ANTIGEN OF "SERUM SICKNESS" TYPE OF HETEROPHILE ANTIBODIES IN HUMAN SERA: IDENTIFICATION AS GANGLIOSIDES WITH N-GLYCOLYLNEURAMINIC ACID

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SUMMARY: Antigen of "serum-sickness" type of heterophile antibodies in pathologic human sera was purified from equine and bovine erythrocyte stroma. The chemical nature of this antigen was glycosphingolipids with N-glycolylneuraminic acid. The antigen of equine erythrocytes was identified as hematoside with N-glycolylneuraminic acid, GlNeu(α,2-3)Gal(β,1-4)Glc(β,1-1) ceramide and the antigen of bovine erythrocytes was N-glycolylneuraminyl-paragloboside, GlNeu(α,2-3)Gal(β,1-4)GlcNAc(β,1-3)Gal(β,1-4)Glc(β,1-1) ceramide. The results indicate that "serum-sickness" antibodies react with a common disaccharide moiety of non-reducing end of the both glycosphingolipids.

The production of heterophile antibodies in sera of patients by receiving therapeutic injection of foreign serum was first described by Hanganutziu (1) and Deicher (2). Thereafter, these antibodies have been called as "serum-sickness antibodies" or "H-D antibodies". The antibodies differ in specificity from other kinds of heterophile antibodies, such as Forssman antibodies (3) and Paul-Bunnell antibodies, "P-B antibodies" of infectious mononucleosis (4). These antibodies react with erythrocytes and sera from various animal species (horse, sheep,

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Abbreviations: GSL, glycosphingolipid; Gal, D-galactose; Glc, D-glucose; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; AcNeu, N-acetylneuraminic acid; GlNeu, N-glycolylneuraminic acid; Cer (ceramide), N-acylsphingosine; PBS, 0.01 M phosphate buffer (pH 7.0) containing 0.15 M NaCl.

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ox and rabbit) and are absorbed with the sediment of guinea-pig kidney homogenate. Recently, "H-D antibodies" were detected in sera from almost of all patients recieved γ-globulin fraction of goat anti-human thymocyte serum (5) and also in sera from some patients suffering from different kinds of diseases (6). The nature of "H-D antigen" has not been still unknown, except some properties being heat-stable, extractable with hot ethanol (7) and precipitable in 75% of ethanol solution (6).

We now present the purification of "H-D antigen" from bovine erythrocytes and equine erythrocytes and demonstrate first that the nature of this antigen is really glycosphingolipid (GSL) with N-glycolylneuraminic acid.

MATERIAL AND METHODS

The preparation of the standard GSLs used in the experiments were described previously (8-11). The isolation of hematoside with N-glycolylneuraminic acid (GlNeu-hematoside) from equine erythrocyte stroma and N-glycolylneuraminylparagloboside (GlNeu-sialosylparagloboside) from bovine erythrocyte stroma was performed as follows. Total lipids were extracted from acetone powder of erythrocyte stroma with chloroform-methanol (2:1, v/v), and acetylated with pyridine-acetic anhydride (2:1, v/v) at 60°C. The acetylated GSLs were isolated by Florisil column chromatography according to Saito and Hakomori (12). After deacetylation, GlNeu-hematoside of equine erythrocytes was purified by silicic acid column chromatography described previously (8) and GSLs with sialic acid (gangliosides) from bovine erythrocytes were obtained by DEAE-Sephadex column chromatography following Ledeen *et al*. (13). With regard to bovine gangliosides, after reacetylation, each ganglioside was isolated from each other by a preparative thin-layer chromatography (TLC) on precoated silica gel plates (E. Merck Laboratories, Inc.) developed one time with a solvent system of chloroform-methanol-water (95:5:0.5, v/v). The gangliosides were eluted from silica gel, deacetylated, neutralized, determined in the hexose content by phenol-sulfuric acid reaction and suspended in PBS containing 0.5 mg/ml sodium taurocholate (Nutritional Biochemical Corp.) for immunological tests according to the previous paper (11).

The analytical TLC on a precoated silica gel plate was carried out with twice development of chloroform-methanol-2.5 M ammonia (60:40:9, v/v) and visualized by spraying with resorcinol reagent. The carbohydrate compositions of "H-D antigen"-active GSLs were analyzed by gas-liquid chromatography (GLC) as previously described (14) for determining the molar ratio of each carbohydrates and sphingosine bases and as described in Ledeen's paper (15) for sialic acid composition.

Sera from thirteen different patients with different diseases were detected to have "serum-sickness" type of antibodies by determining higher levels of hemagglutinin to horse, calf, sheep and rabbit erythrocytes and by testing complete absorption of the hemagglutinin with guinea-pig kidney sediment. One of thirteen sera was obtained from a patient "J. S." who had

received therapeutic injection of horse anti-snake venom.

