Heterogeneity of Hanganutziu-Deicher Antigen Glycoproteins in Different Species Animal Sera

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ABSTRACT. A heterophilic Hanganutziu-Deicher (HD) antigen is present in many animal sera except human and chicken sera. To visualize the antigenic molecules, nine species animal and human sera were analyzed by SDS-PAGE, followed by Western blotting and immunostaining with avian anti-N-glycolyphosphoaminyl-lactosyl ceramide antibody which recognizes the terminal N-glycolyphosphoaminic acid moiety of glycoconjugates as an epitope of the HD antigen. Several HD antigen-active glycoprotein bands were detected in the sera of fetal calf, calf, horse, goat, monkey, rabbit, guinea pig, rat and mouse, except for human serum. The HD antigenic proteins showed heterogeneities in their molecular weights and were not identical with any major band visualized with silver-staining, indicating that they are minor components of serum proteins in each animal. Neuraminidase treatment destroyed the antigenicity of all proteins, confirming that N-glycolyphosphoaminic acid (NeuGc) at the non-reducing terminal of carbohydrate chains is the antigenic epitope in serum glycoprotein molecules as already confirmed in glycosphingolipid (GSL) antigens. The finding of HD-antigenic glycoproteins in animal sera suggests that they also stimulate HD antibody production in patients who received animal antiserum for therapeutic aim.—KEY WORDS: animal serum, glycoprotein, Hanganutziu-Deicher antigen, heterophilic antigen.


Heterophile Hanganutziu and Deicher (HD) antibody was originally detected in sera of patients who had received therapeutic injections of foreign antisera and was found to agglutinate many species of erythrocytes at high titers [1, 2]. N-glycolyphosphoaminic acid (NeuGc)-containing gangliosides were isolated from animal erythrocytes as the HD-antigenic molecules [3], and this sialic acid was found to be an essential epitope of the antigen [4]. In this report, we describe the HD antigen-active glycoproteins demonstrated by an improved method including Western blotting, avidin-biotin complex and alkaline phosphatase (ABC-AP) immunostaining in different species of animal sera.

MATERIALS AND METHODS

Anti-HD3 Antibody: Chicken anti-II’NeuGe-LacCer (HD3) antibody was prepared by immunizing four month-old chiken intramuscularly with 2 ml of emulsion consisting of 1 ml PBS solution containing 1 mg HD3 and 1 mg methylated BSA, and 1 ml complete Freund’s adjuvant. Serum was collected 4 weeks later without any booster injection. The specific antibody was purified by an affinity chromatography using HD3-immobilized octyl-Sepharose 4B column [5].

SDS-PAGE and Immunoblotting: Fresh sera from nine species of animal and human were 20 times diluted with Laemmlis sample buffer (62.5 mM Tris-HCl, pH 6.8, 3% SDS, 5% 2-Mercaptoethanol, 2 mM
EDTA, 10% Glycerol, 0.01% Bromophenol blue) for SDS-PAGE. Fifteen microliter of each serum sample was applied on 4 to 15% gradient polyacrylamide gel [6]. After electrophoresis, one slab gel was stained with 2D-Silver Stain II kit “Daiichi” (Daiiichi Pure Chemicals Co., LTD, Tokyo, Japan). Proteins in another gel were electroblotted on a nitrocellulose membrane (Schleicher & Schuell, Dassel, W. Germany), blocked with 3% Marim (Ajinomoto General Foods, Tokyo, Japan) in TBS (0.1 M Tris-HCl, 0.1 M NaCl, 2 mM MgCl₂, pH 7.5) at 37°C for 1 hr and then immunostained with affinity-purified anti-HD3 antibody at a concentration of 6 μg/ml in TBS at room temperature for 1 hr, after three times washing with TBS, the bound antibody was detected with biotinylated goat anti-chicken IgG (Vector Laboratory Inc., Burlingame, CA, U.S.A.), 1000-folds diluted with TBS at room temperature for 1 hr. After washing similarly, the membrane was further incubated with alkaline phosphatase-conjugated streptavidin (Bethesda Res. Lab., Gaithersburg, MD, USA) 4000-fold diluted with TBS (pH 9.5) containing 50 mM MgCl₂ at room temperature for 15 min. After washing, enzyme localization was visualized by incubation with a substrate solution containing 5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt and Nitroblue tetrazolium chloride (BCIP-NBT kit, Bethesda Res. Lab.) 200-folds diluted with same buffer. One thousand five hundred-folds dilution of normal chicken serum was used as nonspecific control instead of specific antibody.

Neuraminidase treatment: To 10 μl of each diluted serum sample (1 mg protein/ml in PBS), 10 μl of 10 m units of Streptococcal neuraminidase (Seikagaku Kogyo Co., LTD, Tokyo, Japan) or 10 μl PBS were added and incubated at 37°C for 2 hrs, and boiled with equal volume of 2 times concentrated Laemmli’s sample buffer before applying to SDS-PAGE.

