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Production and Characterization of Group-specific Monoclonal Antibodies Recognizing Nonamidated, Glycine- and Taurine-amidated Ursodeoxycholic Acid 7-*N*-acetylglucosaminides

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Ursodeoxycholic acid 7-*N*-acetylglucosaminides (UDCA 7-NAGs) are novel conjugated metabolites whose urine levels are expected to be a specific diagnostic index for primary biliary cirrhosis. To obtain a specific antibody which is useful for developing immunochemical analytical methods of UDCA 7-NAGs, a variety of monoclonal antibodies have been generated. Spleen cells from an A/J mouse, which had been immunized with a conjugate of nonamidated UDCA 7-NAG and bovine serum albumin, were fused with P3/NS1/1-Ag4-1 myeloma cells. After screening by an enzyme-linked immunosorbent assay (ELISA) using a β-galactosidase-labeled antigen, thirteen kinds of antibody-secreting hybridoma clones were established. Binding properties of these monoclonal antibodies were investigated in detail by ELISA. One of these antibodies, Ab-#8 (γ_1, κ) had the most favorable characteristics for clinical application, which was group-specific to the 7-NAG conjugates of nonamidated, glycine- and taurine-amidated UDCAs providing a highly sensitive dose–response curve for each conjugate (midpoint 17 pg per assay for nonamidated UDCA 7-NAG). Cross-reactivities with eleven kinds of bile acids, including some potential interfering metabolites as UDCA 3-sulfate, were negligibly low. By using direct ELISA based on Ab-#8, daily urinary excretion rates of UDCA 7-NAGs of two healthy subjects were determined to be 1030 and 469 μg as GUDCA 7-NAG equivalent. © **1998 Elsevier Science Ltd. All rights reserved.**

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INTRODUCTION

Bile acids are biosynthesized from cholesterol in liver, followed by amidation with glycine or taurine through the carboxyl group on their side chains, before being excreted into the bile. It is well recognized that in hepatobiliary diseases, the concentrations of further conjugated bile acids with sulfuric or glucuronic acid at the 3α-hydroxyl group are significantly increased.

On the other hand, the *N*-acetylglucosaminidation of bile acids had recently been reported [1, 2], the discovery of which stimulated particular interest in the physiological significance of these novel conjugated metabolites. In our laboratory, 7-*N*-acetylglucosaminides (7-NAGs) of nonamidated, glycine- and taurine-amidated ursodeoxycholic acids (UDCA, GUDCA and TUDCA, respectively; Fig. 1) have recently been unambiguously identified in the urine of a patient with primary biliary cirrhosis (PBC) after administration of UDCA [3]. In this study, a newly developed high-performance liquid chromatography (HPLC) with fluorescence detection [4] was used. It has subsequently been demonstrated that such elevated excretion of the 7-NAG conjugates, exceeding ten mg per day, is characteristic of patients with PBC under UDCA therapy [5]. Because these findings strongly suggest that the urine levels of UDCA 7-NAGs after UDCA administration would be a useful index for screening patients with PBC, a feasible and

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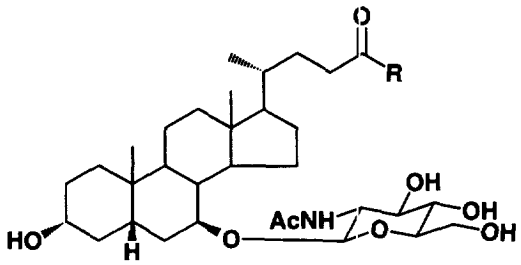
The abbreviation, UDCA 7-NAGs, is used as a generic term for these 7-NAG conjugates, of nonamidated glycine- and taurine-amidated UDCAs.



UDCA 7-NAG : R = OH
GUDCA 7-NAG : R = $NHCH_2COOH$
TUDCA 7-NAG : R = $NH(CH_2)_2SO_3H$
Hapten-BSA conjugate : R = NH-BSA

Fig. 1. Structures of 7-NAG conjugates of UDCA, GUDCA, TUDCA and the hapten-BSA conjugate used for immunization.

reliable assay method, which is suitable for routine diagnosis, is earnestly needed.

