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## USE OF BIOTINYLATED ANTIBODY FOR THE ASSAY OF HANGANUTZIU-DEICHER ANTIBODIES AND ANTIGENS IN FLUIDS AND TISSUES FROM CANCER PATIENTS

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An improved enzyme-linked immunosorbent assay (ELISA) for detection of heterophile Hanganutziu-Deicher (HD) antibodies and antigens, which are frequently detected in sera and/or cancerous tissues from patients with various cancers was developed using biotinylated chicken anti-GM3(NeuGc) antibody and avidin-horse raddish peroxidase conjugate. The N-glycolylneuraminylactosylceramide, GM3(NeuGc) ganglioside was purified from horse erythrocyte membranes. The ELISA procedure required 300 ng GM3(NeuGc) antigen to coat plastic microtiter plates and 190 ng biotinylated antibody per well to give optimum product formation. The technique could detect 6 ng antigen in tissue homogenate as compared to 0.6 ng of the pure compound by inhibition. Chicken anti-GM3(NeuGc) antibody quantitatively inhibited the biotinylated antibody, however, this procedure was not suitable to quantify lower affinity HD antibody in patient sera. Immunostaining specific for HD antigen-positive cells, in tissue sections was by 4  $\mu$ g/ml biotinylated antibody and 200 dilution of Avidin-biotinylated peroxidase complex reagent using pig intestine and lymph node as positive tissues and chicken intestine and lung as negative tissues.

Key Words : ELISA, Hanganutziu-Deicher antibodies ; Tumor-associated antigens

### INTRODUCTION

It has been shown that there are certain specific alterations in glycolipid composition of tumor tissues.<sup>2,4,13,20)</sup> Some glycolipids have been termed as tumor markers.<sup>3)</sup> In addition, the presence of antibodies against these glycolipids have been demonstrated

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in cancer patients such as colonic and melanoma.<sup>10,17)</sup> Glycolipids with N-glycolylneuraminic acid which are normally present in most animals except humans and chickens, have been determined to be Hanganutziu-Deicher (HD) antigens.<sup>17)</sup> However, the antigens are expressed in various human cancers<sup>8,10,20)</sup> as well as in chicken Marek's disease lymphoma.<sup>6,16)</sup> Therefore, detection of HD antigens and antibodies in human tissues and sera has become of diagnostic potential.

HD antibody and antigen assay has been developed using immunological techniques such as hemagglutination inhibition, radioimmunoassay and ELISA.<sup>8,14,20)</sup> Using biotinylated anti-GM3(NeuGc) antibody we have now improved the ELISA procedure to enhance the sensitivity and simplify the procedure and also developed an immunostaining procedure for tissue cells containing HD antigen-active gangliosides or glycoproteins.

#### MATERIALS AND METHODS

Anti-GM3(NeuGc) preparation and biotinylation: The GM3(NeuGc) and GM3(NeuAc) hematosides were purified from equine and bovine erythrocytes respectively.<sup>5)</sup> Specific chicken antisera were prepared by immunizing adult SPF chicken with GM3(NeuGc) as previously described<sup>1)</sup> Four-month-old chickens were immunized intramuscularly with 1 mg of GM3(NeuGc) mixed with an equal amount of methylated bovine serum albumin and 1 ml of complete Freund's adjuvant. Sera were collected after 4 weeks without a booster dose. The globulin fraction was separated by 50% saturated ammonium sulfate precipitation. Anti-GM3(NeuGc) IgG was purified by affinity chromatography using GM3(NeuGc)-coated Octyl-Sepharose 4B gel (Pharmacia Fine Chemicals, Uppsala, Sweden) according to Hirabayashi et al.<sup>8)</sup> The gel was washed and equilibrated with a methanol / 0.1M KCl (1 : 1, v / v) solution. To bind GM3(NeuGc) to the gel, 6 mg GM3(NeuGc) in 10 ml of same solution was added to an equal volume of gel, mixed vigorously and allowed to stand at room temperature for 30 min with occasional shaking. The conjugate was thoroughly washed with 0.01 M phosphate buffer (pH 7.2) containing 0.15 M NaCl (PBS) to remove organic solvents and unbound glycolipids. The GM3(NeuGc)-coated gel was packed in a column (7x10 cm) washed and equilibrated with PBS. The globulin fraction of the 50 ml antiserum in PBS was applied to the affinity column, washed with PBS and then 0.05% Tween 20 containing PBS (Tween-PBS). The antibody (56 mg) was then eluted with 3.0 M KSCN-containing PBS. Purity of the IgG was demonstrated by an immunoelectrophoresis analysis.

