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Note

An antibody to lacto-*N*-biose I*

MASAZUMI TERASHIMA, KOHTARO KATO, TOSHIAKI OSAWA†,
Division of Chemical Toxicology and Immunochemistry, Faculty of Pharmaceutical Sciences, University of Tokyo, Bunkyo-ku, Tokyo 113 (Japan)

TAKU CHIBA, AND SETSUZO TEJIMA
Faculty of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467 (Japan)

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Various plant lectins have been used as probes for the study of structures and functions of cell-surface carbohydrates. However, monoclonal anti-oligosaccharide antibodies have several advantages over such lectins. These include (a) the possibility of forming monoclonal antibodies having various specificities and affinities, (b) a better definition of specificity, (c) a simple preparation of the monovalent derivative (Fab fragment), and (d) a relative lack of toxicity. Recently, the preparation of various monoclonal anti-carbohydrate antibodies has been reported[1–4]. This paper describes the establishment, by the hybridoma technique[5,6], of cell lines producing monoclonal anti-lacto-*N*-biose I antibodies, and their antigenic specificities.

EXPERIMENTAL

Antisera. — Rabbit antisera against mouse IgG, IgGl, IgG2a, IgG2b, and IgG3 were purchased from Miles Laboratories Inc. (Elkart, IN 46515).

Sugar derivatives. — The following free sugars and glycosides were synthesized as previously described: β-D-Gal*p*-(1→3)-D-GlcNAc (Lacto-*N*-biose I, ref. 7); β-D-Gal*p*-(1→4)-D-GlcNAc (*N*-acetyllactosamine, ref. 8); *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside [β-D-Glc*p*NAc-(1→)$OC_6H_4NO_2(p)$, ref. 9]; *p*-nitrophenyl *O*-β-D-galactopyranosyl-(1→3)-[β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→)$OC_6H_4NO_2(p)$, ref. 10], and -(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside [β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→)$OC_6H_4NO_2(p)$, ref. 11], and *p*-aminophenyl *O*-β-D-

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†To whom inquiries should be sent.

galactopyranosyl-(1→3)-[β-D-Gal-(1→3)-β-D-Glc*p*NAc-(1→)$OC_6H_4NH_2(p)$, ref. 11] and -(1→4)-2-acetamido-2-deoxy-β-D-glucopyranoside [β-D-Gal*p*-(1→4)-β-D-Glc*p*-NAc-(1→)$OC_6H_4NH_2(p)$, ref. 11].

*Preparation of lacto-*N*-biose I–BSA.* — β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→)-$OC_6H_4NH_2$ was coupled to bovine serum albumin (BSA) essentially by the method of Westphal and Feier[12]: To a solution of *p*-aminophenyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside[1] (17.8 mg) in water (0.38 mL) was added M hydrochloric acid (0.08 mL). The solution was cooled to 1–2°, and sodium nitrite (3.2 mg) in ice-cold water (0.11 mL) was added. This solution was added slowly, with stirring, to a solution of bovine serum albumin (BSA; 55.5 mg) in cold (1–2°) 0.04M sodium hydroxide (2.77 mL). The solution became deep red. After being kept for 5 h at 1–2°, the pH of the solution was adjusted to 7 to 7.5 with M hydrochloric acid. The solution was dialyzed against distilled water for 3 days, and lyophilized to provide the antigen (65.8 mg) as a red to orange-red powder. The resulting antigen (lacto-*N*-biose I–BSA) contained 28.6 residues of β-D-Gal*p*-(1→3)-β-D-GlcNAc(1→)$OC_6H_4N{=}N$- per molecule of BSA.

Immunization. — BALB/c mice were immunized with the antigen lacto-*N*-biose I–BSA (40 μg) suspended in saline solution (0.1 mL) and emulsified in complete Freund adjuvant (CFA 0.1 mL, Nakarai Chemicals, Ltd., Tokyo) immediately before use. Each mouse received two intraperitoneal injections of the emulsified antigen (0.2 mL each), 2 weeks apart. After 8 days from the last injection, serum from each mouse was tested for anti-lacto-*N*-biose I activity. The mice received a final booster injection on the next day. The spleens were harvested 3 days later.