From these points of view, an enzyme immunoassay (EIA) method for determining UDCA 7-NAGs in human urine has recently been developed in our laboratory using a rabbit polyclonal antibody [6]. The use of monoclonal antibody produced by the B cell hybridoma technology [7] is, however, desirable for standardizing such an immunochemical assay to make it suitable for routine use. This is because the technique unlimitedly provides practically homogeneous antibody molecules with a definite binding property. This paper deals with the production and characterization of a series of monoclonal antibodies which group-specifically recognize these UDCA 7-NAGs.

MATERIALS AND METHODS

Materials

Cholic acid (CA), chenodeoxycholic acid (CDCA) and UDCA were purchased from Nacalai Tesque (Kyoto). All the conjugated bile acids [8–10] as well as the immunogenic conjugate of UDCA 7-NAG and bovine serum albumin (BSA) [6] were those previously prepared in our laboratory. The mouse monoclonal antibody isotyping kit and β-galactosidase (EC 3.2.1.23) from *E. coli* (600–950 u per mg protein) were obtained from Amersham (Tokyo) and Boehringer Mannheim (Tokyo), respectively. Freund's complete adjuvant (FCA) and incomplete adjuvant (FIA) were purchased from DIFCO (Detroit, MI). Rabbit anti-mouse IgG antibody and AffiniPure rabbit anti-mouse IgG + IgM antibody [the second antibodies in the following EIA and enzyme-linked immunosorbent assay (ELISA), respectively] were purchased from Wako Pure Chemical (Osaka) and Jackson ImmunoResearch (West Grove, PA). IgG sorb (a suspension of inactivated *Staphylococcus aureus*) was obtained from Enzyme Center (Malden, MA). 96-Well EIA/RIA plates (No. 3590), cluster dishes and flasks for cell cultures 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 the ORIGEN Hybridoma Cloning Factor (HCF) were purchased from Merck (Darmstadt) and IGEN (Rockville, MD), respectively. Poly-L-arginine·HCl and dimethylsulfoxide (DMSO) were obtained from Sigma Chemical (St. Louis, MO). All other reagents and solvents were of analytical grade.

Mediums

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

Buffers

Buffer A, 0.05 M NaH_2PO_4–Na_2HPO_4 (pH 7.3); buffer B, buffer A containing gelatin (0.1%), NaCl (0.9%) and NaN_3 (0.1%); buffer C, buffer A containing NaCl (0.9%) and NaN_3 (0.02%); buffer D, buffer C containing Tween 20 (0.05%, v/v).

Cell

P3/NS1/1-Ag4-1 myeloma cell [11] was donated by Health Science Research Resources Bank (Osaka).

Immunization

Female BALB/c and A/J mice (five each; 7–8 weeks of age) purchased from Japan SLC (Hamamatsu) were immunized with the BSA conjugate of UDCA 7-NAG at approximately 3-week intervals. The conjugate (50 μg) was injected with the emulsion (0.1 ml) of FCA (primary and the third booster immunization) or FIA (other booster immunizations) and sterile saline (1:1, v/v), subcutaneously in foot pads and at multiple sites on the back. Ten days after the third booster injection, blood was collected from the retrobulbar plexus, and the binding ability of the serum antibodies to UDCA 7-NAG was determined by the EIA procedure (see below). A month after the final booster immunization, the conjugate (50 μg) dissolved in a sterile saline (0.2 ml) was injected intraperitoneally, and spleen cells were prepared 3 days later.

Monoclonal antibody production

The immune spleen cells (1.4×10^8 cells) and 1/5 numbers of the myeloma cells were washed with medium A, mixed together, and then fused with PEG

4000 (40%) in a sterile phosphate-buffered saline containing DMSO (10%, v/v) and poly-L-arginine·HCl (0.001%) (1 ml). After washing with medium A, the fused cells were suspended in HAT medium supplemented with HCF (10%, v/v). This cell suspension was distributed in 96-well cluster dishes (100 μl/well), and cultured overnight under 5% CO_2–95% air at 37°C. The HAT medium (100 μl) was then added to each well, and 3 and 6 days after the fusion, a portion of the culture supernatant (approximately 100 μl) was replaced by a fresh HAT medium. After further culture for 2–3 days, a small portion of hybridoma supernatants was taken from each well and submitted to the screening by the ELISA described below. The antibody-secreting hybridomas were expanded in the HT medium, and then cloned twice by limiting dilution in medium B supplemented with HCF (10%, v/v). Cloned hybridomas were grown in 25 cm^2 culture flasks in medium B (5 ml) until confluence was reached. The cell suspension was centrifuged (1000*g* for 15 min) at 4°C, and monoclonal antibodies contained in the resulting supernatant were used for isotyping or the ELISA for investigating their binding characteristics.

