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**Lectin-dependent inhibition of antigen-antibody reaction: Application
for measuring α 2,6-sialylated glycoform of transferrin**

SSA レクチンによる抗原-抗体反応の阻害：
髄液中 α 2,6-シアル酸化トランスフェリン測定への応用

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1. Abstract

We previously found two isoforms of transferrin, *i.e.*, non-sialylated Tf and α 2,6-sialylated Tf, in cerebrospinal fluid (CSF). The ratio of isoforms (α 2,6-sialylated/non-sialylated Tf) was a marker for idiopathic normal pressure hydrocephalus (iNPH), a dementia-inducing disorder. I have recently found that binding of anti-transferrin antibody (Tf-ab) to α 2,6-sialylated Tf is inhibited by *Sambucus sieboldiana* Agglutinin (SSA) lectin, which recognizes the α 2,6-sialylated epitope. This finding lends itself to automated latex-agglutination immunoassay (ALI), using SSA lectin as an inhibitor specific for α 2,6-sialylated Tf. Namely, concentration of α 2,6-sialylated Tf is estimated by ALI signal difference in the presence and absence of SSA lectin (SSA-ALI). The concentration of α 2,6-sialylated Tf measured by SSA-ALI ($n = 47$) is correlated with those by ELISA ($r^2 = 0.8554$, $p < 0.01$), which was previously developed for measuring α 2,6-sialylated Tf. The ratio of α 2,6-sialylated to non-sialylated, calculated on SSA-ALI signals, shows significant difference between iNPH ($n = 28$) and non-iNPH ($n = 19$) (Mann-Whitney U tests: $p = 0.015$), indicating that the SSA-ALI is a simple and rapid method for measuring the ratio of α 2,6-sialylated/non-sialylated Tf.

2. Introduction

Idiopathic normal pressure hydrocephalus (iNPH) is a dementia-inducing disorder associated with gait disturbance and urinary incontinence (1, 2). The disease is caused by abnormal metabolism in the cerebrospinal fluid (CSF), which induces ventriculomegaly observable by computed tomography (CT) or magnetic resonance imaging (MRI).

We previously reported that cerebrospinal fluid (CSF) contains two transferrin isoforms: one had \square 2,6-sialylated *N*-glycans whereas the other had non-sialylated *N*-glycans. Glycan analyses revealed that the non-sialylated form had biantennary asialo- and agalacto-complex type *N*-glycans, which carried bisecting \square 1,4-Nacetylglucosamine and core \square 1,6-fucose (3, 4). Sialylated form had biantennary complex type *N*-glycans that carry sialyl \square 2,6-galactose residues.

Non-sialylated Tf was decreased in iNPH and the ratio of isoforms (\square 2,6-sialylated/non-sialylated Tf) differentiated iNPH from Alzheimer's disease. For analysis of the isoform ratio, we measured the isoforms by Western blotting (4) or a lectin-probed ELISA (5), in which anti-transferrin antibody (Tf-ab) and lectins were used for detecting protein and glycan portions of Tf, respectively. These methods, however, are not clinically beneficial to use because they are time- and labour-consuming.

Automated Latex-agglutination Immunoassay (ALI) is a rapid and simple method for measuring protein concentration including serum and CSF proteins (6). For

measuring Tf, small latex particles carrying Tf-ab are mixed with Tf in the specimen and the resulting aggregates are photometrically quantified (7). The assay, however, does not distinguish α 2,6-sialylated and non-sialylated Tf, because Tf-ab equally reacts with both isoforms.

In the present study I demonstrate that even polyclonal antibody is inhibited by lectin binding; *i.e.*, SSA (*Sambucus sieboldiana* Agglutinin) lectin, which binds α 2,6-sialylated epitope, inhibits the binding of polyclonal Tf-ab to α 2,6-sialylated Tf. The finding is applied for measuring α 2,6-sialylated Tf by ALI, resulting in confirmation of our previous conclusion that the alteration of Tf isoforms in CSF is a diagnostic marker for iNPH.

