Identification of Distinct Glycoforms of IgA1 in Plasma from Patients with Immunoglobulin A (IgA) Nephropathy and Healthy Individuals*

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Immunoglobulin A nephropathy (IgAN) is the most common form of glomerulonephritis worldwide and is histologically characterized by the deposition of IgA1 and consequent inflammation in the glomerular mesangium. Prior studies suggested that serum IgA1 from IgAN patients contains aberrant, undergalactosylated O-glycans, for example, Tn antigen and its sialylated version, SialylTn (STn), but the mechanisms underlying aberrant O-glycosylation are not well understood. Here we have used serial lectin separation technologies, Western blot, enzymatic modifications, and mass spectrometry to explore whether there are different glycoforms of IgA1 in plasma from patients with IgAN and healthy individuals. Although total plasma IgA in IgAN patients was elevated ~1.6-fold compared with that in healthy donors, IgA1 in all samples was unexpectedly separable into two distinct glycoforms: one with core 1 based O-glycans, and the other exclusively containing Tn/STn structures. Importantly, Tn antigen present on IgA1 from IgAN patients and controls was convertible into the core 1 structure in vitro by recombinant T-synthase. Our results demonstrate that undergalactosylation of O-glycans in IgA1 is not restricted to IgAN and suggest that in vivo inefficiency of T-synthase toward IgA1 in a subpopulation of B or plasma cells, as well as overall elevation of IgA, may contribute to IgAN pathogenesis. Molecular & Cellular Proteomics 13: 10.1074/mcp.M114.039693, 3097–3113, 2014.

Immunoglobulin A (IgA) nephropathy (IgAN)†, also called Berger’s Disease, was first described by Jean Berger in 1968. More than four decades later, IgAN is the most common form of primary glomerulonephritis worldwide and leads to terminal renal failure in 20–40% of patients over 20–25 years. The majority of primary IgAN cases are sporadic, and only a minority of patients appear within family clusters, yet, no heritable gene associated with the disease has been identified (2). Histologically, IgAN is characterized by deposition of IgA1 and inflammatory lesions in the glomeruli. In contrast to IgA2, human IgA1 contains an extra 13 amino acids in its hinge region (HR) to form a ~20 amino acid domain characterizedly rich in Ser/Thr/Pro residues (3). Six of the 9 Ser/Thr residues are usually modified by the mono- and di-sialylated core 1 structure or T antigen [Neu5Acα2–3Galβ1–3(4)Neu5Acα2–6]GalNAcα-Ser/Thr (3). Many studies (4–8) have suggested that undergalactosylated O-glycans, that is, Tn antigen (GalNAcα-Ser/Thr) and its sialylated version, SialylTn (STn, Neu5Acα2–6GalNAcα-Ser/Thr), are enriched in the HR of IgA1 from patients with IgAN in comparison to IgA1 from normal individuals, and might be responsible for the pathogenesis of IgAN. However, the mechanism(s) underlying the undergalactosylation of IgA1 from patients with IgAN is unclear.

Mucin type O-glycosylation (O-glycan) is a common protein post-translational modification of Ser/Thr residues of secreted and transmembrane glycoproteins and can regulate many aspects of their functions and recognition properties (9–14). Within human immunoglobulins only IgA1 and IgD are O-glycosylated in their HR domains (15, 16). The biosynthesis of O-glycans mainly takes place in the Golgi apparatus by serial reactions of a group of glycosyltransferases. In humans, polypeptide-GalNac-transferases (ppGalNAC-Ts) encoded by at least 20 ppGalNAC-Ts, initiate O-glycosylation in the Golgi (17, 18), and possibly in the ER (19, 20), by transferring a GalNAc from UDP-GalNAc to Ser or Thr residues in proteins passing through the secretory pathway to form GalNAcα-Ser/Thr (Tn antigen) (Fig. 1). For the generation of the Tn antigen on IgA1 ppGalNAcT2 may be the key enzyme responsible (21). Typically, the Tn antigen is subsequently efficiently converted to...
UDP-Gal to GalNAc

/H9251

polypeptide

Core 1 [Neu5Ac

STn antigen (Neu5Ac

CMP-Neu5Ac to form

t-synthase. In all cell types, the T antigen is usually sialylated by ST3Gal-I, which transfers the N-acetylneuraminic acid (sialic acid) from

antigen. Because of poor efficiency of ST6GalNAc-I, it is likely that only very high expression of ST6GalNAc-I could outcompete functional

t-synthase. In hematopoietic cells, besides synthesis of sialyl core 1 structures, the

t antigen is usually further modified by core 2

3galactosyltransferase (T-synthase) (22)

Cosmc is the specific molecular chaperone for the

Tn antigen does not

occur in distinct glycoforms and whether differential expres-

sion of these glycoforms might correlate with disease. Here we report the unexpected discovery that human plasma

IgA1 can be separated into distinct glycoforms by serial lectin affinity chromatography: one glycoform contains normal mono/di-sialylated T antigen whereas the other contains exclusive Tn/STn antigens, which was further confirmed by in vitro conversion into T antigen by recombinant human T-synthase and mass spectrometry analysis. We further explored the basis for formation of these distinct glycoforms using cell lines and enzymatic modifications. The identification of the distinct glycoform of plasma IgA1 carrying Tn/STn antigens offers a new direction for future studies aimed at identifying its potential contribution to IgAN.

EXPERIMENTAL PROCEDURES

Plasma Samples and Cell Culture—Blood samples from both

biopsy-proven patients with IgAN and healthy controls were ob-

tained from the Emory Clinic under the approved IRB protocol

(IRB00008410). Information from all donors is reported in Table I. The

plasma, erythrocytes and leukocytes were separated using Lym-

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manufacturer’s protocol. All plasma samples were aliquotted and

stored at −80 °C, or −20 °C during experiments. Dakiki cells (ATCC,

TIB-206) and Tn4 cells (38) were grown in RPMI1640 (Invitrogen,

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room temperature (RT) and then washed three times with PBS-T.

