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Abundance of Galβ1,3GalNAc in O-Linked Oligosaccharide on Hinge Region of Polymerized IgA1 and Heat-Aggregated IgA1 from Normal Human Serum[1]

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Gas-phase hydrazinolysis was used to analyze the glycoform of the O-linked oligosaccharide of human serum IgA1. In our previous report, only one glycoform was obtained from the IgA1 of healthy individuals. However, it was found to be composed of heterogeneous IgA1 components having mutually different glycoforms. First, the IgA1 was separated into two subfractions having different affinities toward jacalin. Among them, the high-affinity subfraction was mainly composed of polymerized IgA1. Comparative study of the carbohydrate chain showed a relative abundance of Galβ1,3GalNAc in the polymerized form. A simultaneous analysis of the N-glycan of these subfractions was also carried out. Three major components, two biantennary and one triantennary oligosaccharides, were obtained from both subfractions and the relative contents of these components were almost the same. On the other hand, IgA1 was artificially polymerized by heating at 63°C for 2 h. The heat-stable IgA1 was separated from the heat-aggregated material on a Sephacryl S-300 column. The obtained heat-stable IgA1 (approximately 20%) was not further aggregated by more heating under the same conditions. The heat-stable IgA1 contained a much higher amount of the sialylated Galβ1,3GalNAc. Thus, it was shown that the degree of completeness of the hinge O-linked oligosaccharide might be correlated with the stability and polymerization process of the IgA1 molecule.

Key words: gas-phase hydrazinolysis, IgA1, IgA nephropathy, MALDI-TOFMS, O-linked oligosaccharide.

Human serum IgA1 is exceptional among human serum glycoproteins because it has O-linked oligosaccharides in its hinge portion in addition to N-linked carbohydrate chains in its structure (*1-3*). In our previous report, the glycoform of the O-linked oligosaccharide of the IgA1 subclass from healthy controls and IgA1 myeloma patients was analyzed by using gas-phase hydrazinolysis. Three glycoforms for IgA1 from myeloma patients and only one glycoform from healthy individuals were found (*4*).

IgA nephropathy is characterized by prominent IgA deposits in the renal mesangium. It is well known that this IgA1 subclass is a dominant deposit in glomeruli in IgA nephropathy (*5, 6*). There are some reports describing the hinge O-linked oligosaccharide of IgA1 from IgA nephropathy patients (*7-9*). A comparison of the jacalin binding ability of IgA1 using the inhibition method with galactose indicated a significantly higher value for IgA1 from IgA nephropathy patients than that from the healthy controls (*10*). An analysis of the O-linked oligosaccharide released from IgA1 using gas-phase hydrazinolysis indicated an increase in the proportion of asialo-Galβ1,3GalNAc on the IgA1 hinge region from an IgA nephropathy patient (*11*). These results suggested the presence of an incompletely glycosylated O-linked oligosaccharide(s) on the IgA1 hinge region in some IgA nephropathy patients.

It is well-known that structural differences in the oligosaccharides in the same glycoprotein (so-called microheterogeneity) can exist in a single glycoprotein molecule (*12*).

In this study, IgA1 purified from the serum of a healthy control was subfractionated and it was found that different fractions exhibited different affinities toward jacalin and different heat stabilities. We then examined the glycoform of the O-linked oligosaccharides on the hinge region of these IgA1 subfractions.

EXPERIMENTAL PROCEDURES

Materials—The following compounds and materials were commercially obtained. Normal human serum was from China Newtech Development and Trade (it was stored at −20°C). Jacalin-agarose was from Vector Laboratories (Burlingame, CA); 2-aminopyridine was from Wako Pure Chem. (Osaka); borane-pyridine complex from Aldrich (Milwaukee, WI); fetuin from GIBCO (Grand Island, NY); and anhydrous hydrazine from Honen Company (Honen, Yokohama). Sephacryl S-300 was from Pharmacia (Phar-

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macia Biotech, Tokyo).