3. Results

3.1 Inhibition of anti-transferrin antibody binding to transferrin by lectins

Human serum transferrin (Tf) has biantennary complex type *N*-glycans that carry Sia α 2,6Gal α 1,4GlcNAc residues, *i.e.*, epitopes for SSA lectin (Fig. 1A). I tested whether or not SSA inhibited binding of anti-transferrin antibody (Tf-ab) to human serum transferrin. ELISA was used for estimating the antigen-antibody reaction. A capture antibody, polyclonal Tf-ab (Cappel), was pre-treated with 1 mM sodium periodate (NaIO₄) to oxidize the exocyclic glycerol side chain of sialic acid, abolishing SSA epitopes on the antibody (8). The periodate-treated antibody was coated on a microtiter plate. Antigen (10 ng/mL of serum Tf) was mixed with a various concentration of SSA lectin, and then the mixture was applied to the plate. After extensive washes, Tf signal was detected with HRP-conjugated anti-human Tf antibody (Bethyl laboratories). As shown in figure 1B, SSA inhibited Tf signal in a dose-dependent manner. The signal (O.D. 1.5) decreased almost linearly in a range of 2 ~ 20 nM of SSA and then saturated in a range of 0.2 ~ 1.86 μ M (ca. O.D. 0.6). Based on the observation, 65 nM of SSA was used in the following inhibition assay (SSA-ELISA). Neither *Maackia amurensis* (MAM) lectin nor BSA inhibited the Tf signal. This result is consistent with the observation that human Tf carries Sia α 2,6Gal α 1,4GlcNAc, an SSA epitope, but not Sia α 2,3Gal α 1,4GlcNAc, a MAM epitope. Human transferrin has two *N*-glycans, which could be partially α 2,6-sialylated. Precise estimation of SSA inhibition signal of these isoforms will need to be examined

with a set of the polyclonal antibodies.

By ELISA, I tested three kinds of antibodies in an SSA inhibition assay; *i.e.*, goat polyclonal Tf-ab from Cappel, rabbit polyclonal Tf-ab from DAKO Co., Ltd. and goat polyclonal Tf-ab from Bethyl laboratories. Their signals associated with serum Tf were inhibited by SSA lectin; ca. 50-60 % inhibition for Cappel antibody (Fig. 1B) and ca. 30% inhibition for DAKO and Bethyl antibodies. The three polyclonal antibodies were more or less inhibited regardless of the species from which each was derived.

3.2 Reactivity of SSA lectin and antibodies against glycoforms of transferrin

To examine glycan specificities in SSA inhibition, I prepared a series of glycan isoforms (glycoforms) of Tf. To abolish SSA epitopes, human serum Tf was treated with 1 mM sodium periodate (NaIO_4) to oxidize the exocyclic glycerol side chain of sialic acid. Tf was also treated with sialidase, yielding asialo-Tf. The asialo-Tf was further treated with galactosidase to prepare agalacto-Tf. As shown in figure 2A, SSA does not inhibit Tf-ab binding to NaIO_4 -treated, asialo- or agalacto-Tf, but does inhibit serum Tf by 62%, suggesting that the terminal α 2,6-sialic acid epitopes on Tf are essential for the inhibition by SSA.

3.3 Reactivity of SSA lectin and antibodies against glycoforms of α 2-macroglobulin

I tested whether SSA inhibition was observed for another α 2,6-sialylated glycoprotein, α 2-macroglobulin (α 2M). SSA inhibition was examined with ELISA,

using anti- α 2M antibody (α 2M-ab) and various glycoforms of α 2M. SSA inhibited α 2M-ab binding to α 2M by 65%, but did not inhibit NaIO_4 -treated, asialo- or agalacto- α 2M (Fig. 2B), suggesting that, regardless of antigen proteins, SSA inhibits antigen-antibody reactions by binding to α 2,6-sialylated-glycans on antigens.