Plasma samples diluted 1:10,000 in PBS-T were incubated in dupli-

the core 1 structure Galβ1–3GalNAcα-Ser/Thr by a single

enzyme, the core 1 β3galactosyltransferase (T-synthase) (22)

which utilizes UDP-Gal as the donor and GalNAcα-Ser/Thr in

proteins as the acceptor. Moreover, the T antigen is usually

further modified, branched and/or elongated to form complex

normal O-glycans, including sialyl Core 1 (or sialyl-T), Core 2

O-glycans and extended Core 1 O-glycans in glycoproteins of

hematopoietic cells (11, 23). Thus, the Tn antigen does not

normalize in significant amounts in cellular glycoproteins

(Fig. 1). Interestingly, biosynthesis of active T-synthase

requires an ER-localized molecular chaperone Cosmc to pre-

vent its aggregation and subsequent proteosomal degrada-

tion (24–26). Defects in Cosmc on Xq24 because of somatic

mutations (27), gene deletion (28), or epigenetic silencing of

its promoter region (29) result in an inactive T-synthase and

consequent expression of the Tn and STn antigens on glyco-

proteins. Such altered O-glycosylation is associated with the

pathology of several human diseases such as Tn syndrome

(30, 31), in which patients have a sporadic acquired mutation

in X-linked Cosmc in hematopoietic precursors, and in neo-

plastic transformations (32, 33). However, in the case of IgAN

no mutation in either Cosmc/T-synthase or other glycosyl-

transferases has been identified, although there are conflict-

ing studies suggesting that compromised transcription of

Cosmc and/or T-synthase, up-regulated expression of ST6GalNAcII, or decreased T-synthase activity in the per-

ipheral blood B cells from patients with IgAN may contrib-

ute to aberrant expression of Tn and STn antigens in IgA1

HR (8, 34–37).

Although previous studies suggested the expression of Tn/

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Carlsbad, CA) containing 20% heat-inactivated fetal bovine serum at

37 °C, 5% CO2.

ELISA Assays—Flat-bottomed 96-well ELISA Microplates (Greiner

bio-one, Frickenhausen, Germany) were coated overnight at 4 °C with

50 µl of 1 µg/ml F(AB′)2 fragment of goat IgG anti-human IgA (Jackson

Immuno-Research Labs, West Grove, PA) in 0.05 M carbonate/bicar-

bonate pH 9.6 buffer. Coated plates were blocked with 1% BSA in

phosphate-buffered saline (PBS)-0.05%, Tween 20 (PBS-T), 1h at

room temperature (RT) and then washed three times with PBS-T.

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phosphate-buffered saline (PBS)-0.05%, Tween 20 (PBS-T), 1h at

room temperature (RT) and then washed three times with PBS-T.

Plasma samples diluted 1:10,000 in PBS-T were incubated in dupli-
cate for 1h at RT, and washed three times with PBS-T. The wells were incubated with peroxidase labeled goat anti-human IgA (λ) (KPL, Gaithersburg, MD) at 0.25 μg/ml in PBS-T solution 1h at RT, and then washed 4 times with PBS-T. The signal was revealed with o-phenylenediamine dihydrochloride (Thermo Scientific, Waltham, MA) according to manufacturer’s instructions. Serial dilutions of commercial human IgA (InvivoGen, San Diego, CA) were used as a standard for quantification. All sample measurements were done twice.

Pull Down Assays—Peanut agglutinin (PNA)-bound Agarose beads were purchased from Vector Labs (Burlingame, CA); Helix pomatia agglutinin (HPA)-beads were generated by coupling free HPA lectin (Sigma Aldrich, St Louis, MO) to Affi-gel 10 beads (Bio-Rad, Hercules, CA) in the presence of GaINAc. Briefly, HPA was resuspended (2 mg/ml) into a 50 mM GalNAc solution and 500 μl of this solution was incubated (1h at RT, in rotation) with 1 ml Affi-gel 10 beads first washed with 10 ml of ice cold water. The beads were then pulled down by centrifugation (1000 rpm, 3min), the supernatant removed, and the beads were incubated 1h with 1 ml of GaINAc solution and 500 μl of this solution was incubated (1h at RT, in rotation) with 1 ml Affi-gel 10 beads first washed with 10 ml of ice cold water. The beads were then pulled down by centrifugation (1000 rpm, 3min), the supernatant removed, and the beads were incubated 1h with 1 ml of 1 M Glycine at RT, in rotation. The Glycine solution was removed and the beads were washed 3 times with 10 ml of PBS, and finally resuspended into 2 ml of PBS and stored at 4 °C.

Plasma samples were diluted 1:2 with PBS and then treated or not with neuraminidase (from Clostridium perfringens, Roche, Germany) (1 μl of neuraminidase for 10 μl of plasma) overnight at 37 °C. PNA and HPA pull downs were performed at RT, 2h, in rotation. After centrifugation (1000 rpm, 3min), the supernatants were collected and designated as Unbound fraction (UB). Following two washes with PBS, the beads were eluted by incubation (15min, RT, in rotation) with an equal volume of the UB fraction of 0.2 M GaINAc for the HPA pull down or 0.2 M Lactose for PNA pull down. The eluted fraction, termed the Bound fraction (B), was collected after centrifugation at 1000 rpm for 3min.

The Anti-Human IgA (α-chain specific)-Agarose beads were purchased from Sigma-Aldrich. Total IgA pull down was performed at 4 °C overnight in rotation. After centrifugation (1000 rpm, 3min) the beads were transferred into a 5 ml Poly-Prep Chromatography Column (Bio-Rad) and washed 3 times with PBS. Beads were eluted 7 times with 100 μl of 0.2 M Glycine pH 2.6. All fractions were pooled, washed and concentrated in water using a centricron unit (Millipore, Billerica, MA), to a final volume of 100 μl. The IgA concentration of each sample was determined by Pierce® BCA Protein Assay Kit (Thermo Scientific).

IgA1-Hinge Region Specific-Tn Antibody Western Blot—Mouse anti-human IgA1 (α1 chain specific) FITC-conjugated and mouse anti-human IgA2 (α2 chain specific) FITC-conjugated were purchased from (Southern Biotech, Birmingham, AL). Rabbit anti-FITC HRP-conjugated antibody was purchased from Dako (Denmark). The goat anti-human IgA (α chain specific) HRP-conjugated antibody was purchased from KPL. PNA HRP-conjugated and HPA HRP-conjugated were purchased from Sigma Aldrich. Goat anti-mouse HRP-conjugated antibody was purchased from KPL.

The 6E5–7Tn antibody was produced as follow. A 20-mer peptide covering the Ser/Thr rich hinge region of IgA1 was synthesized by Schafer-N (Denmark) and in vitro glycosylated using recombinant human ppGalNAcTs GaINAc-T1 and GaINAc-T2 (39) adding in total 7 mols of GaINAc per peptide (VPSTPTTPSPSTPTPS). The glycosylation reactions were monitored by MALDI-TOF mass spectrometry. The IgA1-HR-7Tn glycopeptide was conjugated to KLH (Pierce) using gluteraldehyde as previously described (40, 41), and conjugation efficiency was evaluated using comparative titration analysis of the KLH-conjugated peptide versus the corresponding non-conjugated glycopeptide in an ELISA. Female Balb/c mice were injected subcutaneously with 20 μg KLH-conjugated glycopeptide in a total volume of 200 μl (1:1 mix with Freund’s (complete (first immunization only) and incomplete adjuvant (Sigma) for subsequent injections) under the Danish Animal Experiments Inspectorate approved protocol. Mice received 3 immunizations 14 days apart and blood was collected from the orbital sinus 1 week following the third immunization. After fusion of splenocytes from the immunized mouse with NS-1 myeloma cells, the hybridoma clones were screened based on positive reactivity to the glycopeptide immunogen and negative reactivity to the non-glycosylated peptide and various other control Tn-peptides in a direct ELISA. The monoclonal mouse IgG1 specifically reacting to the IgA-HR-7Tn glycopeptide, designated as 6E5–7Tn, was generated, and the purified 6E5–7Tn (from hybridoma media using Protein-G column) was used for the experiments.