Preparation of enzyme-labeled antigen

UDCA 7-NAG was converted into its *N*-succinimidyl ester according to the reported method [12]. A solution of β-galactosidase (500 μg) in buffer A (200 μl) was added to the ester (10 mol eq to the enzyme) in dioxane (200 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 d, and the resulting solution was adjusted to contain 100 μg/ml of the β-galactosidase-labeled antigen in buffer B, which was stored at 4°C until use.

EIA

Serially diluted immune serum with buffer B and the solution of enzyme-labeled antigen (100 ng) in buffer B (each 100 μl) were mixed and incubated at 4°C for 2 h. Diluted second antibody (1:200 with buffer B) (100 μl) and a 5% suspension of IgG sorb (50 μl) were then added to the mixture, and incubated at 4°C for an additional 2 h. After the addition of buffer B (1.5 ml), the mixture was centrifuged at 4°C (1000*g* for 10 min). The supernatant was aspirated off, and the pellet was washed once with buffer B (1.5 ml). The bound enzymic activity retained on the resulting precipitate was measured using *o*-nitrophenyl β-D-galactopyranoside as a substrate [13].

ELISA for screening hybridomas

A solution of the second antibody diluted 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 3 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 3 times with buffer D, to which diluted hybridoma supernatants (1:6 with buffer B) (100 μl) were then added. After incubation at room temperature for 30 min with continuous shaking, the solutions were discarded and the wells were washed 3 times with buffer D. The enzyme-labeled antigen (100 ng) dissolved in buffer B (100 μl) was then added and incubated as above. After washing in the same manner, a buffer B containing *o*-nitrophenyl β-D-galactopyranoside (0.06%), $MgCl_2$ (0.2%) and 2-mercaptoethanol (0.7%, v/v) was distributed (100 μl) and the plates were incubated at 37°C for 30 min. This enzymic reaction was terminated by the addition of 1 M Na_2CO_3 (100 μl), and the absorbance at 415 nm was measured using a MPR A4i microplate reader (Toson).

ELISA for characterizing monoclonal antibodies

The second antibody-coated 96-well microtiter plates were prepared as above. For determining the optimum dilution of the hybridoma supernatant, serially diluted supernatant with buffer B (100 μl), buffer B (100 μl), and 50% (v/v) ethanol (50 μl) were added to the wells. These mixtures were then incubated at room temperature for 1 h. After washing the wells as above, the enzyme-labeled antigen (50 ng) dissolved in buffer B (100 μl) was added and incubated at room temperature for 3 h. The wells were washed again, and the bound enzymic activity was measured as above. Dose–response curves were constructed as follows: a solution of enzyme-labeled antigen (50 ng) in buffer B (100 μl) and a series of standard UDCA 7-NAG (0–1000 pg) dissolved in 50% (v/v) ethanol (50 μl) were added to the wells which were vortex-mixed for 30 s. The hybridoma supernatant diluted with buffer B (100 μl) was added to the mixture, vortex-mixed, and incubated at room temperature for 3 h. The wells were then washed and the bound enzymic activity was measured as above (enzymic reaction = 1 h). In the cross-reaction study, the competitive antigen–antibody reaction was performed by replacing UDCA 7-NAG with various related compounds. Cross-reactivity of each analog was expressed as the relative amount required to reduce the initial bound enzymic activity by half, where the mass of UDCA 7-NAG was arbitrarily taken as 100% [14].