3.4 Reactivity of SSA lectin and antibodies against crude CSF

I next applied SSA inhibition assay for human crude CSF. As shown in figure 3A, human CSF contains both α 2,6-sialylated and non-sialylated Tf (an upper and lower band, respectively, on an immunoblot). The crude human CSF was applied to an SSA-agarose column to fractionate α 2,6-sialylated Tf and non-sialylated Tf, which were recovered in lactose-eluted and flow-through fraction, respectively (Fig. 3A). SSA inhibition is detected with α 2,6-sialylated Tf in the eluted fraction, but not detected with non-sialylated Tf in flow-through fraction (Fig. 3B). These results suggest that SSA inhibition is applicable for measuring α 2,6-sialylated Tf in fractionated CSF. In addition, the ELISA signal in human crude CSF was partially inhibited by adding SSA, indicating that SSA inhibition is applicable even for crude CSF.

3.5 SSA-ALI as a high throughput method for diagnosing iNPH

Next I examined whether SSA also inhibits Tf-ab binding to α 2,6-sialylated Tf in an automated latex-agglutination immunoassay (ALI), which is a rapid and simple method

for measuring protein concentration (6). For measuring Tf, small latex particles carrying Tf-ab are mixed with Tf in the specimen and the resulting aggregates are photometrically quantified. In the assay, specimen was mixed with SSA in a flow cell, and then Tf-ab-coated latex beads were added. For SSA inhibition in ALI, the polyclonal Tf-ab from DAKO was used due to its highest sensitivity. ALI signals with serum Tf were inhibited by SSA lectin in a saturable manner (Fig. 4). Double reciprocal plot analysis revealed that the maximal inhibition is 77% and half maximal inhibition is observed at 8.4 μ M of SSA lectin. Based on this observation, 62.5 μ M of SSA lectin was used in the following inhibition assay (SSA-ALI). A calibration line for quantifying \square 2,6-sialylated Tf was linear at least in a range of 1.25 ~ 20 μ g/ML (Fig. 5A). I then applied SSA-ALI to crude human CSF. Dilution linearity was observed in a range of 0.125 ~ 0.5 μ L of crude CSF. When 0.25 and 0.5 μ g of \square 2,6-sialylated Tf were added to 0.1 μ L of CSF, signal recoveries were $95.7 \pm 11.6 \%$ and $97.5 \pm 10.5 \%$ of the expected value, respectively, suggesting that the interfering substances in CSF, if any, are negligible. Day-to-day variation of the assay was less than 8%. By SSA-ALI, I measured concentration of \square 2,6-sialylated Tf in crude CSF from 47 individuals. The specimen was also measured by a lectin-probed ELISA (5), which was developed for estimating concentration of \square 2,6-sialylated Tf in our previous study. The results of SSA-ALI with 47 individuals were correlated with those of the lectin-probed ELISA ($R^2 = 0.8554$, $p < 0.01$) (Fig. 5B). Thus the SSA-ALI could be a simple and rapid method for measuring \square 2,6-sialylated isoform of Tf in CSF. In addition, the SSA-ALI was successfully applied to diagnosis of a dementia-inducing disorder, idiopathic normal

pressure hydrocephalus, which shows abnormal CSF metabolism and increase of \square 2,6-sialylated /non-sialylated Tf ratio (Fig. 6).

4. Discussion

Fucosylated alpha-fetoprotein (AFP) is an early marker of hepatocellular carcinoma (9, 10). It is notable that non-fucosylated AFP is not the specific marker, indicating that glycoform-specific detection of AFP is essential for accurate diagnosis. The diagnostic isoform, fucosylated AFP, is recognized by *Lens culinaris* Agglutinin (LCA) lectin (10). Suzuki *et al.* found that LCA lectin inhibited the binding of anti-AFP monoclonal antibodies to the fucosylated AFP (11). Only a few monoclonal antibodies were inhibited by LCA, but most were not. Indeed, only two out of thirty MoAbs were inhibited; one was inhibited by 36% and the other was 59% (12). They speculated that LCA binding to the core fucose sterically hindered neighboring antigen epitopes that were recognized by the limited number of monoclonal antibodies (11). It is notable that SSA lectin binds to the most distal glycan epitopes, terminal α 2,6-sialic acids, whereas LCA binds proximally. Tf glycan complexed with SSA at the terminal α 2,6-sialic acids may be flexible enough to hinder a large part of antigen epitopes, which are recognized by a polyclonal Tf-ab. Further analysis is required to clarify the molecular mechanisms of lectin inhibition of antibody binding.