For biotin conjugation, the antibody was dialysed against 0.1 M NaHCO<sub>3</sub> and adjusted to a concentration of 1 mg / ml. Sixty  $\mu$ l of freshly prepared N-hydroxysuccinimidobiotin in dimethyl sulfoxide (Wako Pure Chem. Ind. Ltd), was added for each 1 ml of the protein solution.<sup>9)</sup> After mixing thoroughly, it was left at room temperature for 4 hr, then dialysed against PBS with 0.01% NaN<sub>3</sub> overnight to

remove unbound biotin and stored at  $-80^{\circ}\text{C}$  before use.

ELISA procedures : ELISA was performed using 96 well flat bottom plate (Nunc, Inter Med. Denmark) as previously described<sup>12)</sup> with some modification for the avidin-biotin enzyme complex (ABC reagent), (Vector Laboratories Inc., Burlingame, CA). The optimal ELISA conditions used were as follows : Each well was coated with 300 ng GM3(NeuGc) dissolved in 50  $\mu\text{l}$  methanol by evaporation. Non-specific binding sites were blocked with 0.2 ml of 1% ovalbumin containing PBS for 1 hr at  $37^{\circ}\text{C}$ . After washing thrice with 0.2 ml Tween 20-PBS, 190 ng of the biotinylated antibody in 1% egg albumin-containing PBS (50  $\mu\text{l}$ ) was added to each well and allowed to react for 1 hr at  $37^{\circ}\text{C}$ . After the reaction, each well was washed twice in Tween-PBS and once with 1% egg albumin containing 50 mM carbonate buffer (pH 9.6). Preformed ABC reagent diluted 100 fold with Tween-PBS was added and allowed to react for 30 min at  $37^{\circ}\text{C}$ . The wells were washed thrice with Tween-PBS and finally 0.2 ml of the substrate of 0.2 mM 2,2'-azino-di-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 0.004%  $\text{H}_2\text{O}_2$  and 50 mM sodium citrate buffer (pH4.0) was added. The enzyme reaction was performed for 30 min at  $37^{\circ}\text{C}$  and the product was determined immediately by measuring absorbance at 405 nm using Titertek Multiskan, (Flow Lab Inc.).

Antibody amount in human sera was determined by measuring inhibition of the reaction between biotinylated antibody and immobilised GM3(NeuGc) antigen. Two fold diluted human sera were added to 380 ng of the biotinylated antibody (50  $\mu\text{l}$ ) and 50  $\mu\text{l}$  of the mixture was transferred to antigen coated wells. A standard curve was prepared with unbiotinylated antibody instead of serum and ELISA was completed as above.

Similarly, for antigen detection, GM3(NeuGc) was serially diluted with PBS and normal human liver homogenate. Volumes of 50  $\mu\text{l}$  of the antigen solution were mixed with 50  $\mu\text{l}$  (380 ng) of biotinylated antibody. After incubation for 1 hr at  $37^{\circ}\text{C}$ , 50  $\mu\text{l}$  of the mixture was transferred to each antigen coated well and ELISA was completed as above. In preparation of human tissue homogenate containing mainly particulate fraction, 10% normal liver homogenate was made by warring blender in 25% sucrose containing 2 mM  $\text{CaCl}_2$ .<sup>21)</sup> The crude homogenate was filtered through cotton gauze, layered over 30% sucrose and centrifuged at 800 g for 10 min. The 25% layer was again centrifuged at 20,000g for 1 h. The pellet was suspended in PBS with a glass homogenizer and stored at  $-20^{\circ}\text{C}$  till use.

Immunostaining of tissue antigen : Normal intestine and mesenteric lymph nodes obtained from healthy pigs as HD antigen-positive tissues and normal intestines and lung tissues from SPF chickens were fixed with Bouin's solution.<sup>18,19)</sup> Consecutive 5  $\mu\text{m}$ -thick paraffin sections were prepared from each sample and used as substrate for biotin-avidin-immunostaining according to Hsu et al.<sup>14)</sup> After paraffin removal from each section with solvent systems of xylene and ethanol series, the section was first

