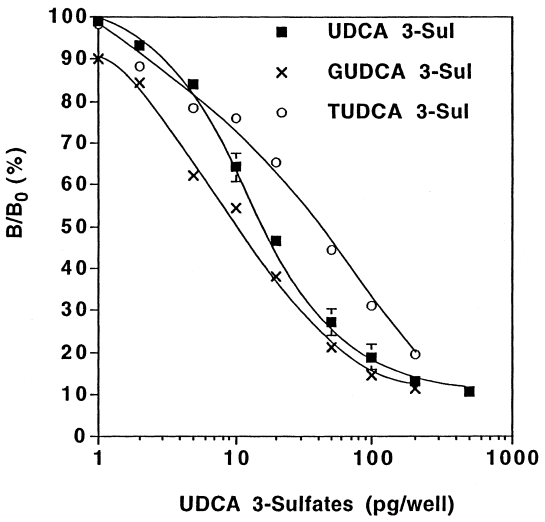

Fig. 2. Dose-response curves for 3-Suls of UDCA (■), GUDCA (×), and TUDCA (○) in the ELISA using the monoclonal antibody Ba-10.

3.3. Measurement of UDCA 3-Suls in human urine

The urine UDCA 3-Suls levels of three healthy male volunteers, who had not taken exogenous UDCA, were determined by the ELISA using the antibody Ba-10, after serial dilution of the urine specimens (Table 3). Good parallelism was observed between the assay values and the dilution ratio, indicating that this assay system is valid for determining the urine levels without complicated pretreatment of the specimens. The mean daily urinary excretion ratio of the UDCA 3-Suls of five male volunteers (without administration of UDCA) was 131 ± 61.2 (SD) μg (nonamidated UDCA 3-Sul equivalent), which was even lower than the excretion ratio of UDCA 7-NAGs of the same volunteers (287.2 ± 171.4 μg; GUDCA 7-NAG equivalent) determined simultaneously by a specific ELISA which had been established in our laboratory [20] (Fig. 3). The time course of the urinary excretion UDCA 3-Suls during daily administration of UDCA was then monitored by the ELISA (Fig. 4). Before administration, the UDCA 3-Suls levels in the first urine were 0.21

Table 3
Urine levels of UDCA 3-Suls obtained by ELISA using the antibody Ba-10 after serial dilution (μg/day)[a]

Urine dilution[b]	Subject		
	A	B	C
1:25	149.5 ± 21.2	70.0 ± 3.6	79.8 ± 6.1
1:50	143.0 ± 11.2	70.0 ± 1.5	71.5 ± 8.3
1:100	130.0 ± 15.0	76.0 ± 7.8	85.8 ± 4.0
1:200	156.0 ± 16.2	80.0 ± 12.1	92.4 ± 8.4

[a] Calculated as nonamidated UDCA 3-Sul equivalent (Mean ± SD; $n = 4$).
[b] Urine specimens were serially diluted with 50% ethanol, and subjected to the ELISA.

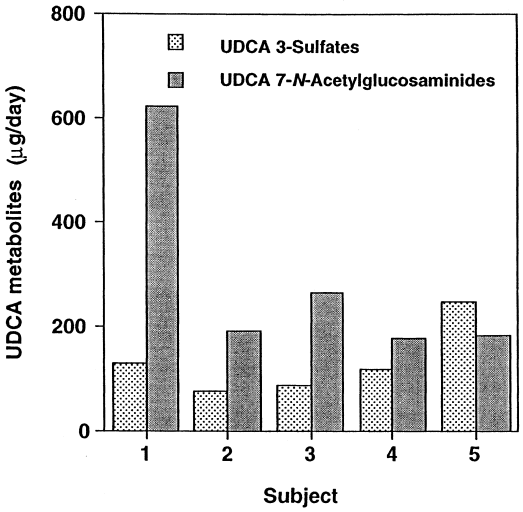

Fig. 3. Daily urinary excretion of UDCA 3-Suls (dotted bars) and 7-NAGs (shaded bars) in five healthy male volunteers.

and 0.076 μg/ml for the volunteers A and B, respectively, which were compatible with the reported data [23], but significantly increased after the administration of UDCA and exceeding 5 μg/ml at the fifth day. The daily urinary excretion ratios on day 5 was 6.0 and 10.2 mg for the volunteers A and B, respectively, which were significantly higher than the ratio for the volunteers not taking exogenous UDCA.