Fusion. — Spleen cells (2×10^8 cells) of the immunized mice were fused according to the method of Watanabe and Ohara[13] with the mutant BALB/c myeloma cell-line MOPC 21 NSI/1 (3×10^7 cells), which was kindly provided by Dr. Milstein (Molecular Research Council, Cambridge, U.K.). Fusion was performed in the presence of 45% poly(1,2-ethanediol) (1 mL, mol. wt. 4 000; Sigma Chemical Co. St. Louis, MO 63178) in Dulbecco's modified Eagle's medium containing poly(L-arginine) (5 μg/mL, Sigma). Cells were washed and suspended (3×10^6 cells/mL) in RPMI-1640 medium containing 10% fetal calf serum, 2mM L-glutamine, mM pyruvate, and 0.05mM 2-mercaptoethanol (CM), and then dispensed in volumes of 0.8 mL into the wells of a multi-tissue, culture plate (Falcon 3008; Falcon Plastics Co., Oxnard, CA 93030). Twenty-four hours later, HAT medium[14] (CM supplemented with hypoxanthine, aminopterine, and thymidine, 0.7 mL) was added to each well. HAT medium (~50% by volume) was additionally fed at 1–3 day intervals. Aminopterine was omitted from the HAT medium after day 15. About three weeks after fusion, hydrid cells were transferred into CM and maintained.

Solid-phase radio immunoassay. — Hybrids producing anti-lacto-*N*-biose I antibodies were screened and cloned by limiting dilution in a microtiter well (Falcon 3042). The hybridoma clones were assayed for IgG because it is well known that a hyperimmunized mouse mainly produces IgG.

Antigen-coated plates were prepared as follows: lacto-*N*-biose I–BSA [20

μg/mL in 10mM sodium phosphate buffer containing 0.15M sodium chloride (PBS)] was incubated in wells of a U-bottomed polyvinyl plate for 2 h at 37°. To avoid nonspecific binding of antibodies to the lacto-*N*-biose I–BSA-coated well, the plates were incubated with 10% BSA in PBS for 1–2 h at 37°.

The antibody binding assay was performed as follows: (a) The supernatant culture medium of each cloned hybridoma was mixed with an equal amount of 10% BSA in PBS, and incubated for 1 h at 37° in order to neutralize the antibody directed toward the carrier (BSA). (b) A sample (50 μL) of the mixture was incubated for 1 h at room temperature in a well precoated with lacto-*N*-biose I–BSA. Nonbound immunoglobulin was removed from the well by washing five times with distilled water. (c) ^{125}I-Rabbit anti-mouse IgG in PBS (50 μL, $\sim 5 \times 10^4$ c.p.m.) was added to each well and incubated for 1.5 h at room temperature. The residual, nonbound anti-mouse IgG was removed from the well by washing with water. (d) Each well was cut out and counted in a gamma counter.

To determine the immunoglobulin class of each monoclonal antibody, the wells were incubated with rabbit anti-mouse IgG1, IgG2a, IgG2b, or IgG3, instead of lacto-*N*-biose I–BSA.

Determination of antigenic specificity. — Inhibition of passive hemagglutination was carried out with sheep erythrocytes (SRBC) coated with lacto-*N*-biose I–BSA (lacto-*N*-biose I–SRBC). Lacto-*N*-biose I–BSA was absorbed on SRBC by the method of Gronowicz *et al.*[15], modified as follows: Lacto-*N*-biose I–BSA (one part, 0.5 mg/mL) was mixed with 0.25mM chromium chloride (10 parts) and packed, washed SRBC (one part), and the mixture was incubated for 1 h at 30°. The erythrocytes were washed and suspended in PBS. Sugar inhibitors were serially diluted in microtiter plates, and then preincubated with a four-fold minimum hemagglutinating dose of antibody solution for 1 h at 37°. The suspension of lacto-*N*-biose I–SRBC was added to the wells, and the incubation was continued for 30 min at 37°, and examined for agglutination.

RESULTS AND DISCUSSION

*Production of anti-lacto-*N*-biose I hybridomas.* — The fusion of the NSI/1 myeloma with spleen cells from a BALB/c mouse immunized with lacto-*N*-biose I–BSA yielded 11 positive wells for anti-lacto-*N*-biose I antibodies out of 93 culture wells. One of the 11 positive wells was cloned by limiting dilution, yielding seven anti-lacto-*N*-biose I-producing clones (LNB-3, -6, -8, -11, -13, -18, and -19). All of the monoclonal antibodies from the seven clones were identified as belonging to the IgG1 subclass by the binding tests described in the Experimental section. Since the clones obtained from other ten positive wells were unstable and the titers of the antibodies from these clones were low, we did not investigate further these clones.