For all lectin (HPA and PNA) and Western (IgA, IgA1, IgA2, and 6E5–7Tn) blots, samples were boiled for 5 min in reducing conditions and run into 4–20% Mini-PROTEAN® TGX™ precast gels (Bio-Rad, Richmond, VA) and then dried- transferred onto a nitrocellulose membrane using the iBlot® Blotting System and the iBlot® Gel Transfer Stacks, Mini Nitrocellulose (Invitrogen).

For the PNA, HPA, and IgA blots, the membranes were blocked 1h at RT with a Tris-Buffered Saline (TBS) solution containing 0.1% Tween 20 and 5% (w/v) BSA and then incubated with HPA- or PNA-HRP 1 μg/ml in TBS, 0.1% Tween 20, 5% (w/v) BSA for 1h at RT, or incubated with goat anti-human IgA HRP-conjugated antibody at 0.2 μg/ml in TBS for 10 min at RT. The membranes were finally washed four times with TBS, 0.1% Tween 20.

For the IgA1, IgA2, and 6E5–7Tn blots, the membranes were blocked 1h at RT with TBS solution containing 0.1% Tween 20 and 5% (w/v) non-fat dried milk and incubated with mouse anti-human IgA1 or IgA2 FITC-conjugated at 1 μg/ml or with mouse 6E5–7Tn antibody at 1 μg/ml in TBS, 0.1% Tween 20, 5% (w/v) milk overnight at 4 °C. The membranes were then washed four times with TBS, 0.1% Tween 20 followed by incubation with rabbit anti-FITC HRP-conjugated for IgA1 or IgA2 at 1 μg/ml or with goat anti-mouse HRP-conjugated antibody at 1 μg/ml in TBS, 0.1% Tween 20, 5% (w/v) non-fat dried milk 1h at RT. The membranes were finally washed four times with TBS, 0.1% Tween 20.

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Quantification of Lectin and Statistical Analysis on Western Blot Data—Lectin and Western blot films were scanned using a Cannon scanner and band intensities were quantified using an Alphatech system. The intensity of each band was measured and subtracted from the background value. Statistical differences were analyzed using an unpaired Student’s t test, p value < 0.05 were considered significant.

Conversion of Tn Antigen in IgA1 to T Antigen in Vitro by Recombinant T-Synthase—Soluble recombinant human T-synthase was produced and purified as described previously (26). For the T-synthase reaction on IgA1: 10 μg of plasma-purified IgA from IgAN patient P7 and from control individual C16 were incubated with guanidinium hydrochloride (6 M final) or with the same volume of water for 1h at RT. Samples were subsequently washed with water and concentrated with Centrifugal Filter Units (4 ml, 10,000 Da cut-off, Amicon® Ultra, Millipore, Billerica, MA) to a final volume of 50 μl. Then 6

| TABLE I | IgAN patients and healthy controls information |
| Age (years) | Gender ratio (M:F) |
|----------|------------------|
| IgAN patients | 44.57 ± 13.34(S.D.) | 8:6 |
| Healthy controls | 32.33 ± 16.94(S.D.) | 6:9 |
μl of recombinant T-synthase, UDP-Gal (Calbiochem, San Diego, CA) (final concentration 1.4 μM) or water, and MnCl₂ (final concentration 20 μM) were added to the IgA concentrated solution. The mixture was incubated overnight at 37 °C.

Semiquantitative PCR—Total RNA was extracted from 2 × 10⁶ Dakiki or Tn4 cells with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to manufacturer’s instructions. The primer pairs were synthesized by Integrated DNA Technologies (Coralville, IA) and are as follows:

Cosmc, forward: GCTCCTTTTTGAAAGGTTGTG reverse: TACTGCCAGCCAAAAGACTCA, T-synthase, forward: AAGGTGACCACCCAGCCTAA, reverse: CTTTGAGCTTGTTGCTGCTT, ST6GalNAcI, forward: GCAACACACAGCAAAGGCTCCTATCCCAA, reverse: TGTCAGGACCTTGTGCACCAAGGAGTATG, ST6GalNAcII, forward: GGAATGTGCCGTTGAGTTGCTCAAG, reverse: AGCGAATCAACCCCTACCAAGTGCAGCACCCCTC. The reaction mixes were composed of 4 μl of cDNA for a final volume of 20 μl. PCR amplifications were performed using the following cycles program: 98 °C 30s, then 37 cycles of 98 °C 10s, 60 °C 20s, and 72 °C 10s, followed by a final extension of 5min at 72 °C. PCR products were separated on a 2% agarose gel.

N- and O-Glycan Removal and Mass Spectrometry Analysis—IgA from pooled IgAN patient and healthy control individual plasma samples were purified as described above and IgA concentration determined (Pierce® BCA Protein Assay Kit, Thermo Scientific).

N-glycans were released from ~70 μg of purified IgA in a total volume of 90 μl using the PNGase F enzyme (New England Biolabs, Ipswich, MA), and 1 μl of cDNA for a final volume of 20 μl. PCR amplifications were performed using the following cycles program: 98 °C 30s, then 37 cycles of 98 °C 10s, 60 °C 20s, and 72 °C 10s, followed by a final extension of 5min at 72 °C. PCR products were separated on a 2% agarose gel.

RESULTS

Plasma Concentration of IgA is Increased from IgAN Patients, and O-Glycans on Plasma IgA From Both IgAN Patients and Control Donors Similarly Contain Tn and STn Structures—A common feature reported in IgAN patients is an increase of plasma IgA as compared with healthy controls (42). We determined the concentration of plasma IgA by ELISA, and found that, consistent with earlier studies, IgA was present at an average of 4.77 mg/ml (± 1.09 mg/ml) from 14 IgAN patients, whereas in control plasma from 14 healthy donors we found 2.97 mg/ml (± 0.70 mg/ml) IgA, thus representing a low, but significant ~1.6 fold elevation of IgA in patients with IgAN (Fig. 2A).

In profiling the O-glycans on glycoproteins such as IgA in plasma samples, we next examined their recognition by lectins that can discriminate between Tn and T antigens. Helix pomatia agglutinin (HPA) binds with high affinity to terminal α-GalNAc residues on O-glycans, such as the Tn antigen (43, 44). In previous studies, HPA has been used to identify the presence of Tn-containing glycans in total IgA1 by ELISA (45, 46). In our study we prepared immobilized HPA in order to test whether there are specific glycoforms of IgA1 enriched in Tn and/or STn. Plasma samples were either untreated or treated with neuraminidase to expose potential T and Tn antigens. For the other patient or control samples, the removal of sialic acids resulted in only a slight increase in IgA1 staining in the HPA’ fraction. These results indicated that among the IgA1 molecules that have under-galactosylated O-glycans, the Tn and STn antigens are common, and that IgA1 from both IgAN patients and control individuals contained IgA1 expressing the Tn and STn epitopes.