4. Discussion

Immunoassays of bile acid metabolites based on a specific monoclonal antibody should be useful as a standard diagnostic tool for various hepatobiliary diseases. The use of monoclonal antibodies produced by the hybridoma technology [24] certifies reproducibility of assay values, because these antibodies are constantly supplied with a definite binding property. However, no monoclonal antibody against a bile acid derivative has been reported, until we have recently generated an antibody which group-specifically recognizes non-amidated, glycine- and taurine-amidated UDCA 7-NAG [20]. Indeed, this monoclonal antibody enabled us to establish a simple and reliable ELISA system for human urine UDCA 7-NAGs. We expected that ELISA would be useful for diagnosing PBC, because these 7-NAG metabolites are suggested to be characteristic of the patients with this disease [25].

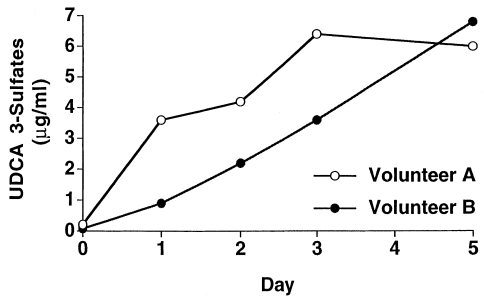

Fig. 4. Concentrations of UDCA 3-Suls in the first urine from healthy male volunteers A (○) and B (●) during daily oral administrations of UDCA. (a) Measured by the ELISA using the antibody Ba-10. (b) Volunteers took 200 mg of UDCA three times in a day (total 600 mg).

In this paper, we have succeeded in producing the monoclonal antibody Ba-10 having a useful binding property for clinical application, which is group-specific to the nonamidated and amidated UDCA 3-Suls. In course of the establishment of desirable antibody-secreting hybridomas, we found a very large difference in cross-reactivity with CDCA 3-Sul (a crucial interfering metabolite of the assay) [7,9] between the antibodies derived from the BALB/c mouse and those from the A/J mouse, and this difference was well correlated to the difference in the cross-reactivities of their serum antibodies. These results indicate that for selecting an adequate spleen donor, it would be helpful to examine not only the titer of the serum antibody against a desirable antigen, but also the cross-reactivities with some crucial interfering compounds.

The present ELISA system using the antibody Ba-10 was sensitive enough for practical use, and allowed the determination of urine UDCA 3-Suls levels even before the administration of UDCA. The minimum detectable amount was approximately 2 pg (4.2 fmol as nonamidated UDCA 3-Sul equivalent), and thus the ELISA is much more sensitive than the conventional immunoassays of various bile acids based on polyclonal antibodies [22], the majority of which exhibited a sensitivity over 10 pmol per assay. Excellent group-specificity of the antibody Ba-10 allowed us to measure the total amount of 3-sulfates of the nonamidated and amidated UDCA in human urine without any complicated sample pretreatment. A few attempts have been reported so far for determining UDCA 3-Suls in body fluids using gas chromatography (GC) [26,27], GC/mass spectrometry (MS) [28] or high-performance liquid chromatography (HPLC) [29]. Recently, a method for the separation and determination of bile acid 3-sulfates including UDCA 3-Suls by LC/electrospray ionization MS has also been developed in our laboratory [30]. Although these chromatographic techniques are advantageous for separately determining the nonamidated, glycine- and taurine-amidated forms of UDCA 3-Sul, complicated sample pretreatment procedures, i.e., solid-phase extraction of polar conjugated metabolites and their subsequent group separation using an anion exchanger are required. In gas chromatographic techniques, the solvolysis of the sulfuric acid moiety and derivatization into a volatile compound are also necessary. Moreover, none of these methods

provided sufficient sensitivity for measuring the UDCA 3-Suls in healthy volunteers before the administration of the exogenous UDCA. The present monoclonal antibody Ba-10 would also be useful for simplifying such a pretreatment, namely, an immunoaffinity extraction procedure [31], based on the antibody immobilized on a suitable solid support, would enable an efficient and feasible group-specific extraction and concentration of UDCA 3-Suls from any biological fluids.

UDCA 3-Suls have been recognized as one of the major metabolites of UDCA in urine regardless of the intake of exogenous UDCA both in healthy subjects and patients with hepatobiliary diseases [7–9,11]. On the other hand, it has been reported that significant levels of UDCA 7-NAGs in the urine have been detected only in patients with PBC under UDCA therapy; thus, it still remains unclear whether the NAG conjugates are excreted in healthy subjects or not [25,32]. In our present study, UDCA 7-NAGs could be detected even in the urine of healthy volunteers before taking UDCA, and furthermore, these concentrations were higher than those of the UDCA 3-Suls, revealing a new aspect of the metabolism of bile acids. The combined use of the present ELISA system of the UDCA 3-Suls and the ELISA of UDCA 7-NAGs would offer some helpful information not only for diagnosing hepatobiliary diseases but also for studying bile acid metabolism.