Initially, the hemagglutinating titer of anti-lacto-*N*-biose I antibody in the hybridoma-culture medium was determined with lacto-*N*-biose I–SRBC as target cells. The titers of the supernatant, hybridoma culture-medium were 1:18–1:16.

TABLE I

INHIBITION OF ANTI-LACTO-*N*-BIOSE I ANTIBODY–INDUCED HEMAGGLUTINATION BY VARIOUS SUGARS[a]

Inhibitory sugars	*Clones*						
	3	6	8	11	13	18	19
β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→O)$C_6H_4NO_2$	0.0041	0.0041	0.0013	0.0013	0.0013	0.0013	0.0013
β-D-Gal*p*-(1→3)-D-GlcNAc	0.37	0.37	0.37	0.37	0.12	0.37	0.37
β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→O)$C_6H_4NO_2$	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0
β-D-Gal*p*-(1→4)-D-GlcNAc	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0
β-D-Gal*p*-(1→4)-D-Glc	> 30	> 30	> 30	> 30	> 30	> 30	> 30
β-D-Glc*p*NAc-(1→O)$C_6H_4NO_2$	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0	> 3.0
D-GlcNAc	> 30	> 30	> 30	> 30	> 30	> 30	> 30
D-Gal	> 30	> 30	> 30	> 30	> 30	> 30	> 30

[a]Minimum concentration (mM) of sugars required to inhibit a four-fold minimum-hemagglutination dose of antibody.

When the hybridoma cells, LNB-13, the supernatant culture-medium of which showed the highest titer *in vitro* (1:16), were injected (5×10^6 cells/mouse) into BALB/c mice primed with Pristance (2,6,10,14-tetramethylpentadecane; Tokyo Kasei, Tokyo, Japan) to obtain ascitic fluid, the titer of the ascitic fluid was nearly 100 times higher than that of the supernatant culture-medium. The ascitic fluid from each mouse averaged 10–12 mL.

Antibody specificity determined by passive-hemagglutination inhibition. — The specificity of the anti-lacto-*N*-biose I hybridoma antibodies was determined with hybridoma culture medium. Several sugars and their derivatives were tested initially for their ability to inhibit agglutination of lacto-*N*-biose I–SRBC. The antibodies did not agglutinate SRBC coated with BSA instead of lacto-*N*-biose I–BSA, and BSA did not inhibit the hemagglutination of lacto-*N*-biose I–BSA-coated cells with the antibodies. Lacto-*N*-biose I completely inhibited the hemagglutination caused by the hybridoma antibodies (Table I); none of the other sugars tested showed any inhibition. Monosaccharides such as D-galactose, 2-acetamido-2-deoxy-D-glucose, and β-D-Glc*p*NAc-(1→)$OC_6H_4NO_2(p)$ were found to lack inhibitory activity. In addition, the observation that *N*-acetyllactosamine and β-D-Gal*p*-(1→4)-β-D-Glc*p*-NAc-(1→O)$C_6H_4NO_2(p)$ did not inhibit the hemagglutination indicates that the antibodies are specific for the β-D-Gal*p*-(1→3)-D-GlcNAc sugar sequence, but not for the β-D-Gal*p*-(1→4)-D-GlcNAc sequence. Finally, β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→O)$C_6H_4NO_2(p)$ inhibited the hemagglutination 100 times more than lacto-*N*-biose I, indicating that the antibodies recognize the antepenultimate phenyl residue as well as the β-D-Gal*p*-(1→3)-D-GlcNAc sugar sequence.

Perlmutter *et al.*[16] have suggested that a subclass-specific regulatory mechanism exists in mice immunized by carbohydrate antigens. They reported that murine IgG responses against several bacterial-carbohydrate antigens were largely restricted to the rare IgG3 subclass, and their data agreed with those reported by Young *et al.*[1]. However, our anti-lacto-*N*-biose I IgGs were found to belong to an IgG1 subclass.

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