Measurement of UDCA 7-NAGs in human urine

Urine samples after 24 h were collected from two healthy male volunteers (A and B; 39 and 23 y old, respectively). For direct measurement, these urines were serially diluted with 50% (v/v) ethanol and the aliquots (50 μl) were submitted to ELISA using Ab-#8, according to the procedure for constructing of dose–response curves (see above). The urine levels of UDCA 7-NAGs were determined as "GUDCA 7-

NAG equivalent" using a dose–response curve for GUDCA 7-NAG. On the other hand, a portion of the urine samples (1.0 ml) was submitted to Sep-Pak C_{18} cartridges (Waters Chromatography, Millipore, Milford, MA) as described previously [3]. A fraction containing UDCA 7-NAGs was collected, and the solvent was evaporated off under a N_2 gas stream. It had been confirmed that 7-NAGs of UDCA, GUDCA and TUDCA were all quantitatively recovered through this extraction step using standard compounds. The residue was dissolved in 50% ethanol (1.0 ml), serially diluted and measured by ELISA as described above.

RESULTS

Cell fusion and monoclonal antibody production

For the purpose of diagnosing PBC, it is advantageous to measure the total amount of the 7-NAG conjugates of UDCA, irrespective of the amidation status at the carboxyl group on the side chain. For this reason, a hapten-carrier conjugate, in which UDCA 7-NAG molecules were directly linked with BSA via their own carboxyl group (hapten/BSA molar ratio 7), was used for the immunogen (Fig. 1). The opportunity for obtaining antibody-secreting hybridomas seems to be related with the strength of the humoral immune response against the target antigen which should be reflected by the serum antibody titer. Although BALB/c mice are the commonest spleen donors in cell fusion, some cases have been reported where another mice strain afforded successful results for generating the monoclonal anti-steroid antibody [15–18]. Indeed, one of the authors recently reported that A/J mice provided a much better result than BALB/c mice for producing monoclonal antibodies against the active form of vitamin D_3 [19]. Thus, we immunized both BALB/c and A/J mice, and the titers of the anti-UDCA 7-NAG antibody in serum were compared by the EIA after the third booster immunization. Absorbance at 415 nm due to bound enzymic activity by 10000-fold diluted serum was 0.29 ± 0.12 for BALB/c mice and 0.56 ± 0.08 for A/J mice (enzymic reaction 30 min; mean $\pm$ SD of five individuals). An inhibition test was further performed by the addition of unlabeled UDCA 7-NAG to determine the affinity of elicited antibodies. The A/J-4 mouse, which showed both high titer and large inhibition values, was given the final immunization, and its spleen cells were fused with the P3/NS1/1-Ag4-1 myeloma cells [11] using the 40% PEG 4000 supplemented with DMSO and poly-L-arginine, which were reported to increase fusion efficiency [19]. About ten days after the fusion, hybridomas were observed in over 90% of the microwells. The screening by the ELISA, examining the binding ability to the β-galactosidase-labeled antigen, demonstrated that 84 kinds of hybridomas obviously secreted an antibody to UDCA 7-NAG. Twenty hybridomas were selected from these antibody-positive cells based on the antibody titer of their culture supernatant and the inhibition experiment (data not shown). Cloning of these hybridomas by limiting dilution resulted in thirteen kinds of the antibody-secreting hybridoma clones each derived from different microwells in the initial culture plates.

Table 1. Immunochemical and binding properties of monoclonal antibodies

Antibody	Titer	Midpoint (pg/assay)	Isotype
Ab-#8	1:1500	17	γ_1, κ
Ab-#11	1:2000	21	γ_1, κ
Ab-#14	1:1000	45	γ_1, κ
Ab-#31	1:1000	39	γ_1, κ
Ab-#39	1:1000	43	γ_1, κ
Ab-#44	1:1500	32	γ_1, κ
Ab-#46	1:2000	48	γ_1, κ
Ab-#53	1:2000	21	γ_1, κ
Ab-#61	1:400	1300	γ_{2b}, κ
Ab-#65	1:1500	52	γ_{2a}, κ
Ab-#70	1:1200	860	γ_{2a}, κ
Ab-#72	1:400	285	γ_{2b}, κ
Ab-#81	1:1000	76	γ_1, κ

Characterization of monoclonal antibodies

Isotypes of heavy- and light-chains of each monoclonal antibody were determined as shown in Table 1. The binding characteristics of these antibodies were investigated by the competitive ELISA system. 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 units by a one hour enzymic reaction (Table 1). The absorption due to nonspecific binding in the absence of antibody was below 3% of the B_0 value.