dipped in 0.3% H<sub>2</sub>O<sub>2</sub> in methanol to block endogenous peroxidase. After washing three times by dipping it in renewed cold PBS agitated by magnetic stirrer for 5 min each, whole area of the tissue was overlayed with biotinylated chicken HD antibody diluted with 1% ovalbumin-PBS, to which additional 0.5 M NaCl was added, at a concentration of 3.8  $\mu$ g/ml (400 fold dilution of the original concentration) and allowed to stand at room temperature for 1 hr in a moist chamber. After washing similarly, the section was stained at room temperature for 2 hr with streptavidin-biotinylated peroxidase, (ABC regant, Amersham) diluted 200 fold with PBS. After similar washes with cold PBS, the section was preincubated at room temperature for 30 min in substrate solution containing 30 ng 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chem. Ind. Ltd) in 0.05 M Tris-HCl buffer (pH 7.6), and then 30  $\mu$ l of 30% hydrogen peroxide was added to start the enzyme reaction. The reaction was stopped within additional 5 to 10 min when the tissue color developed slightly by quickly rinsing it with tap water. It was then stained with 0.2% methyl green for 3 to 5 min before light microscopic observation.

#### RESULTS

Establishment of ELISA procedure: Avian antibody against GM3(NeuGc) was first

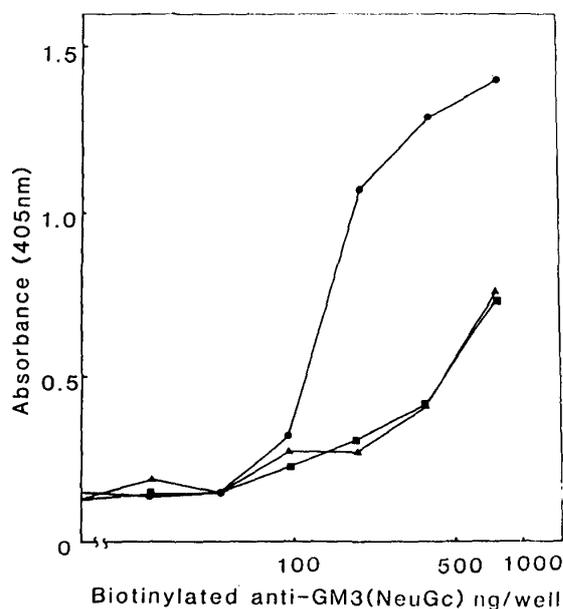


Fig. 1. Titration of the biotinylated chicken anti-GM3(NeuGc). Two-fold serially diluted antibody preparations were added to each well coated with 2.0  $\mu$ g of GM3(NeuGc) (●), GM3(NeuAc) (▲) and no antigen (■). Other conditions were as described in Materials and Methods.

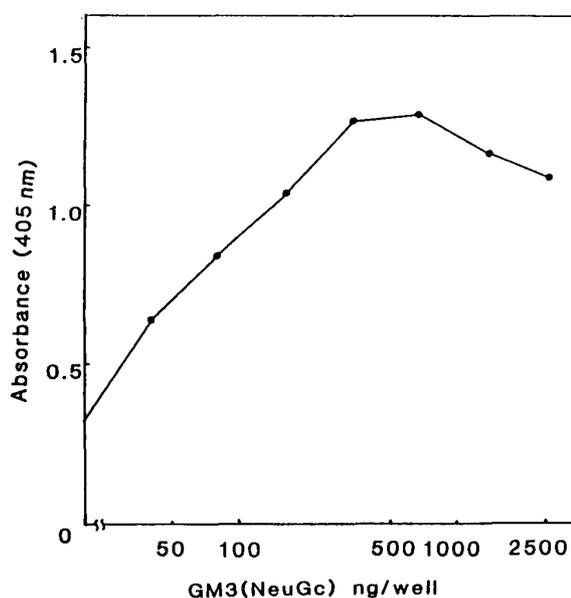


Fig. 2. Titration of GM3(NeuGc) antigen for microplate coating. Different amounts of GM3(NeuGc) in methanol were coated per well and reacted with 190 ng of the biotinylated antibody as shown in Fig. 1.

purified and biotinylated. The titration of the antibody preparation was performed to determine the optimal ELISA conditions. Addition of 190 ng of biotinylated antibody gave a strong reaction with GM3(NeuGc) but not with GM3(NeuAc) as shown in Fig. 1. About 600 ng of the antigen coated per well gave the maximum reaction (Fig. 2). However, to achieve a high inhibition sensitivity, an amount of 300 ng was considered suitable.

An inhibition curve was obtained for the purified unbiotinylated chicken anti-GM3(NeuGc) (Fig. 3). From the calibration curve, patient serum (NS) in which HD antibody was detected by a previous ELISA<sup>14)</sup>, was highly positive as compared to normal human serum. Another patient serum (TK) was also positive for the HD antibodies. However, both patient sera did not completely inhibit biotinylated antibody even at high concentrations indicating that human HD antibodies have a lower affinity than the biotinylated antibody prepared from chicken antisera.