A variety of methods have been reported to measure distinct types of glycoforms of Tf: Western blotting (4), lectin-probed ELISA (5), an anion exchange chromatography (13), isoelectric focusing (14), and capillary electrophoresis (15). The SSA-ALI we developed is more time- and labour-saving than these other methods.

SSA inhibition assay was applied to detect α 2,6-sialylated α 2M as well as α 2,6-sialylated Tf at least in ELISA. The assay may be applicable for other glycoprotein markers that have α 2,6-sialylated glycan. For example, α 2,6-sialylated carcinoembryonic antigen is elevated in serum of colon cancer patients with liver metastases (16). Haptoglobin with α 2,6-sialylated glycans is significantly increased in serum of patients with prostate cancer versus those with benign prostate diseases (17). Measurement of these α 2,6-sialylated glycoproteins by SSA inhibition assay or SSA-ALI would be useful for diagnosing these diseases.

5. Materials and Methods

5.1 Patients

This study included 28 patients with iNPH, including 14 males and 14 females (median age 76 years; range 64 ~ 85 years). The patients were diagnosed using the Clinical Guidelines for iNPH issued by the Japanese Society of Normal Pressure Hydrocephalus (18). The non-iNPH group consisted of 19 patients, including 10 males and 9 females (median age 76 years; range 64 ~ 85 years). Briefly, the diagnosis of possible iNPH was based on the clinical symptoms and signs of gait disturbance, dementia, and urinary incontinence, as well as computed tomography (CT) and magnetic resonance imaging (MRI) findings of enlarged ventricles (Evans index of > 0.3). A bolus infusion test and tap test (removal of 30 mL of CSF) were performed routinely as necessary to determine probable iNPH in all patients. The tap test-negative patients were classified as non-iNPH. The probable iNPH patients were subjected to a CSF shunt operation. The diagnosis of definite iNPH was based on improvement of the symptoms at a month after the operation. The study identified 28 patients who fit the above-mentioned criteria. Shunt non-responders were classified as non-iNPH patients.

This study, including the process of securing informed consent, was approved by the Ethics Committee of Fukushima Medical University (approval number 613), which is guided by local policy, national laws, and the World Medical Association Declaration of Helsinki.

5.2 Lectins

SSA and MAM were purchased from J-Oil Mills, Kanagawa, Japan.

5.3 Transferrin and α 2-Macroglobulin isoforms

Serum Tf and plasma α 2M purified from human blood were purchased from Sigma-Ardrich, St. Louis, MO, USA. Serum Tf or plasma α 2M was treated with sialidase (Nacalai Tesque, Kyoto, Japan) to remove terminal sialic acids, generating asialo-Tf or asialo- α 2M (19). Asialo-Tf or asialo- α 2M was further treated with galactosidase (Seikagaku Corporation, Tokyo, Japan) to remove terminal galactoses, yielding agalacto-Tf or agalacto- α 2M.

5.4 Western blotting and lectin-probed ELISA

For detecting Tf isoforms, Western blotting was optimized as described before (4) and a lectin-probed ELISA was previously developed (5). Briefly, Tf-ab was coated on a 96-well C8 Maxisorp immunomodule plate (Nunc, Roskilde, Denmark). CSF samples were appropriately diluted with TBST containing 0.5 mM EDTA and applied to the plate and incubated at 4°C overnight. After three washes with TBST, the plate was incubated sequentially with a biotin-SSA (Seikagaku Corporation, Tokyo, Japan) and a horseradish peroxidase-labeled streptavidin (Takara-Bio Inc., Kyoto, Japan). After three washes with TBST, the plate was incubated with the TMB substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, USA) and the absorbance at 450 nm was measured in each well by a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