Almost all antibodies, except for Ab-#61, #70 and #72, afforded highly sensitive dose–response curves for UDCA 7-NAG [Fig. 2(a)], as shown by their midpoint values (<100 pg), which are the amount of antigen required to inhibit the bound enzymic activity by half (Table 1). Among them, Ab-#8, #11 and #53 provided a very sensitive response whose midpoint was 17 or 21 pg per assay. The cross-reactivities with five bile acids were then determined in order to evaluate the applicability of these antibodies to biological specimens (Table 2). As we initially expected, all antibodies exhibited very large cross-reactivities with both the 7-NAG conjugates of GUDCA and TUDCA: for example, Ab-#8 exhibited 45% and 50%, respectively. The three antibodies, Ab-#61, #70 and #72, which provided less sensitive dose–response curves, showed even higher reactivity with these amidated UDCA 7-NAGs (220–320% and 280–390% for GUDCA 7-NAG and TUDCA 7-

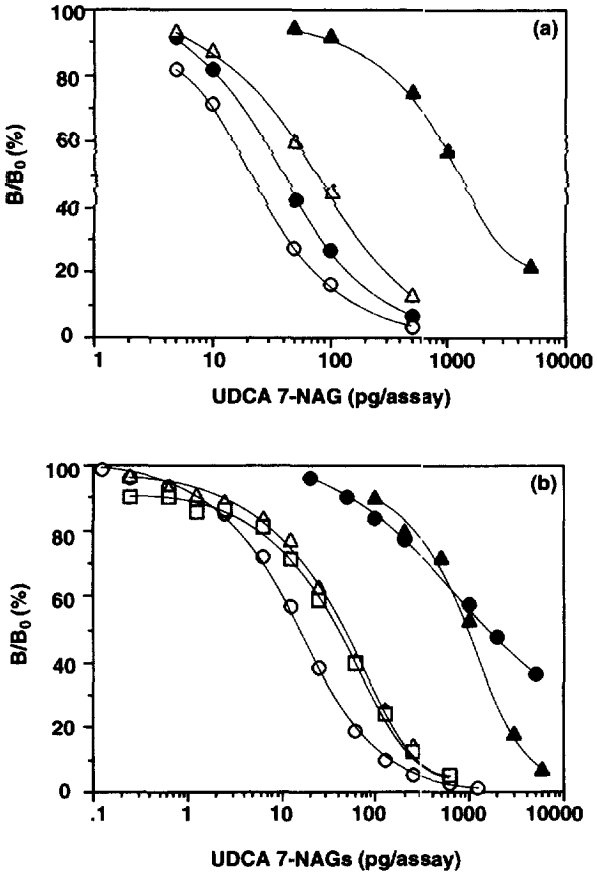


Fig. 2. Dose–response curves of (a) UDCA 7-NAG in the ELISA using monoclonal antibodies Ab-#8 (○), #46 (●), #61 (▲) and #81 (△); (b) UDCA 7-NAG (○), GUDCA 7-NAG (△) and TUDCA 7-NAG (□) in the ELISA using the monoclonal antibody Ab-#8, UDCA 7-NAG in the ELISA obtained from the A/J-4 mouse (●), and GUDCA 7-NAG in our previous EIA based on a rabbit antiserum (▲)[6].

NAG, respectively). On the other hand, very low cross-reactivities were observed with UDCA 3-sulfate (3-S, <0.01%), which is a conceivable major UDCA metabolite in urine after UDCA administration [20] and thus could be a potential interfering substance in the selective measurement for UDCA 7-NAGs. Negligibly low cross-reactivities with 7-unconjugated UDCA (≤0.93%) and GUDCA (≤3.3%) suggest that the sugar moiety plays a very important role in the specific binding between these antibodies and UDCA 7-NAGs.