Parts of "H-D antibodies" were isolated from the mixed sera of two patients "C. S. and T. H." by affinity chromatography described in Marcus's paper (16). Five ml of "H-D serum" was absorbed at cold room with 10 ml of 7.5% polyacrylamide-2.5% bisacrylamide gel containing 10 mg of equine GlNeu=hematoside, 10 mg of egg lecithin and 100 mg of cholesterol. The bound protein was recovered by elution with 3 M KSCN, dialysis against PBS and concentration to approximately 200 μg/ml. The protein solution was diffused in 1% agarose gel with rabbit antisera to different human immunoglobulin chains, γ-chain, μ-chain, α-chain, κ-chain and λ-chain (Behring Inst.) and normal rabbit serum. Sera from four different patients with infectious mononucleosis which contain high levels of "P-B antibodies" were kindly donated from Dr. Kano (School of Medicine, State University of New York) and were tested for cross-reactivity of "H-D antigen"-active gangliosides by Dr. Matuhashi (Institute for Medical Science, University of Tokyo).

Hemagglutination inhibition was performed as follows. Prior to experiment, 0.2 ml of diluted H-D serum "J. S." containing 2 units of hemagglutinin was mixed with 0.2 ml of each inhibitor solution and kept for 12 hours at 4°C before 0.2 ml of 0.2% suspension of equine erythrocytes was added to. After one hour incubation again at cold room, each tube was centrifuged at 2,000 rpm for 2 min. Hemagglutination was read by gently shaking.

RESULTS

Glycolipid fraction of bovine erythrocytes was isolated into neutral glycolipids and gangliosides by DEAE-Sephadex column chromatography. "H-D antigen" activity was detected in only ganglioside fraction. Then, the ganglioside fraction was reacetylated and applied on preparative TLC plates. Seven different fractions those were named A, B, C, D, E, F and G in order of decreasing Rf values were isolated as shown in Fig. 1 and tested for immunological reactivity after deacetylation. The most potent inhibitor in equine erythrocyte-hemagglutination was fraction D. The compound D inhibited hemagglutination at the concentration of 0.9 μg/ml as hexose content. The compound was the most abundant glycolipid of bovine erythrocytes and moved slightly slower than AcNeu-sialosylparagloboside from human erythrocytes in analytical TLC. The compound was detected to have N-glycolylneuraminic acid, glucosamine, galactose, glucose and sphingosines at the molar ratio of 0.8:1.0:2.0:1.0:1.0, respectively. The GLC analysis of carbohydrate composition and movility of TLC were identical with the major ganglioside of

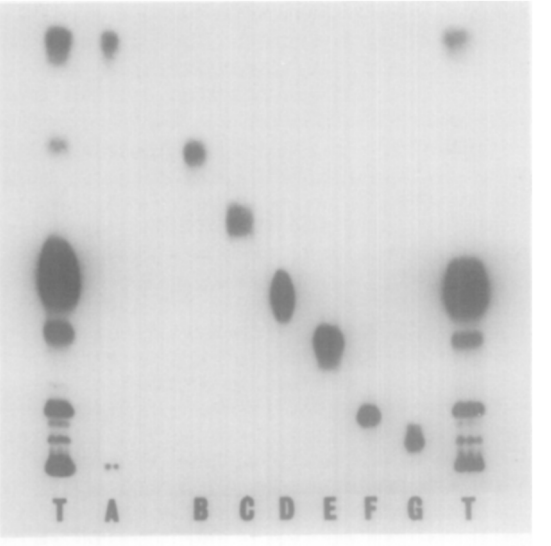

Fig. 1 TLC of each acetylated ganglioside of bovine erythrocytes. Total gangliosides (T) were separated into seven different fractions (A to G) by preparative TLC. An analytical TLC was carried out by developing with chloroform-methanol-water (95:5:0.5, v/v) and visualizing with resorcinol reagent.

bovine erythrocytes which had been already established by Wiegandt et al. (17), that is GlNeu-sialosylparagloboside. Fraction A and C, also inhibited hemagglutination at the concentration of 1.9 μg/ml. The compound A was identical with GlNeu=hematoside of equine erythrocyte (18) in movility on TLC analysis and sugar components by GLC analysis. However, the fraction C was recovered too small in amount to be analyzed in carbohydrate composition. The other fractions seemed to given only one band by analytical TLC and gave no inhibition at the concentration of 15.0 μg/ml. Fraction B and E were analyzed by GLC to have the identical sugar composition of AcNeu-hematoside and AcNeu-sialosylparagloboside, respectively. The potent inhibitors, fraction D and A each gave a clear precipitin line with "H-D serum" and fraction C gave a weak precipitin line, in agarose-gel diffusion, but the other fractions did not show precipitin reaction.