Due to the recent application of UDCA therapy to some diseases including chronic hepatitis and PSC, the opportunity of prescribing UDCA will increase. The present ELISA will be useful for re-examining the metabolic fate of UDCA, and for investigating the site-directed delivery of UDCA [6]. The ELISA could also be used to monitor the metabolic fate of the UDCA 3-Suls themselves.

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References

[1] R. Poupon, Y. Chretien, R.E. Poupon, F. Ballet, Y. Calmus, F. Darnis, Is ursodeoxycholic acid an effective treatment for primary biliary cirrhosis?, Lancet 1 (1987) 834–836.

[2] Y. Calmus, P. Gane, P. Rouger, R. Poupon, Hepatic expression of class I and class II major histocompatibility complex molecules in primary biliary cirrhosis: effect of ursodeoxycholic acid, Hepatology 11 (1990) 12–15.

[3] A. Crosignani, P.M. Battezzati, K.D.R. Setchell, M. Camisasca, E. Bertolini, A. Roda, M. Zuin, M. Podda, Effects of ursodeoxycholic acid on serum liver enzymes and bile acid metabolism in chronic active hepatitis: a dose-response study, Hepatology 13 (1991) 339–344.

[4] O. Chazouilleres, R. Poupon, J.P. Capron, E.H. Metman, D. Dhumeaux, M. Amouretti, P. Couzigou, D. Labayle, J.C. Trinchet, Ursodeoxycholic acid for primary sclerosing cholangitis, J. Hepatol. 11 (1990) 120–123.

[5] D.L. Earnest, H. Holubec, R.K. Wali, C.S. Jolley, M. Bissonette, A.K. Bhattacharyya, H. Roy, S. Khare, T.A. Brasitus, Chemoprevention of azoxymethane-induced colonic carcinogenesis by supplemental dietary ursodeoxycholic acid, Cancer. Res. 54 (1994) 5071–5074.

[6] C.M.P. Rodrigues, B.T. Kren, C.J. Steer, K.D.R. Setchell, The site-specific delivery of ursodeoxycholic acid to the rat colon by sulfate conjugation, Gastroenterol. 109 (1995) 1835–1844.

[7] P. Back, Urinary bile acids, in: K.D.R. Setchell, D. Kritchevski, P.P. Nair (Eds.), The Bile Acids: Chemistry, Physiology, and Metabolism vol. 4; Methods and Applications, Plenum Press, New York, 1988, pp. 405–440.

[8] K.D.R. Setchell, D. Watson, W.F. Balistreri, H. Yamashita, A simple, rapid and non-invasive test of compliance to oral ursodeoxycholic acid (UDCA) therapy-detection of UDCA-sulfate: a specific urinary metabolite, Hepatology 14 (1991) 261A.

[9] H.-U. Marschall, H. Matern, H. Wietholtz, B. Egestad, S. Matern, J. Sjövall, Bile acid *N*-acetylglucosaminidation. In vivo and in vitro evidence for a selective conjugation reaction of 7β-hydroxylated bile acids in humans, J. Clin. Invest. 89 (1992) 1981–1987.

[10] K. Nakamura, M. Yoneda, A. Kimura, K. Tamori, S. Yokohama, Y. Sato, T. Kato, T. Hasegawa, H. Saito, M. Aoshima, M. Fujita, I. Makino, Increase of sulfated ursodeoxycholic acid in the serum and urine of patients with chronic liver disease after ursodeoxycholic acid therapy, J. Gastroenterol. Hepatol. 11 (1996) 385–390.

[11] K. Nakamura, Investigation of sulfate group position of urinary sulfated ursodeoxycholic acid in patients during ursodeoxycholic acid administration, Acta Hepatol. Jpn. (Kanzo) 35 (1994) 814–821.

[12] J. Goto, H. Kato, F. Hasegawa, T. Nambara, Synthesis of monosulfates of unconjugated and conjugated bile acids, Chem. Pharm. Bull. 27 (1979) 1402–1411.

[13] J. Goto, H. Kato, K. Kaneko, T. Nambara, Synthesis of monosulfates of cholic acid derivatives, Chem. Pharm. Bull. 28 (1980) 3389–3394.