5.5 Lectin inhibition assay by ELISA

Tf-ab (Cappel; ICN Pharmaceuticals, Aurora, OH, USA) and α 2M-ab (R&D Systems, Minneapolis, MN) were treated with 1 mM sodium periodate to oxidize the exocyclic glycerol side chain of sialic acid (8), thus abolishing SSA epitopes on the antibody. The

periodate-treated antibody was coated on a 96-well C8 Maxisorp immunomodule plate. Samples were appropriately diluted with TBST, mixed with 65 nM of SSA lectin as an inhibitor of Tf-ab or α 2M-ab binding (or BSA as a negative control), and incubated at room temperature for 1h. The mixture was applied to the plate and incubated at 4°C overnight. After five washes with TBST, the plate was incubated with HRP-conjugated anti-human Tf antibody (Bethyl laboratories, Montgomery, TX, USA) or α 2M-ab (GeneTex, San Antonio, TX) for 1h. After washing 5 times with TBST, the plate was incubated with the TMB substrate, and the absorbance at 450 nm was measured. In similar assay, MAM lectin was examined for their inhibitory potencies in Tf-ab binding.

5.6 Fractionation of Tf isoforms in human CSF

The crude human CSF was applied to an SSA-agarose (J-Oil Mills) column to fractionate α 2,6-sialylated Tf and non-sialylated Tf, which were recovered in lactose-eluted and flow-through fraction, respectively. After removal of lactose, both fractions were subjected to SSA-ELISA assay.

5.7 SSA inhibition assay by automated latex-agglutination immunoassay

The antibody-sensitized latex particles were prepared as follows. The 190 nm polystyrene latex particles suspended in Buffer A (25 mM HEPES pH 7.5, 1 mM EDTA 2Na, 0.05% NaN₃) were mixed with the equal volume of Buffer A containing the polyclonal Tf-ab (Dako, Glostrup, Denmark) that was pretreated with 1 mM sodium periodate. The final concentrations of the antibody and the particles were 1.5 mg/mL and 0.25% (w/v), respectively. The mixture was stirred at room temperature for 1 h. The antibody-sensitized particles were centrifuged at 59,000 x g for 40 min. The precipitated particles were suspended by sonication in 0.5% Block Ace in Buffer B (25 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA-2NA, 0.05% NaN₃), and then centrifuged at 59,000 x g for 40 min. The precipitated particles were resuspended with Buffer B at the final concentration of 0.05%.

The immuno-agglutination reaction was started by mixing 2 µL of sample solution with 100 µL of the particle reagents and 100 µL of SSA (or BSA) in Buffer B. The immuno-agglutination reaction was monitored at a wavelength of 570 nm with a Hitachi Autoanalyzer H7180 (Hitachi, Tokyo, Japan). Human serum Tf was used as a standard.

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7. Abbreviations:

AFP, alpha-fetoprotein; ALI, automated latex-agglutination immunoassay; α 2M-ab, anti- α 2-macroglobulin antibody; CSF, cerebrospinal fluid; Gal, Galactose; iNPH; idiopathic normal pressure hydrocephalus, LCA, *Lens culinaris* Agglutinin; MAM, *Maackia amurensis*; SSA, *Sambucus sieboldiana* Agglutinin; Tf-ab, anti-transferrin antibody

Conflict of interest

None declared.

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9. Figures and Figure legends