To detect minute amounts of antigens in human cancerous tissue, we added various amounts of GM3(NeuGc) to normal liver homogenate and then calculated the recovery rate of the antigen detected. A 50% inhibition was achieved with 0.6 ng and 6 ng GM3(NeuGc) dissolved in PBS and the homogenate respectively (Fig. 4). This difference is most likely due to nonspecific masking of the antigen by molecules in the liver homogenate.

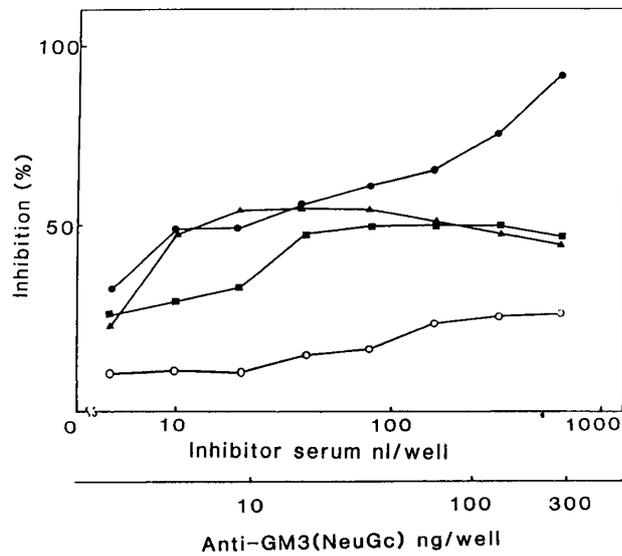


Fig. 3. Inhibition of the biotinylated antibody by HD antibody-positive patient sera as compared to unbiotinylated chicken antibody. Fifty  $\mu$ l of serially diluted purified anti-GM3(NeuGc) antibody (●), HD antibody-positive human sera NS (▲), TK (■) and normal human serum (○) were mixed with 50  $\mu$ l (380 ng) biotinylated antibody. Fifty  $\mu$ l of the mixture were added to antigen coated wells and ELISA completed as in previous figure.

Immunostaining of tissue antigens: Suitable conditions of biotin-avidin-immunostaining for the antigenic tissue localization studies were examined by using pig intestines and mesenteric lymph node as the antigen-positive tissues while chicken intestines and lung were used as the antigen-negative tissues (Table 1). Fifty (4  $\mu$ g/ml) to four hundred fold dilutions of biotinylated first antibody could stain HD antigen-positive cells as shown in Fig. 5. Fifty or one hundred dilution of the first antibody needed 400 dilution of ABC reagent to minimize non-specific staining, but 200 or 400 dilution of the first antibody needed 200 ABC dilution to stain the positive cells without non-specific staining. One percent ovalbumin and 0.5 M NaCl used for dilution of the first antibody were effective to minimize non-specific staining. In pig intestine, lymphocytes, monocytes and granulocytes in lamina propria were stained (Fig. 5A, B) and in pig mesenteric lymph node some population of lymphocytes located at the cortex were stained (Fig. 5C). Chicken intestine (Fig. 5F) and lung were not stained in any part.

#### DISCUSSION

Enzyme-linked immunoassay system for chicken antisera to gangliosides has

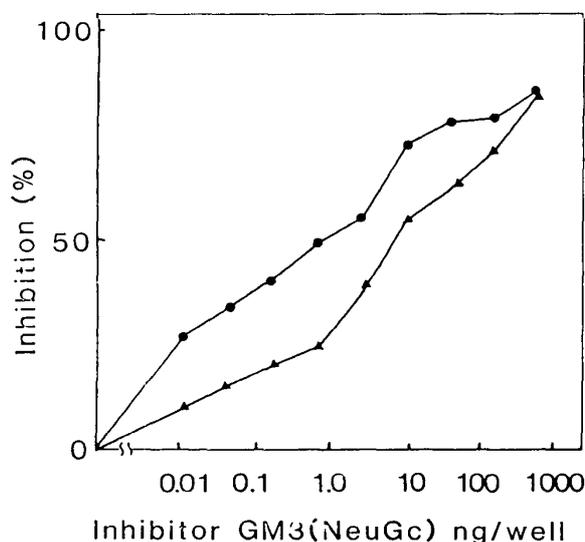


Fig. 4. Inhibition of the biotinylated antibody by GM3(NeuGc) and GM3(NeuGc)-added normal human tissue homogenate. Fifty  $\mu$ l of serially diluted GM3(NeuGc) in PBs (●) and liver homogenate (▲) were mixed with 50  $\mu$ l (380 ng) biotinylated antibody and incubated for 1 hr 37°C. Fifty  $\mu$ l of the mixture were added to antigen coated well and ELISA was done as shown in previous figures.