We next evaluated the percentage of Tn/STn-containing IgA1 among the total IgA1 population with the same method
O-Glycans of Human IgA1

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Fig. 2. Analysis of Tn/STn content of IgA1 from plasma: A. Total IgA plasma level (mg/ml) of IgAN patients and healthy control individuals, determined by ELISA (**, p < 0.01). B. Western blot analysis of plasma samples after HPA-bead pull down, immunblotted with anti-human IgA1 (κ1 chain specific) antibody. All samples were treated with (+) or without (−) neuraminidase prior to HPA chromatography. Results of 14 IgAN patient samples (labeled P) are shown in the two sets of upper panels, where the top panels show the HPA− fractions and the bottom panels show the HPA+ fractions. Results of 14 control samples (labeled C) are shown in the two sets of lower panels, where the top panels show the HPA− fractions and the bottom panels show the HPA+ fractions. C. Western blot analysis of plasma samples after HPA-bead pull down, immunblotted with anti-human IgA1 (κ1 chain specific) antibody. All samples were treated with neuraminidase prior to experiments. An equal volume of plasma sample was used as the input (I) and used for HPA pull down experiments. All subsequent unbound (UB) and bound (B) fractions were loaded into the gel for analysis. D. Quantification of HPA−-IgA1 staining (B) and total IgA1 (I). All observable bands in I and B fractions were quantified. The HPA−-IgA1 fraction intensity is expressed as a percentage of the IgA1 in the corresponding input fraction. The difference of HPA−-IgA1 percentage between the IgAN patients and control samples was NS. Vertical black lines between Western blot lanes indicate that lanes were not contiguous on the same gel or from different gels. ELISA assays were performed in duplicate, twice independently.
of HPA pull-down. IgA1 staining intensity in the bound glycoforms (HPA\(^{-}\)) was compared with the input and expressed as a percentage of the total IgA1 (Fig. 2C, 2D). In Fig. 2C, higher molecular weight bands stained with anti-human IgA1 (\(\alpha_{1}\) chain specific) antibody were consistently seen in plasma as the amount of IgA1 heavy chain (55 kDa) increased and probably represented the oligomers of IgA/IgA1 and/or their incompletely reduced forms. These bands were considered as IgA1-specific signal in the signal quantification. Both groups of samples showed similar content of bound IgA1 (16.50% of HPA\(^{-}\)-IgA1 for IgAN patients samples and 19.31% for control individuals) (Fig. 2D). Thus, overall in IgAN patients, whereas there is a 1.6-fold elevation in total IgA, the relative percentage of IgA1 with the HPA\(^{-}\) glycoform after desialylation in IgAN patients is similar to that in controls.

IgA1 Isolated from IgAN Patients and From Healthy Controls Exhibit Similar PNA and HPA Reactivity—The above results appear to conflict with many studies on IgA1 O-glycosylation suggesting that the presence of Tn and/or STn antigens on IgA1 is a characteristic change in IgA nephropathy (42, 47, 48). To further test our results indicating that Tn and/or STn antigens are found in IgA1 from IgAN patients and controls, we analyzed affinity purified IgA from plasma samples of all IgAN patients and controls by lectin blots with HPA and with peanut agglutinin (PNA) after treatment with neuraminidase. PNA binds with high affinity to the non-sialylated T antigen in glycoproteins (49), whereas HPA recognizes the Tn antigen by recognition of the \(\alpha\)-linked GalNAc (43, 44). To assess and validate our method to semi-quantify the lectin staining intensity, we chose one pair of IgAN patient and control samples for each lectin staining: The P14 and C19 samples were used for HPA lectin blots and P3 and C10 samples for PNA lectin blots, repeating the experiment three times for each sample. As shown in Fig. 3A the relative intensity of PNA staining versus the IgA1 input measured between samples was constant with low measurement variations between replicates. A similar result for HPA/IgA1 staining was shown in Fig. 3B although the higher intensity of HPA with a higher variation was observed in P16 sample, but nevertheless, the trend of differences between samples remained the same. These results demonstrate that the method used is reliable to semi-quantify and compare the relative HPA and PNA staining between patients with IgAN and healthy individual samples. As expected, all purified desialylated IgA samples contained a glycoform that was stained with PNA (Fig. 3C, 3D top panels), and most of the samples from IgAN patients were also stained with HPA (Fig. 3C mid panel). Consistently, most of the samples from healthy individuals were also stained with HPA (Fig. 3D mid panel) in a similar fashion to the samples from IgAN patients. In order to quantitatively evaluate the PNA-
HPA-reactive IgA1, the staining intensity of each sample was measured and normalized by its corresponding IgA1 intensity. As shown in Fig. 3E,3F, normalized PNA and HPA intensities for patient group and controls were plotted. Although variations were observed between individuals, there were no significant differences in either HPA or PNA staining between samples from IgAN patients and controls. Because only the HR of IgA1 but not IgA2 is O-glycosylated (50), our results showed no overall differences in the types of O-glycosylation of IgA1 between IgAN patients and healthy control individuals, and Tn and STn structures naturally occur on plasma IgA1. Consistent with our results in Fig. 2, this data, which are contrary to prior reports that Tn/STn antigens on IgA1 are the characteristics of IgA1 from IgAN, further confirm that these antigens are present on IgA1 of both IgAN patients and control donors.

Plasma from IgAN Patients and Healthy Controls Contain Two Pools of IgA1 With Distinct Glycoforms that can be Distinguished by PNA or HPA Lectins—The above results suggest that a fraction of IgA1 in the IgA pool from plasma samples of both IgAN patients and control donors might contain O-glycans with Tn and STn structures. Human IgA1 HR contains nine potential O-glycosylation sites with four to six of them usually occupied (3). Thus, we questioned whether the O-glycans on the IgA1 with Tn/STn antigens were composed of homogeneous or heterogeneous structures. For example, a homogeneous aberrant O-glycosylated IgA1 population would contain Tn/STn glycans on all the glycosylation sites and lack the core 1 (or T antigen) based O-glycans, whereas a microheterogeneous IgA1 population would contain all types of glycans on the same IgA1 molecule including Tn/STn and core 1 based O-glycans. To address this question, we devised an experimental approach that incorporated serial lectin chromatography. Plasma samples were first treated with neuraminidase and then chromatographically separated by HPA-beads into the HPA-bound (HPA+) and HPA-unbound (flow through, HPA−) materials. These two materials were then further chromatographed on PNA-beads to obtain PNA-bound (PNA+) and PNA-unbound (PNA−) materials. These two materials were then further chromatographed on PNA-beads to obtain PNA-bound (PNA+) materials, which would represent PNA-bound (PNA+) materials HPA−PNA− (B,B) and HPA−PNA− (U,U), as well as PNA-unbound (PNA−) materials HPA−PNA− (B,U), HPA−PNA− (U,U), as schematically illustrated in Fig. 4A. These subsequent fractions were analyzed by Western blot using anti-IgA1 antibody.