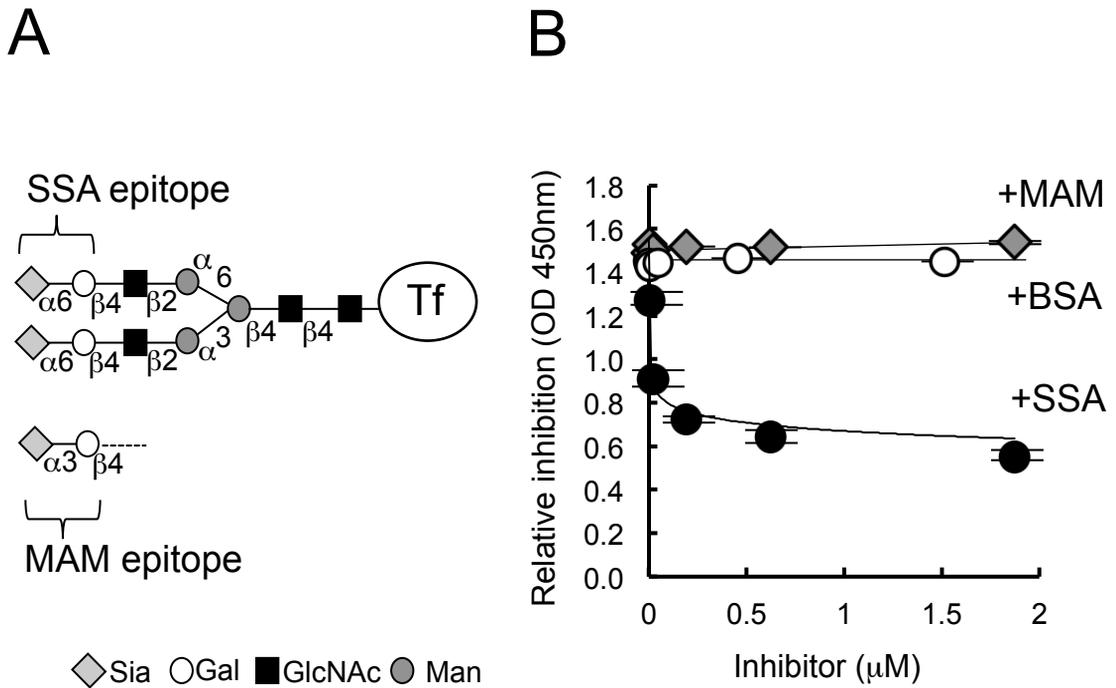


Fig. 1. Lectin epitopes on *N*-glycans of Tf and lectin-dependent inhibition of Tf-ab binding to Tf in ELISA.

Human serum Tf has biantennary complex type *N*-glycans that carry sialyl- α 2,6-galactose β 1,4GlcNAc residues, *i.e.*, SSA epitopes (A) : Sia, sialic acid; Gal, galactose; GlcNAc, *N*-acetylglucosamine; and Man, mannose. An MAM epitope, a sialyl- α 2,3-galactose β 1,4GlcNAc residue, is also illustrated. Effect of lectin addition on Tf-ab binding to Tf was examined in ELISA (B). ELISA signals were plotted in the presence of SSA (closed circle), MAM (gray square) or BSA (open circle) (Mean \pm S.D.). Inhibition was examined in the range of 2 nM \sim 1.86 mM of lectins.