already been reported<sup>9,14</sup>), as well as thin-layer chromatography immunostaining and radiomunoassay.<sup>11,15</sup> Antibodies to gangliosides have also been demonstrated in sera of tumor bearing patients.<sup>2</sup> The old ELISA using enzyme-labeled anti-human immunoglobulin to detect the antibodies was an effective screening technique but of poor quantitative application. In the present study we modified the ELISA procedure by using biotinylated anti-GM3(NeuGc) for the detection and determination of the tumor associated-HD antigen and its antibodies in human tissues and sera respectively. Avidin, a glycoprotein found in egg white has a very high affinity for biotin molecule. Immunoglobulin or peroxidase covalently coupled to biotin can bind to avidin which has four active sites for biotin. The avidin multivalence amplifies the antigen antibody reaction and lattice-like complex is formed.

In our system, 300 ng and 190 ng of antigen coated and biotinylated antibody respectively were optimum to give adequate product formation. The high affinity of biotin for the avidin conjugated with horse radish peroxidase amplified the reaction to enhance sensitivity. This reduced the necessary antigen amount for coating a plate well from 2.5  $\mu$ g to 300 ng.<sup>9</sup> This high sensitivity also accounts for the background reaction in no-antigen and GM3(NeuAc)-coated wells at high antibody concentrations.

TABLE 1 Biotinylated antibody and ABC reagent titration in immunostaining

Biotinylated ab Dilution	ABC reagent Dilution	Specific <sup>a</sup> Staining	Non-specific <sup>b</sup> Staining
50	100	++ <sup>c</sup>	+
50	200	++	+
50	400	+	-
100	100	++	+
100	200	+	+
100	400	+	-
200	100	ND <sup>d</sup>	ND
200	200	ND	ND
200	400	+	-
400	100	++	+
400	200	+	-
400	400	+	-

a : Immunostaining intensity of HD antigen-positive cells in pig intestine preparation.

b : Immunostaining background in chicken intestine preparation.

c++ : strong staining; + : weak staining; - : no staining.

d : Not determined.

At high antigen concentrations used for coating there was an apparent decrease in reaction. This is due to an inhibition of biotinylated antibody by release of excess antigen loosely bound to plate during subsequent washings.

The small amount of antigen coated per well and the assay's high sensitivity achieved appreciable inhibition of the biotinylated antibody. For 50% inhibition, 0.6 ng of pure antigen and 6 ng antigen in tissue homogenate indicated the detectable range. Radioimmunoassay for the same ganglioside could detect 31 ng<sup>14)</sup> and TLC immunostaining using (<sup>125</sup>I) protein A 100 ng.<sup>15</sup> By comparing the inhibition of human sera with that of purified chicken anti-HD3, it is possible to perform quantitative tests. However, the human and chicken antibodies seem to have variable affinities for the HD antigen. Human antibodies could inhibit about 50 % biotinylated antibody with relatively low affinity but not higher affinity of the rest of antibody.

The HD molecule is a novel tumor-associated glycolipid whose antigenic determinant (NeuGc) is present in sugar chains of various gangliosides and glycoproteins.<sup>1)</sup> A sensitive detection assay for HD antigens and antibodies is vital in research and a potential tool in clinical diagnosis. Since chicken and human antibodies show different affinities for the antibody, a biotinylated human monoclonal antibody would be more ideal whenever the latter become available.

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Fig. 5. Immunostaining of pig and chicken tissues with a biotinylated antibody and ABC reagent system.

A. Pig intestine was stained with 400 fold dilution of biotinylated anti-GM3(NeuGc) antibody and then with 200 fold dilution of ABC reagent (magnification x100).

B. Same as A (magnification x400). An arrow indicates a positive cell.

C. Same specimen was stained with only 100 fold dilution of ABC reagent (magnification x400). Some cells look stained in a black and white picture, but the colour was blue and they are definitely negative cells.

D. Pig mesenteric lymph node was stained as done in Fig. 5A section (magnification x400). Arrow shows a positive cell.

E. Same specimen was stained with only 100 fold of ABC reagent (magnification x400).

F. Chicken intestine was stained as done in Fig. 5A section (magnification x400).