RESULTS

Sera from fetal calf, calf, horse, goat, monkey, rabbit, guinea pig, rat, mouse and human were analyzed by SDS-PAGE followed by silver-staining (Fig. 1) and then immunoblotted by using anti-HD3 antibody (Fig. 2). Several protein bands were stained in each serum except human serum (Fig. 2A). Their molecular masses were summarized in Table 1. The bands of 55 KD and/or 65 KD were detected with normal chicken serum, indicating they are nonspecific bands (Fig. 2B). These nonspecific bands were resistant to neuraminidase treatment while all HD antigenic bands disappeared by the treatment (Fig. 3). HD antigen-active proteins were heterogeneous in each serum and were minor components in comparison with the protein bands stained with the silver-staining kit (Fig. 1 and Table 1). The major HD antigens in fetal calf serum were 300

![Fig. 1. The protein bands in animal and human sera detected by silver staining. The sera from human and 9 species animal were analyzed by SDS-PAGE using 4 to 15% gradient polyacrylamide gel. The gel was then stained with a silver-staining kit.](image-url)
Fig. 2. Immunoblotting of HD antigenic glycoproteins from animal sera. Human and 9 species animal serum proteins were analyzed by SDS-PAGE using 4 to 15% gradient polyacrylamide gel, and then blotted to nitrocellulose membrane. Immunostaining was done with chicken anti-HD3 antibody (A) or normal chicken serum (B) as described in “MATERIALS AND METHODS”.

Fig. 3. Disappearance of HD antigenic glycoprotein bands by neuraminidase treatment. 10 μl of each diluted serum (1 mg/ml in PBS) were mixed with 10 μl of 10 m unit neuraminidase (+) or with 10 μl PBS (−). After incubation at 37°C for 2 hrs, the mixtures were analyzed by SDS-PAGE, and then immunoblotted as described in “MATERIALS AND METHODS”.

and 170 KD, however, adult calf serum had other two bands of 110 and 45 KD. The HD antigens in several strains of murine sera (C3H/He, BALB/c, NZB) showed the same pattern, meanwhile there was also no difference between different individuals of the
Table 1. The molecular mass of HD antigenic bands detected in animal and human sera

<table>
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<th>Species of sera</th>
<th>Fetal calf</th>
<th>Calf</th>
<th>Horse</th>
<th>Goat</th>
<th>Monkey</th>
<th>Human</th>
<th>Rabbit</th>
<th>Guinea pig</th>
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Molecular mass is represented as kilodalton.

same strain (data not shown). No HD antigen could be detected in human serum as expected, but African Green Monkey serum had abundant amounts of antigenic proteins with molecular weight of 170, 140, 100 and 70 KD.

DISCUSSION

Hanganutziu-Deicher (HD) antibodies were first demonstrated in sera from patients who had received therapeutic injections of horse anti-tetanus toxin [1, 2]. The patient sera gave a precipitin line against horse serum as well as several species animal sera. The sera also hemagglutinated several species animal erythrocytes such as sheep, bovine and horse erythrocytes, and the hemagglutinin was absorbed with guinea pig kidney homogenate [7]. This Davidson's absorption test distinguished HD antibodies from other heterophilic antibodies such as Forssman antibody [8] and Paul-Bunnell antibody [9]. The nature of HD antigens in animal sera was searched for many years, but it was quite difficult because HD antibody-positive patient sera gave a fused precipitin line against whole protein region from γ-globulin to albumin isolated by electrophoresis in agar gel [10]. Bovine serum proteins isolated by DEAE cellulose column chromatography reacted with HD serum (Naiki et al. unpublished data).

Previously, we isolated HD antigens from bovine erythrocytes as N-glycolylneuraminic acid-containing gangliosides [3] and Merrick et al., isolated it from bovine erythrocytes as glycoprotein [11]. However, presence of specific antigens such as glycoproteins in animal sera has been still unknown so far.

In the present paper, we analyzed the HD antigen-active glycoproteins in various species animal sera by using one of most sensitive immunodetection methods. Several antigen-active glycoproteins were detected in each serum of calf, horse, goat, monkey, rabbit, guinea pig, rat and mouse. These glycoproteins of each serum showed marked heterogeneity in their molecular weight and their contents. Fetal calf and mouse serum antigens were absorbed with the agarose gels conjugated with Concanavalin A (Con A), Lens culinaris agglutinin (LCA) and Ricinus communis agglutinin (RCA), while rabbit serum antigens with Con A, RCA, Wheat germ agglutinin (WGA)-conjugated agarose gels, indicating that the sugar chains of HD glycoproteins are different in each animal (Wang et al. unpublished data). However, neuraminidase treatment destroyed the antigenicity of all species glycoproteins, it suggested that all antigenic glycoproteins have N-glycolylneuraminic acid at the non-reducing terminal of the carbohydrate chains as the HD antigenic epitope. Up to now, we were
not clear about the reasons for the HD antigen’s heterogeneity in their molecular weights and contents among different species of animals, but the various core proteins of the HD antigen are supposed to be an important reason. In this paper, we succeeded in the separation of several glycoprotein antigens by using SDS-PAGE and immunoblotting. Previously, no researcher has used SDS treatment. Therefore, glycolipid antigens should non-covalently bind to all proteins in serum to result unsuccessful isolation of specific glycoproteins. In fact, we detected two different HD antigen-active gangliosides in fetal calf serum [3]. Sera from other species animals such as bovine, sheep and horse should contain the ganglioside antigens which are non-covalently binding to serum proteins. Both glycoprotein and glycosphingolipid antigens can stimulate HD antibody production in patients who received foreign serum.

Recently, we found in many papers [12–14] that HD antibodies can be detected with high frequency in sera from patients with various cancers, who had never received animal serum. Eight different HD antigenic gangliosides were already identified in different cancerous tissues. However, detection of cancerous tissue and serum glycoprotein antigens has not been performed. The immunoblotting technique used in this paper may be useful to isolate and detect glycoprotein antigens from human cancerous tissues and serum immunocomplex. We already confirmed that it was quite useful to detect glycoprotein antigens from one of human gastric cancer cell lines which did not have ganglioside antigens but showed HD antigen-positive staining by flow cytometric analysis [15]. In further studies, if monoclonal antibodies to HD glycoproteins from animal and human cancerous origins can be established, it will be greatly helpful for investigating their detail structures.

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