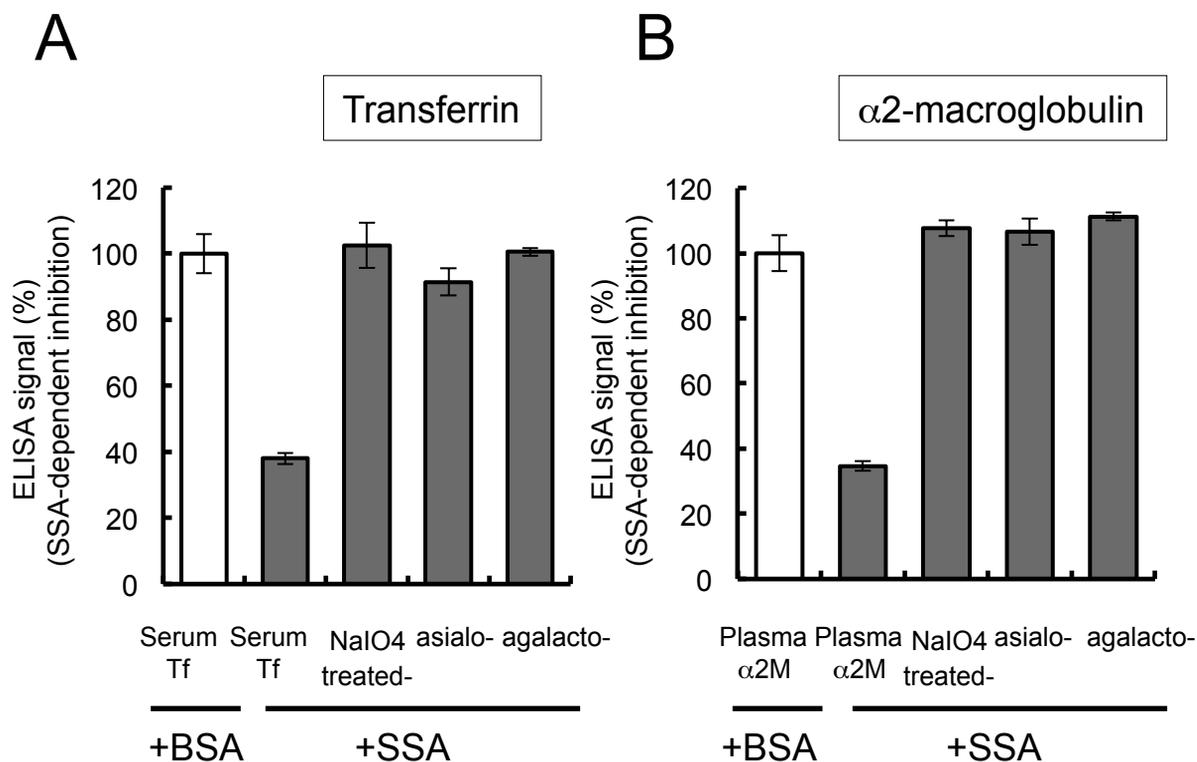


Fig. 2. Glycan specificity of SSA-dependent inhibition of antigen-antibody reactions in ELISA.

SSA was tested for inhibition of antibody binding to glycan-isoforms of Tf (A) or α2M (B). The proteins were treated with sodium periodate to oxidize sialic acid (NaIO₄ treated-). The proteins were also treated with sialidase to remove sialic acid (asialo-), and further treated with galactosidase to remove galactose (agalacto-). SSA Inhibition (%) is defined using BSA as a control (Mean ± S.D.).

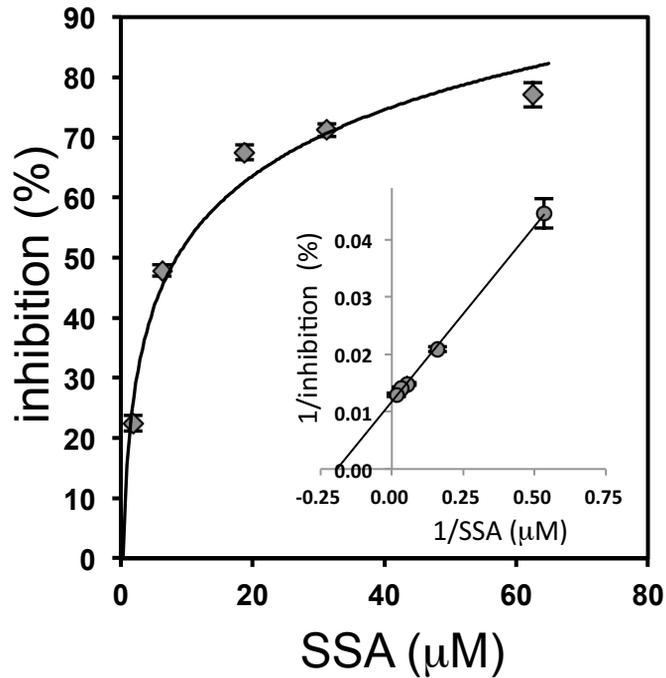
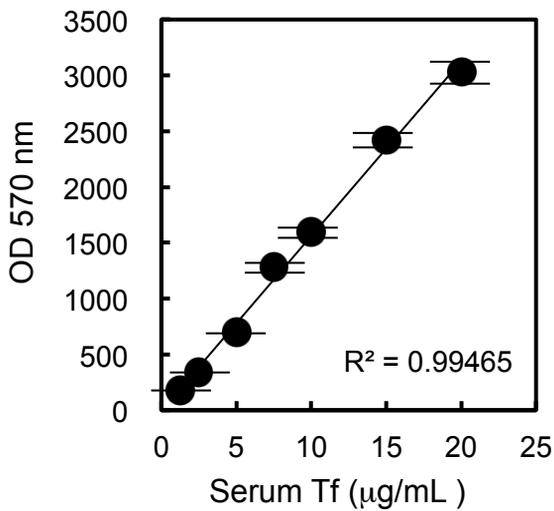
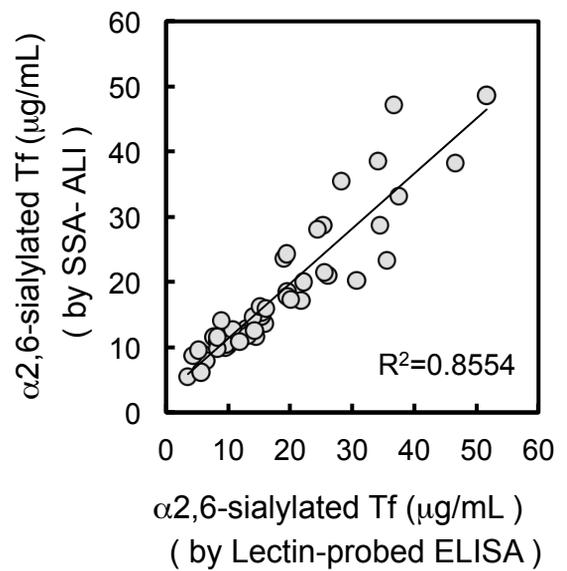