Based on these results mentioned, we concluded that Ab-#8 is the most practical antibody among the thirteen monoclonal antibodies generated here, because it afforded the highest sensitivity in the ELISA system, satisfactory large cross-reactivities with 7-NAGs of GUDCA and TUDCA, and essentially no cross-reactivity with UDCA, GUDCA or UDCA 3-S (<0.01%). The cross-reactivities of Ab-#8 with eight additional bile acids were determined as follows: CA, CDCA and GUDCA 3-S, <0.01%; glycine-amidated CA and CDCA, <0.1%; UDCA 3-NAG, 0.36%; GUDCA 3-NAG, 0.29%; and UDCA 3-glucuronide, <0.02%. Negligibly low cross-reactivities with UDCA 3-NAG and GUDCA 3-NAG indicate this antibody well recognizes the site for conjugation with NAG. Highly sensitive dose–response curves were obtained with Ab-#8 for the 7-NAGs of GUDCA and TUDCA as well as UDCA 7-NAG, all of which covered approximately 1–200 pg per assay as a measurable range [Fig. 2(b)]. The detection limit for the 7-NAGs of UDCA and GUDCA, defined as the amount of bile acids required to give a B/B_0 of 2SD below, were 0.5 and 0.7 pg per assay, respectively. The dose–response curve for GUDCA 7-NAG was obviously more sensitive than that of previous EIA with a rabbit antiserum [6] and ELISA with a A/J-4 mouse antiserum corrected before fusion [Fig. 2(b)]. The affinity constant (K_a) of Ab-#8 for UDCA 7-NAG was estimated to be $5.4 \times 10^9\ M^{-1}$ (mean of two experiments) by a reported method based on an ELISA which measures free antigen after the reaction between the antigen and immobilized antibody has been completed in another microtiter plate [21].

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

Antibody	UDCA 7-NAG	GUDCA 7-NAG	TUDCA 7-NAG	UDCA	GUDCA	UDCA 3-S
Ab-#8[b]	100	45	50	<0.01	<0.01	<0.01
Ab-#11	100	31	39	0.01	<0.01	<0.01
Ab-#14	100	41	50	0.02	0.02	<0.01
Ab-#31	100	42	45	0.02	0.01	<0.01
Ab-#39	100	47	54	0.03	0.02	<0.01
Ab-#44	100	43	49	0.01	0.01	<0.01
Ab-#46	100	59	63	0.02	0.02	<0.01
Ab-#53	100	41	47	0.01	<0.01	<0.01
Ab-#61	100	220	280	0.93	3.3	<0.05
Ab-#65	100	42	52	0.03	0.03	<0.01
Ab-#70	100	270	390	0.64	2.5	<0.17
Ab-#72	100	320	340	0.36	2.0	<0.06
Ab-#81	100	51	45	0.03	0.04	<0.01

[a]Calculated by 50% displacement method[14].
[b]Cross-reactivities of Ab-#8 with 8 additional compounds are described in the text.

Table 3. Urine levels of UDCA 7-NAGs obtained by ELISA using monoclonal antibody Ab-#8[a]

Urine dilution	Sample A[b]		Sample B[b]	
	direct	extraction[c]	direct	extraction[c]
1:200	–	–	490	420
1:400	988	988	510	437
1:800	1092	1144	427	392
1:1600	1165	1040	470	370
1:3200	873	998	448	412
Mean	1030	1043	469	406

[a] μg/day as GUDCA 7-NAG equivalent.
[b] Collected from healthy male volunteers.
[c] Extracted with a Sep-Pak C_{18} cartridge.

Measurement of UDCA 7-NAGs in human urine

The urine levels (GUDCA 7-NAG equivalent) of two healthy volunteers were determined by ELISA using the monoclonal antibody Ab-#8 (Table 3). Direct measurement afforded similar assay values to those obtained after extraction with Sep-Pak C_{18} cartridges. In addition, acceptable parallelism was observed between assay values and the dilution ratio of the urine. Daily urinary excretion rates measured by the direct assay were 1030 and 469 μg for subjects A and B, respectively.

DISCUSSION

It has widely been recognized that monoclonal antibodies produced by the hybridoma technology are valuable analytical reagents which enable us to standardize various immunological techniques involving immunoassay and immunoaffinity purification procedures. Many attempts have consequently been made so far for generating monoclonal antibodies against various steroids which are useful as diagnostic indicators. To our knowledge, however, no monoclonal antibodies against bile acid derivatives have been produced although such antibodies should be of great utility for diagnosing various hepatobiliary diseases. From these points of view, we have undertaken to produce monoclonal antibodies against UDCA 7-NAGs, which are expected to be a valuable diagnostic tool for PBC.