Eight randomly selected plasma samples from each group, IgAN patient and control, were treated with neuraminidase.
prior to HPA-bead chromatography. To measure the capacity of the lectin-column and avoid the potential problem of overloading, in a preliminary experiment, HPA− fraction (flow through) from the HPA column was reloaded onto a new HPA column, and HPA bound and unbound fractions were blotted with anti-IgA1. The HPA+ IgA1 was only present in the first-round chromatography, but not in the second-round fraction, demonstrating that the conditions used could exclusively and quantitatively isolate the HPA+ IgA1 from both IgAN patients and control individuals consisted of in Fig. 4. Unexpectedly, the results shown in Fig. 4 demonstrated that the majority of plasma samples from both IgAN patients and control individuals were present only in the U,U lane, as predicted, corresponding to the double negative material (HPA−PNA−), as revealed by anti-IgA2 antibody (Fig. 4C). Unexpectedly, the results shown in Fig. 4D demonstrated that the majority of plasma samples from both IgAN patients and control individuals consisted of mainly two glycoforms of IgA1: one being HPA−PNA− expressing only Tn/STn antigens and lacking the T antigen, and the other being HPA+PNA+ expressing only T antigen with or without sialic acid. Some of the IgA1 was HPA+PNA−, which may represent a glycoform with complex O-glycans beyond sialylT, such as core 2-related structures, or non O-glycosylated, that in either case would not bind either HPA or PNA. This material was not further examined. Interestingly, in addition to those two glycoforms of IgA1, only sample P2 contained some of the HPA+PNA+ (B,B) material, which would represent microheterogeneous structures (a combination of Tn/STn and T or Sialylated T). These results indicate that plasma IgA1 molecules from both patients with IgAN and control donors are distinguished by two major glycoforms: one expressing Tn/STn (B,U fraction), whereas the other expressing normal O-glycans (U,B and U,U fractions). We found that both of the glycoforms were present within plasma IgA1 from both patients with IgAN and controls. Moreover, there were no significant differences in terms of ratios of these two distinct glycoforms of IgA1 between samples from patients with IgAN and healthy individuals.

**Tn Antigen on IgA1 from Control and IgAN Patient Samples Can be Converted to T Antigen In Vitro by Recombinant Human T-Synthase**—To further verify that the HPA−PNA− IgA1 glycoform contains the Tn/STn structures and lacks the T antigen, we incubated purified IgA1 with soluble human recombinant T-synthase to test whether the Tn structure could be enzymatically converted in vitro to the T antigen. Afterward, we determined the reactivity of the treated sample to HPA and PNA, as well as to a monoclonal antibody 6E5–7Tn, which recognizes the Tn antigen in the context of the HR of IgA1 (unpublished data). As shown in Fig. 5A, 5B, 6E5–7Tn bound well to the IgA1 in P2 plasma and to purified plasma IgA1 from both control C16 and IgAN patient P7. The P2 plasma sample was treated with neuraminidase to ensure that a maximum of Tn antigen sites were exposed. The specific expected signal, at ~55 kDa corresponding to the 6E5–7Tn on IgA1, was seen as a thin band because of compression by the massive amount of albumin in the plasma. Without neuraminidase treatment, the 6E5–7Tn antibody was also able to recognize the Tn antigen in the context of IgA1-HR, suggesting here and as in Fig. 2B that the HR of IgA1 was mostly represented by Tn antigens and not STn antigens. Consistently, the 6E5–7Tn antibody also bound to the HPA−PNA− glycoforms of IgA1 from P3 plasma, but did not bind to the HPA−PNA+ glycoform from the C14 sample, in accordance with our previous HPA staining results. Importantly, 6E5–7Tn staining was inhibited by addition of synthetic IgA1 hinge region glycopeptides carrying four to ~six T antigens, but not by 20 mM GalNAc (Fig. 5A, 5B), demonstrating the specificity of the antibody. The bands at ~50 kDa and ~100 kDa visible in Fig. 5B, as well as very mildly in Fig. 5A, for the P2 plasma sample, were considered as nonspecific plasma-related signal. Indeed, these two bands were only observed in the plasma sample and not in any of the purified IgA samples. Importantly, the staining of these two bands was unaffected by the presence of IgA1-HR Tn-glycopeptides, whereas the 55 kDa specific signal corresponding to the α chain of IgA1 was abolished.

To test as substrates of T-synthase, the two purified IgA samples, P3 (IgAN patient) and C16 (control), were treated with or without guanidinium hydrochloride (Gdm-HCl) which denatures proteins such as IgA1, and allowed us to explore the efficiency of the T-synthase toward native versus denatured IgA1. In the presence of T-synthase and the donor UDP-Gal, but without treatment with Gdm-HCl, IgA1 was stained by both HPA and 6E5–7Tn (Fig. 5C, 5D). However, when both IgA samples were denatured with Gdm-HCl and incubated with T-synthase in the presence of UDP-Gal, staining of both HPA and 6E5–7Tn on IgA1 was lost (Fig. 5C, 5D). Given the fact that the T-synthase is a specific enzyme that recognizes only the Tn antigen acceptor, these results demonstrate that the Tn antigen was present in the IgA1 from both patients with IgAN and the healthy controls. Interestingly, staining of HPA and 6E5–7Tn was decreased but not completely abolished when the IgA1 samples were not denatured by treatment of guanidinium hydrochloride, indicating that the conformation of IgA1 might hinder the recognition of IgA1 by T-synthase. Taken together, these results confirm that the Tn/STn antigen occurs on a fraction of IgA1 in plasma from both patients with IgAN and healthy donors.