Fig. 4. Dose dependency of SSA concentration on inhibition of Tf-ab binding to Tf in ALI.

Inhibition (%) is defined as difference in ALI signals between the presence and absence of SSA. A dose dependency was examined in the range of 1.9 mM - 62.5 mM of SSA and the results are plotted in a double reciprocal manner in an insert .

A**B****Fig. 5. SSA-dependent inhibition of Tf-ab binding to Tf in ALI**

Based on SSA-dependent inhibition of ALI signals, a calibration line is prepared in a range of 1.25 - 20 mg/mL, using serum Tf as a standard (A). α2,6-Sialylated Tf in crude CSF (n = 47) was measured by SSA-ALI or lectin-probed ELISA, which was previously developed for measuring α2,6-sialylated Tf. ALI data are plotted against lectin-probed ELISA data (B).

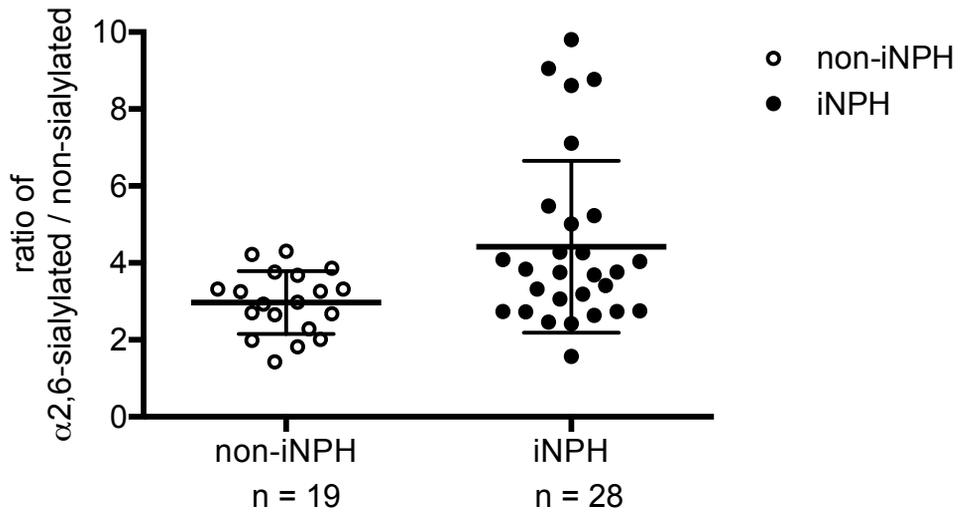


Fig. 6. Ratio of $\alpha 2,6$ -sialylated Tf/non-sialylated Tf in CSF from iNPH and non-iNPH. The ratio of $\alpha 2,6$ -sialylated Tf to non-sialylated Tf was plotted. Non-iNPH (n = 19) is represented with open circles and iNPH (n = 28) is represented with closed circles. Mean + S.D. is indicated with an error bar. The ratio of non-iNPH and iNPH are significantly different ($p = 0.015$, Mann-Whitney U tests).