Table 1. Hemagglutination inhibition by different glycosphingolipids

Glycolipids	Chemical structures	Lowest conc. that give complete inhibition (μg/ml)
Lactosylceramide	Gal(β,1-4)Glc-Cer	125[2]
Globoside	GalNAc(β,1-3)Gal(α,1-4)Gal(β,1-4)Glc-Cer	-[1]
Forssman	GalNAc(β,1-3)Gal(α,1-4)Gal(β,1-4)Glc-Cer |(α,1-3) GalNAc	-
Paragloboside	Gal(β,1-4)GlcNAc(β,1-3)Gal(β,1-4)Glc-Cer	-
AcNeu-hematoside	AcNeu(α,2-3)Gal(β,1-4)Glc-Cer	125[2]
GlNeu-hematoside	GlNeu(α,2-3)Gal(β,1-4)Glc-Cer	2.0
AcNeu-sialosylparagloboside	Gal(β,1-4)GlcNAc(β,1-3)Gal(β,1-4)Glc-Cer |(α,2-3) AcNeu	-
GlNeu-sialosylparagloboside	Gal(β,1-4)GlcNAc(β,1-3)Gal(β,1-4)Glc-Cer |(α,2-3) GlNeu	2.0
G_{M2} ganglioside	GalNAc(β,1-4)Gal(β,1-4)Glc-Cer |(α,2-3) AcNeu	-
G_{M1} ganglioside	Gal(β,1-3)GalNAc(β,1-4)Gal(β,1-4)Glc-Cer |(α,2-3) AcNeu	-
G_{D1a} ganglioside	Gal(β,1-3)GalNAc(β,1-4)Gal(β,1-4)Glc-Cer |(α,2-3) |(α,2-3) AcNeu AcNeu	-

[1] Negative at a concentration of 125 μg/ml [2] Weak inhibition

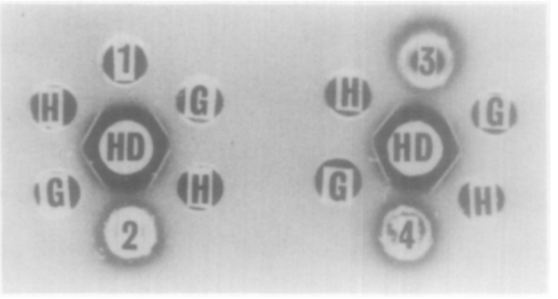

Fig. 2 Double diffusion test of "H-D serum" and "P-B sera" with equine GlNeu-hematoside and bovine GlNeu-sialosylparagloboside. 1 to 4, Sera from four different patients with infectious mononucleosis; HD, Sera from a patient "Z. M." containing "H-D antibodies"; G, GlNeu=sialosylparagloboside from bovine erythrocytes (250 µg/ml); H, GlNeu=hematoside from equine erythrocytes (250 µg/ml).

GlNeu-hematoside was also purified from equine erythrocyte stroma as the only "H-D antigen"-active substance. The compound was the most abundant GSL and had N-glycolylneuraminic acid, galactose, glucose and sphingosines at the molar ratio of 1.0: 0.9:1.0:0.9, respectively. The compound was the strongest inhibitor in hemagglutination test as well as GlNeu-sialosyl-paragloboside of bovine erythrocyte as shown in Table 1. The other compounds used, the structures of which were given in Table 1, gave no inhibition at the concentration of 125 µg/ml except that only lactosylceramide and AcNeu-hematoside gave weak inhibition at the maximum concentration employed. Only GlNeu=hematoside and GlNeu-sialosylparagloboside, each gave a clear precipitin line with serum containing "H-D antibodies" and the both precipitin lines seemed completely fused as shown in Fig. 2. The other compounds shown in Table 1 gave no precipitin reaction. Four different sera containing "P-B antibodies" reacted with neither GlNeu-hematoside nor GlNeu-sialosylparagloboside (Fig. 2). The other twelve "H-D sera", all gave positive reaction with

GlNeu-hematoside in gel diffusion. The GlNeu-hematoside-binding proteins of the sera isolated by affinity chromatography were demonstrated to be really immunoglobulins by positive reaction in gel diffusion with antisera to human immunoglobulin γ-chain (IgG), κ-chain (Light chain) and λ-chain (Light chain). But no precipitin reaction was obtained with antisera to human immunoglobulin μ-chain (IgM) and α-chain (IgA).

DISCUSSION

As above mentioned, the chemical structures of the two different "H-D antigen" active GSLs were first proved in this communication. The common disaccharide moiety of the two compounds suggested that "H-D antibodies" possibly react with the terminal disaccharide part, GlNeu(α,2-3)Gal. Various species of animals, equine, bovine, sheep, rabbit, pig, elk, dog, cat, mouse, hamster and primate monkey except chicken, have N-glycolylneuraminic acid residues (19, 20) in complex carbohydrates such as mucin, glycoproteins, immunoglobulins and gangliosides of erythrocyte membrane, serum and/or secretions. In addition, some of these compounds are known to have "H-D antigen" activity (21). But in human being, N-glycolylneuraminic acid have never been detected in any substances from body fluids and many organs (20). Therefore, if some substances containing such an antigen site invade into human by injection of foreign antisera, infection of microorganisms, absorption of milk and meats from abnormal intestinal sites, or inhalation of animal scurf, "H-D antibody" production shall be readily stimulated. However, it is future problem why such antibodies can be elevated in only pathologic human sera. Other investigators reported that the preparation of infectious mononucleosis antigen from erythrocyte stroma of various species of animals still possessed "H-D anti-

gen" activity (6, 21). But the present "H-D antigen" active glycolipids both did not have any "P-B antigen" activity.

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