**IgA1-Secreting B Cell Line Dakiki Exclusively Produces IgA1 With Core 1-Based Glycoform**—The Dakiki cell line has been previously characterized as an IgA1-secreting B cell line (29, 51). Using ELISA-based assays with the *Helix aspersa* agglutinin (HAA) lectin, previous studies reported that IgA1 secreted by the Dakiki cells was galactose-deficient and mostly sialylated, that is, STn O-glycans (51). Thus, we reasoned that the Dakiki cell line would be useful to explore the mechanism underlying the STn on the HR of IgA1. We first examined
the expression of Cosmc, T-synthase, ST6GalNAcI, and ST6GalNAcII, whose corresponding chaperone and glycosyltransferases are involved in the formation of T, STn, and sialylT antigen structures respectively (Fig. 1). As shown in Fig. 6A, Cosmc/T-synthase, whose products are responsible for the formation of T antigen, and ST6GalNAcII whose product is primarily responsible for synthesizing the sialylT and disialylT structures, are expressed in Dakiki cells, whereas ST6GalNAcI, which encodes a sialyltransferase primarily responsible for synthesis of STn antigen on O-glycoproteins, is not expressed. As demonstrated in previous studies (29, 38), the human B cell line Tn4 cells do not express Cosmc and ST6GalNAcI genes, but do express ST6GalNAcII. Tn4 cells, which have an epigenetically silenced Cosmc (29), expressed Tn antigen but not core 1-based (T antigen) structures (Fig. 6B), whereas total Dakiki cell extracts exhibited strong PNA staining only after neuraminidase treatment, but were only weakly stained with HPA. These results demonstrate that Dakiki cells express normal sialylated core 1-based O-glycans in cellular glycoproteins. Isolated IgA1 from the media of Dakiki cells was quantitatively bound by PNA after desialylation, but not by HPA, demonstrating that IgA1 secreted from Dakiki cells contains normal O-glycans, and little if any Tn/STn antigen (Fig. 6C). This is interesting in light of our finding that Dakiki cells do express ST6GalNAcII, confirming prior studies (29, 51), yet do not synthesize significant amounts of STn on glycoproteins including IgA1. PNA staining of IgA1 isolated from Dakiki was similar to that of IgA1 isolated from both sets of plasma samples whereas HPA staining was totally absent (Fig. 6C, 6D). Consistent with this result, and in contrast to the IgA1 from patients with IgAN and healthy controls, IgA1 from Dakiki cells was exclusively bound by PNA-beads but not by HPA-beads (Figs. 6E, 2B). Taken together, these results demonstrated that the IgA1 secreted from Dakiki cells lacks either Tn or STn antigens, and expresses normal sialylated core 1-based O-glycans. These results also strongly argue against a role for ST6GalNAc-II in STn formation on IgA1 as suggested by earlier studies (8, 51, 52). Furthermore, the results show that in this cell line a single major glycoform (HPA+ H11002 PNA+H11001) of IgA1 is generated. Thus, the formation of different glycoforms of IgA1 is rather a cellular phenomenon and not inherent to all IgA1 molecules.
To further test our conclusions regarding O-glycosylation and different glycoforms of IgA1, we investigated the O- and N-glycans on the distinct glycoforms of IgA1 by MS analysis. We chose to analyze the total O-glycans after their release from the intact IgA or IgA1 rather than from HR glycopeptides, because the heterogeneity of the total HR glycopeptides makes the full analysis of all glycopeptides difficult, and inefficiencies and instabilities occur regarding glycopeptides in typical MALDI-TOF ionization approaches (53). Because some samples were in very limited amounts, we pooled seven plasma samples from IgAN patients (P3, 4, 5, 7, 8, 11, and 12) and seven plasma samples from healthy individuals (C9, 14, 16, 18, 19, 20, and 24). The seven IgAN patient plasma samples selected correspond to the plasma samples used in Fig. 4D with the exception of the sample P2, which significantly contains the HPA/H1PNA/IgA1 glycoform. For the control samples, seven of the eight control samples used in Fig. 4D were randomly selected, because none of them significantly contain the HPA/H1PNA/IgA1 glycoform. Total IgA from these two pools of samples was purified. The N-glycans released from purified IgA of the IgAN patients and controls, as well as the purified IgA1 of Dakiki cells, were analyzed by MS. The N-glycan profile from controls and IgAN patients showed a very similar pattern (Fig. 7A two upper panels), with compositions that suggest they are mostly mono- or di-sialylated biantennary N-glycans, with or without a core fucose. Purified IgA from plasma include both IgA1 and IgA2, and we cannot rule out the possibility of contributions of minor amounts of N-glycans from other glycoproteins, including IgG and IgM. Nonetheless, no differences in the N-glycan profiles were observed between IgAN patients and control groups. Interestingly, N-glycans of IgA1 produced in Dakiki cells had compositions consistent with non-sialylated, non-fucosylated biantennary N-glycans (Fig. 7A lower panel), suggesting sialyltransferases acting on IgA1 N-glycans are not expressed or not functional in these cells. It should be noted that unlike IgA from plasma, the IgA1 from Dakiki cells is much more homogeneous, as these cells clonally produce copious amounts of IgA1 without significant contamination of other glycoproteins.

Next, we analyzed the whole O-glycan profile of IgA1 purified from pooled plasma of IgAN patients and control individuals, as well as IgA1 secreted by Dakiki cells. IgA1 is one of very few glycoproteins in human plasma to carry O-glycans, therefore, and unlike the N-glycans, the O-glycans profile from purified IgA1 would represent the O-glycans carried mainly, if not exclusively by IgA1. The analyses in Fig. 7B show that the total O-glycan profiles of IgA1 from pooled IgAN patient and healthy control plasma, which is the mixture of all glycoforms, and from Dakiki cells were similar; the majority of the O-glycans were primarily mono-sialylated core 1 structure, with minor amounts of core 1 (T antigen), di-sialylated core 1 structures and, unexpectedly, di-sialylated core 2 structures which have been previously reported (54).

Overall, we did not observe significant differences in N- and O-glycan MS profiles between IgA1 from IgAN patients and
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from healthy controls. It should be noted that the released monosaccharide GalNAc from glycoproteins, which represents the Tn antigen, cannot be observed on MALDI under these conditions, because its molecular weight is <400 Daltons and causes it to be buried in the background noise of the matrix used for MS. Next, we confirmed the existence of the two glycoforms observed with the serial lectin chromatography as seen in Fig. 4. The pooled IgA from IgAN patients and healthy controls were treated with neuraminidase and then separated by chromatography on HPA. The presence of IgA was confirmed in both Unbound (UB, HPA−) and Bound (B, HPA+) fractions (Fig. 7C). Based on the IgA staining in Fig. 7C, an equivalent amount of HPA+ and HPA− IgA was then taken for MS analysis (Fig. 7D). The HPA Unbound (UB, HPA−) fraction of IgA1 from both IgAN patients and healthy controls contain glycans with the composition indicating they are primarily of the core 1 structure (Fig. 7D upper panels), whereas the HPA Bound (B, HPA+) fraction of IgA1 was highly deficient in such core 1-based structures (Fig. 7D bottom panels). Only very minor signals corresponding to the core 1 structure could be observed at m/z 518. The intensity of these signals, mostly buried in the matrix noise, represents merely about 3% of the signal intensity observed at m/z 518 in the Fig. 7D upper panels for the HPA− samples. In addition, these low signals corresponding to m/z 518 are buried in the matrix and could not be verified as the core 1 (T antigen) structure because it could not be analyzed by fragmentation by MS/MS. These data further indicated that the HPA+ glycoform exclusively carries Tn or STn in both IgAN patients and control individuals.