[14] J. Goto, K. Suzaki, T. Nambara, Synthesis of 3-glucuronides of conjugated and nonconjugated bile acids, Chem. Pharm. Bull. 28 (1980) 1258–1264.

[15] T. Niwa, T. Koshiyama, J. Goto, T. Nambara, Synthesis of *N*-acetylglucosaminides of unconjugated and conjugated bile acids, Steroids 57 (1992) 522–529.

[16] G. Köhler, S.C. Howe, C. Milstein, Fusion between immunoglobulin-secreting and nonsecreting myeloma cell lines, Eur.J. Immunol. 6 (1976) 292–295.

[17] H. Hosoda, Y. Sakai, H. Yoshida, S. Miyairi, K. Ishii, T. Nambara, The preparation of steroid *N*-hydroxysuccinimide esters and their reactivities with bovine serum albumin, Chem. Pharm. Bull. 27 (1979) 742–746.

[18] A.F.S.A. Habeeb, Determination of free amino groups in proteins by trinitrobenzenesulfonic acid, Anal. Biochem. 14 (1966) 328–336.

[19] H. Hosoda, T. Karube, N. Kobayashi, T. Nambara, Enzyme labeling of steroids by the *N*-succinimidyl ester method.

Preparation of horseradish peroxidase-labeled antigen for use in enzyme immunoassay, Chem. Pharm. Bull. 33 (1985) 249–255.

[20] N. Kobayashi, H. Oiwa, J. Goto, Production and characterization of group-specific monoclonal antibodies recognizing non-amidated, glycine- and taurine-amidated ursodeoxycholic acid 7-*N*-acetylglucosaminides, J. Steroid Biochem., Molec. Biol. 64 (1998) 171–177.

[21] G.E. Abraham, Solid-phase radioimmunoassay of estradiol-17 β, J. Clin. Endocrinol. Metab. 29 (1969) 866–870.

[22] A. Roda, E. Roda, D. Festi, C. Colombo, Immunological methods for serum bile acid analysis, in: K.D.R. Setchell, D. Kritchewvski, P.P. Nair (Eds.), The Bile Acids: Chemistry, Physiology, and Metabolism vol. 4; Methods and Applications, Plenum Press, New York, 1988, pp. 269–314.

[23] N. Yamaga, H. Kohara, Conjugation types of 7β-hydroxylated bile acids detected in healthy human urine, J. Biochem. Tokyo 116 (1994) 1123–1126.

[24] G. Köhler, C. Milstein, Continuous cultures of fused cells secreting antibody of predefined specificity, Nature 256 (1975) 495–497.

[25] A. Kimura, K. Nakamura, I. Makino, Measurement of bile acid *N*-acetylglucosaminides in serum and urine of patients with chronic liver diseases during ursodeoxycholic acid treatment, Jpn. J. Gastroenterol. 92 (1995) 224–232.

[26] I. Makino, K. Shinozaki, S. Nakagawa, K. Mashimo, Measurement of sulfated and nonsulfated bile acids in human serum and urine, J. Lipid Res. 15 (1974) 132–138.

[27] C.B. Campbell, C. McGuffie, L.W. Powell, The measurement of sulphated and non-sulphated bile acids in serum using gas-liquid chromatography, Clin. Chim. Acta 63 (1975) 249–262.

[28] N. Murata, T. Beppu, H. Takikawa, H. Otsuka, T. Kasama, Y. Seyama, Determination of sulfated and nonsulfated bile acids in serum by mass fragmentography, Steroids 42 (1983) 575–592.

[29] J. Goto, H. Kato, Y. Saruta, T. Nambara, Separation and determination of bile acid 3-sulfates in human bile by high-performance liquid chromatography, J. Chromatogr. 226 (1981) 13–24.

[30] S. Ikegawa, T. Yanagihara, N. Murao, H. Watanabe, J. Goto, T. Niwa, Separatory determination of bile acid 3-sulfates by liquid chromatography/electrospray ionization mass spectrometry, J. Mass Spectrom. 32 (1997) 401–407.

[31] S. Miyairi, H. Shimada, N. Awata, J. Goto, T. Nambara, Novel immunoaffinity extraction for liquid chromatographic determination of major metabolites of 4-acetoxy-2-(4-methylphenyl)benzothiazole in plasma, J. Pharmaceut. Biomed. Anal. 12 (1994) 389–395.

[32] H.-U. Marschall, B. Egestad, H. Matern, S. Matern, J. Sjövall, *N*-Acetylglucosaminides: a new type of bile acid conjugate in man, J. Biol. Chem. 264 (1989) 12989–12993.