To enlarge the probability for obtaining desirable monoclonal antibodies, it seems to be very important to choose an adequate animal as a spleen donor, which has shown a high response to the target antigen after immunization. Although both BALB/c and A/J mice strains exhibited significant humoral response against the immunogen that we used here, the A/J-4 mouse was selected as the suitable spleen donor based on careful examinations. The fusion experiment successfully afforded very many antibody-secreting hybridomas, from which thirteen kinds of promising clones have been established.

It should be noted here that, during the screening of desirable hybridomas, we found some hybridomas secreting the antibodies which were bound to the β-galactosidase-labeled UDCA 7-NAG, but that binding was not inhibited by the addition of unlabeled UDCA 7-NAG. This finding is compatible with the fact that the majority of the established monoclonal antibodies afforded dose–response curves with much higher sensitivity than that obtained with the antiserum from the spleen donor, A/J-4 mouse [Fig. 2(b)]. It is conceivable that two kinds of antibody subpopulations would have been present in this polyclonal antibody: high affinity antibody subpopulations like Ab-#8, and undesirable subpopulations whose binding can not be inhibited by an unlabeled antigen. To eliminate these by-product hybridomas which secrete the latter antibodies, without using a corresponding tritium-labeled compound (not commercially available in this case), an adequate inhibition test with an unlabeled target hapten was very important in the screening steps.

Among the thirteen kinds of monoclonal antibodies, Ab-#8 was evaluated to be the most useful one. This antibody was practically group-specific to the 7-NAG conjugates of UDCAs with a different amidation status at the side chain. This binding property is reasonable considering the chemical structure of the immunogen used here, that is, UDCA 7-NAG molecules were coupled to BSA through their own carboxyl group on the side chain: anti-hapten antibodies generally do not substantially recognize a partial structure on the hapten molecule which is close to the coupling site with the carrier protein. Moreover, Ab-#8 provided highly sensitive dose–response curves for the 7-NAGs of UDCA, GUDCA and TUDCA in the ELISA procedure, which are approximately 100-fold more sensitive than that of our previous EIA based on a rabbit antiserum [6] by comparison of their midpoints [Fig. 2(b)].

Taking into account the previous reports indicating that the glycine-amidated form is the major component among the UDCA 7-NAGs in the urine of patients with PBC [3, 5], it would be convenient to measure the total amount of these 7-NAG conjugates as a GUDCA 7-NAG equivalent using a dose–response curve for GUDCA 7-NAG for routine screening of this disease. As a preliminary examination for clinical application, we measured the daily urinary excretion rates of the GUDCA 7-NAG equivalent for two healthy volunteers, and obtained the values of 1030 and 469 μg (Table 3). Although a slight overestimation was observed for sample B (approximately 1.2 times higher), the direct assay values were acceptably in agreement with those obtained after extraction, showing good parallelism with the sample dilution ratio. Thus, it is expected that the ELISA

allows us a very simple and direct measurement which is available for the purpose of the routine diagnosis of PBC. The assay results obtained here are interesting, because it has remained obscure whether 7-NAG conjugated bile acids are excreted in healthy subjects [2, 5]. The present "monoclonal ELISA" is much more feasible than the previous EIA, and should allow simultaneous measurement of a large number of urine samples. In addition, only a small volume of urine sample (~100 μl) should be required, because of its extremely high sensitivity. Further validation of this assay system is now underway in our laboratory.

The Ab-#8 would be also available for an immunoaffinity purification system, which selectively extracts those UDCA 7-NAGs from biological specimens; this method will be useful for developing a separatory determination of each 7-NAG conjugate by liquid chromatography-mass spectrometry, which should be suitable for determining such highly polar bile acid metabolites. In this case, corresponding [^{18}O]-labeled compounds can be employed as excellent internal standards for estimating the recovery rates through the purification step [22]. We are also interested in the recognition mechanism of anti-hapten monoclonal antibodies. A set of monoclonal antibodies generated in this study would be a good model for analyzing the structure–function relationship of the antibody paratope and a steroidal epitope.

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