Finally, to unequivocally confirm the presence of Tn antigen on HPA−-IgA1 fraction, we utilized the recombinant T-synthase to convert the Tn on HPA−-IgA1 to the core 1 structure, as we did in Fig. 5, and then the O-glycans were released and analyzed by MS. By definition, the O-glycan structure that can be converted by the T-synthase into the core 1 structure in the presence of the sugar donor UDP-Gal is the Tn antigen, that is, GalNAcα-Ser/Thr. We purified total IgA from a control donor (C12), treated the material with neuraminidase, and then isolated the HPA−-IgA1 glycoform by chromatography on immobilized HPA. After denaturation with Gdm-HCl, the HPA−-IgA1 was incubated with T-synthase with or without UDP-Gal, and the O-glycans were then released, permethylated and analyzed by MS. Fig. 7E ensured that a similar amount of IgA was being analyzed in both conditions, in the presence and absence of UDP-Gal for T-synthase reactions. Thus, the same amount of material was used to perform MS analysis. As expected, in the absence of UDP-Gal, no core 1 disaccharide structure was detected in the HPA−-IgA1 fraction (Fig. 7F left panel). However, when the same HPA−-IgA1 fraction was incubated with UDP-Gal and with the T-synthase, the core 1 disaccharide was synthesized, released and detected by MS (Fig. 7F right panel). Taken together, these results demonstrate that the existence of two major glycoforms of IgA1, one expressing exclusively the Tn/STn antigens, and the other being exclusively normal O-glycans (Fig. 8), and that both glycoforms exist in plasma of both patients with IgAN and in normal controls.

DISCUSSION

In the present study we investigated the occurrence of the Tn/STn antigens on IgA1 of patients with IgAN and healthy individuals, and whether there were disease-specific glycoforms of IgA1. We utilized a combination of quantitative and qualitative approaches to examine potential differences of plasma IgA1 in their O-glycan structures using lectin blots, lectin chromatography, Western blot with monoclonal antibody, enzymatic conversion and mass spectrometry. These approaches substantially differ from those in previous studies in the field, which did not employ methods to separate potential glycoforms using specific lectins, but instead used lectin-based ELISA or MS of HR glycopeptides of total IgA (46, 48, 55–59). Here, our results have both demonstrated unexpectedly that total plasma IgA1 exists as two major and separable glycoforms, one expressing primarily the Tn/STn antigens, and the other expressing normal sialylated and galactosylated O-glycans, and that the relative amounts of IgA1 glycoforms expressing Tn and/or STn antigens occur at a similar percentage in a minor fraction of plasma IgA1 from patients with IgAN and healthy donors. Furthermore, IgA1 secreted from the B cell line Dakiki is exclusively expressed with normal sialylated galactosylated O-glycans and lacks Tn/STn antigens, despite the expression of ST6GalNAcII in these cells. Our findings strongly argue that expression per se of Tn/STn on plasma IgA1 is not a hallmark for IgAN.

Overall our results are consistent with several historical studies that have detected the presence of IgA1 containing the Tn antigen in plasma of both IgAN patients and control individuals in their published data (42, 45, 46, 48, 56, 58, 60). Several studies have also reported minimal or even no differences between galactosylation of IgA1 from IgAN patients and IgA1 from control donors, showing in their data similar reactivity with lectins (HAA, HPA or VWA) specific to galactose-deficient O-glycans (48, 60, 61). Yet, the conclusions of

Fig. 7. MS analysis of IgA1 glycoforms O-glycans: A, MS analysis of the N-glycans and B, O-glycans of purified IgA1 from plasma of control individuals and IgAN patients, and Dakiki cell culture media. C, Western blot analysis with anti-IgA antibody of HPA Unbound (UB, HPA−) and HPA Bound (B, HPA+) fractions following HPA chromatography of pooled IgAN patients and control plasma. D, MS analysis of the O-glycans of HPA− and HPA+ fractions of plasma IgA1 from controls and IgAN patients. E, Western blot analysis with the anti-IgA antibody of the HPA− fraction of C12 sample IgA1 after incubation with T-synthase, with or without UDP-Gal. F, MS analysis of the O-glycans of the HPA− fraction of C12 sample IgA1 after incubation with T-synthase, with or without UDP-Gal.
many of these reports identify galactose-deficient IgA1 as a hallmark of IgAN and as a marker to discriminate between IgAN patients and healthy individuals. Here, we show that after removal of sialic acids from IgA1, HPA can be used to efficiently pull down a fraction (19%) of plasma IgA1 from all of the IgAN patient samples tested and, unexpectedly, from all of the control samples as well (Fig. 2B). Without neuraminidase treatment, only 3 of 14 IgAN patients and 5 of 14 control samples did not exhibit significant IgA1 pull down with HPA, suggesting that the Tn antigen in some IgA1 glycoforms in those plasma is mostly sialylated and occurs as the STn antigen. In addition, the purified IgA1 from all control and IgAN patient samples exhibited direct and similar HPA reactivity after desialylation. Therefore, based on our results, we conclude that there is no significant difference in the Tn/STn content in plasma IgA1 between patients with IgAN and healthy controls.

Consistent with several earlier findings (42, 48), we found that plasma of IgAN patients contains significantly more IgA (1.6-fold higher) than plasma of control individuals (Fig. 2A). This differential level of IgA1 expression was the only significant difference between those two groups that we could identify. Of course, our results imply that the absolute amount of Tn antigen-containing IgA1 glycoforms is, on average, also elevated ~1.6-fold in the plasma of IgAN patients, thus, this overall elevation of the Tn/STn-containing glycoforms of IgA1 could play a role in the pathogenesis. We also noted that some healthy individuals have a higher level of plasma galactose-deficient IgA1 (the HPA+/PNA− glycoforms) than some IgAN patients. Therefore, the sole expression of Tn antigen on plasma IgA1 is unlikely to be useful as a unique diagnostic feature for IgAN.

An unexpected finding in our study is the identification of two major and separable glycoforms of IgA1 molecules in plasma of all donors (Fig. 8). By performing serial HPA- and PNA-chromatography on plasma IgA1 of IgAN patients or healthy individuals, we found that IgA1 molecules from 15 of the 16 samples tested could be easily separated into two unique fractions in their reactivity to only one or the other lectin (HPA+/PNA− or HPA−/PNA+), and only one of those 16 samples contained a third glycoform that was the HPA+/PNA− glycoform of IgA1 (Fig. 4D). We confirmed the existence of the HPA+/PNA− glycoform by demonstrating it could be readily converted to the core 1 structure by recombinant human T-synthase in vitro. We also verified by MS analysis that no core 1 based structures were detected in the HPA+/PNA− glycoform of IgA1, whereas core 1 O-glycans were found in the HPA−/PNA+ glycoform (Fig. 7D, 7F). These data demonstrate that IgA1 occurs in relatively homogeneous and separable glycoforms in terms of O-glycan galactosylation, and can be readily distinguished and isolated by chromatography on HPA and PNA, and that both glycoforms occur in IgAN patients and healthy controls.

Several past studies have analyzed the O-glycans of IgA1, but the approaches have differed from ours. Previous studies generally utilize the unfractionated IgA1 material, that is often
digested by trypsin, followed by MS analysis of the 33-mer tryptic glycopeptide corresponding to the hinge region (55, 62–65). Such studies have suggested a rather wide heterogeneity in the O-glycan content of serum IgA1 from IgAN patients and healthy individuals with up to 57 differentially glycosylated hinge region glycopeptides; accordingly, most of the IgA1 would be predicted by such studies to be mixed glycoforms and would be predicted to be bound by both PNA and HPA lectins after desialylation. To our knowledge, our work is the first attempt to separate IgA1 glycoforms from the same individual’s plasma with two lectins thought to discriminate “normal” (PNA+) and “aberrant” (HPA+) O-glycosylated IgA1.

Indications of these glycoform homogeneities were further confirmed when we investigated the O-glycosylation of IgA1 secreted by the immortalized B cell line Dakiki. Unlike the plasma samples tested, IgA1 purified from the culture media of Dakiki cells showed reactivity only to PNA after desialylation, suggesting that only sialylated core 1-based O-glycans are present in the IgA1 secreted by these cells (Fig. 6). Dakiki cells represent a clonal expansion of B cells and therefore it is not surprising that they produce a homogeneous population of IgA1. The presence of a single major glycoform with galactosylated O-glycans on IgA1 from Dakiki cells demonstrates that IgA1 in general can be efficiently galactosylated by T-synthase in cells to form the core 1-based O-glycans. This raises the question as to the mechanism for formation of the undergalactosylated glycoforms in plasma we designated as HPA+/PNA−.

Although our work clearly demonstrates that the so-called aberrant O-glycosylation of IgA1, that is, expression of the Tn and STn antigens, occurs in all individuals including healthy controls, the cause of this phenomenon is not yet known. We can envision three scenarios that might explain the presence of two glycoforms of IgA1 in the plasma of individuals. In Scenario I there may be two different populations of IgA1-producing B cells or plasma cells with one “major” population that produces IgA1 with “normal” core 1 based O-glycans, whereas another “minor” population is unable to add galactose to the O-glycans of IgA1 and secretes IgA1 carrying Tn/STn antigens. In Scenario II the IgA1-producing cells simultaneously produce both HPA+/PNA− and HPA−/PNA+ glycoforms of IgA1, whereby for either kinetic or other reasons, one portion of IgA1 molecules made by the cells is not O-galactosylated, whereas another portion is O-galactosylated. In Scenario III the IgA1 produced in a particular cell may be folded into such a structural form that the hinge region is only accessible to the ppGalNACTs but not to the T-synthase. Our results with the Dakiki cells tend to favor Scenario I, because the cells produce a single glycoform that is HPA+/PNA−. We note that in some samples there was a small amount of a heterogeneous HPA+/PNA− glycoforms, which might arise if there is a limited amount of T-synthase activity or an excess amount of IgA1 in cells, also taking into account the evidence suggesting that the IgA1 hinge region is not a good substrate for T-synthase, or T-synthase has a very high $K_{m}$ to the Tn antigen on the hinge region of IgA1.

If the different glycoforms we observed arise by Scenario I, it may suggest that the minor population of B cells or plasma cells which produce the HPA+/PNA− (Tn/STn) glycoform of IgA1 may have a deficiency in T-synthase activity, either temporary down-regulation of Cosmc and/or T-synthase, or persistently compromised T-synthase activity, such as silencing and mutation of Cosmc as discussed below. The poor kinetic activity of T-synthase to the IgA1 coupled with deficiency of T-synthase could lead to a deficiency of galactose on IgA1 yet O-glycosylation of cellular glycoproteins could be relatively normal. Consistent with this are some studies proposing that patients with IgAN show decreased expression of Cosmc and/or T-synthase (8, 34, 36). However, we observed no significant differences in the percentage of IgA1 glycoforms lacking O-galactosylation between patients with IgAN and healthy controls. It is also possible that there could be site-specific differences in O-glycosylation among the two glycoforms that we observed independent of their total content of galactose or Tn/STn antigens, and that such site-specific differences might contribute to disease pathogenesis. In any case, using our affinity isolation approaches, such site-specific studies should be done on isolated glycoforms of IgA1 from both normal controls and IgAN patients.

Finally, our results also strongly argue that ST6GalNAC-II does not compete with the T-synthase to promote the formation of the STn antigen, because we observed that ST6GalNAC-II is expressed in the Dakiki cells, yet IgA1 secreted from these cells is negative to HPA after desialylation. In addition, Dakiki cells do not express, or so little if any, STn on their glycoproteins (Fig. 6B). Suzuki and others (8, 51) concluded, using indirect approaches, that up-regulated ST6GalNACII is responsible for the synthesis of STn on IgA1. ST6GalNAC-Ii, however, has been primarily described to sialylate the core 1 and α2,3sialyl-core 1 structures to form NeuAca2,6GalNAc- within sialyl-core 1 and disialyl-core 1 structures (66–68), whereas ST6GalNAc-I is mainly involved in sialylation of the Tn, but not T, antigen to form the STn (69, 70). Consistent with these data, we also observed that Tn4 cells only express the Tn antigen, but no significant amounts of the STn antigen, although they express ST6GalNACII, but not ST6GalNACI (Fig. 6A). Lastly, we observed in preliminary studies that the recombinant human ST6GalNAc-II is only able to sialylate core 1 and α2,3sialyl-core 1 structures on IgA1 HR peptides in vitro, but not the Tn antigen on IgA1 HR peptides. Taken together, these data strongly indicate that ST6GalNAc-II is not the sialyltransferase responsible for the synthesis of the STn antigen on IgA1.

In conclusion, we have demonstrated that plasma IgA1 from patients with IgAN and healthy control individuals similarly contain IgA1 with Tn/STn antigens. However, we found that IgA1 in plasma from both IgAN patients and healthy
controls exists as two distinct glycoforms, one with Tn/Stn O-glycans as a minor fraction, and the other as the major fraction containing the normal siaiylated and galactosylated O-glycans (Fig. 8). Thus, the Tn antigen, considered as a marker of pathological conditions or neoplastic transformations, naturally occurs in healthy individuals on IgA1 at a similar level as compared with patients with IgAN, and therefore should not be considered as a unique feature for IgA in IgAN. The only significant difference we found between the IgAN patient samples and the control samples is the total plasma IgA concentration, being 60% higher in average in IgAN patient samples and the control samples is the total IgAN. The only significant difference we found between the control cell O-glycosylation of IgA1 in IgA Nephropathy.

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