Preparation and Subfractionation of IgA1 by Jacalin-Agarose Affinity Chromatography—Unless otherwise stated, the jacalin-agarose affinity chromatography was carried out at room temperature. Ten ml of serum was applied to the jacalin column (10 ml) and thoroughly washed with 0.1 M Tris-HCl buffer, pH 7.6, containing 0.02% sodium azide. The column was first eluted with 0.8 M glucose (50 ml) and then with 0.8 M galactose (50 ml) in the above buffer. The absorbance of the eluate at 280 nm was read to detect the protein. Nonspecifically bound materials were eluted with glucose. Bound IgA1 fraction was eluted by stepwise or linear gradient elution using 0.8 M galactose. IgA1 from serum treated at 63°C for 2 h was also fractionated on the jacalin-agarose column with a linear gradient elution. The obtained IgA1 subfractions were dialyzed against distilled water and lyophilized. For the preparation of the heat-stable IgA1, purified IgA1 dissolved in PBS was treated at 63°C for 2 h and then fractionated on a Sephacryl S-300 column (1.5×62 cm) equilibrated with 50 mM ammonium bicarbonate. The purified IgA1 subfractions were lyophilized and used for further examination. The release of the *O*-linked oligosaccharides from IgA1 was performed by hydrazinolysis, basically according to Patel *et al.* (*13*).

Release of Intact O-Linked Oligosaccharide from IgA1 by Gas-Phase Hydrazinolysis—The procedures for releasing *O*-linked oligosaccharides from IgA1 were described in our previous report (*4*). Briefly, the solution containing IgA1 was dialyzed against distilled water. The isolated IgA1 was dried under reduced pressure at 50°C. Approximately 2 mg of dried sample was treated with anhydrous hydrazine at 65°C for 6 h using Hydraclub S-204 (Honen). After the reaction, hydrazine was absorbed in concentrated sulfuric acid under reduced pressure. The dried sample was suspended in 250 μl of saturated sodium bicarbonate and then 25 μl of acetic anhydride was added for acetylation. After a 30-min incubation at room temperature, this treatment was repeated. To desalt the solution, the sample was applied to a Dowex 50W column (X8, H^+ form, 3 ml) (Muromachi Kagaku Kougyou, Tokyo) and washed with 15 ml of distilled water. The eluate containing the released oligosaccharide was then dried. Release of the *N*-linked oligosaccharide was carried out according to the method reported by Takasaki *et al.* (*14*).

Pyridylamination of Released Oligosaccharide—The dried sample was pyridylaminated as previously reported (*15*). Briefly, the sample was dissolved in 20 μl of 2-aminopyridine (2-AP) solution (2.2 g/ml acetic acid) warmed with hot water. The reaction mixture was heated at 90°C for 1 h. Then 20 μl of the 20% pyridine borane complex in the above 2-AP solution was added and the mixture was heated at 80°C for 80 min. The pyridine borane complex was evaporated twice with 400 μl of 75% methanol and then with 500 μl of 80% methanol. The obtained pyridylaminated (PA) oligosaccharide was fractionated by HPLC.

HPLC Analysis of PA Oligosaccharide—The PA oligosaccharide was fractionated using a PALPAK Type N column (0.46×25 cm) (Takara Shuzo) at a flow rate of 1.0 ml/min at 40°C. Two solvents, A and B, were used. Solvent A was composed of 50 mM acetic acid adjusted to pH 7.3 with triethylamine and acetonitrile (15 : 85, v/v). Solvent B was composed of 1 M acetic acid adjusted to pH 7.3 with triethylamine and acetonitrile (50 : 50, v/v). The column was equilibrated with solvent A. After sample injection, the proportion of solvent B was increased linearly to 55% over 110 min. The PA oligosaccharides were detected by fluorescence measurement (excitation 310 nm, emission 380 nm).

Two-dimensional analysis of PA *N*-linked oligosaccharide was carried out as follows. First, the PA asialo *N*-linked oligosaccharide was fractionated using a TSK-GEL AMIDE-80 column (0.46×25 cm) (Tosoh) at a flow rate of 1.0 ml/min at 40°C. Two solvents, C and D, were used. Solvent C was composed of 3% acetic acid adjusted to pH 7.3 with triethylamine and acetonitrile (35 : 65 v/v). Solvent D was composed of 3% acetic acid adjusted to pH 7.3 with triethylamine and acetonitrile (50 : 50, v/v). The column was equilibrated with solvent C. After sample injection, the proportion of solvent D was increased linearly to 100% for 50 min. Next, the PA asialo *N*-linked oligosaccharide was fractionated using a Shim-pack CLC-ODS column (0.6×15 cm) (Shimadzu) at a flow rate of 1.0 ml/min at 55°C. Two solvents, E and F, were used. Solvent E was 10 mM sodium phosphate buffer, pH 3.8. Solvent F was solvent E containing 0.5% 1-butanol. The column was equilibrated with a mixture of solvents E and F (80 : 20, v/v). After sample injection, the ratio of solvent F was increased linearly up to 50% for 60 min. All the above HPLCs were carried out using a Shimadzu LC-9A chromatograph equipped with an RF-535, a fluorescence spectromonitor. The PA oligosaccharides were detected by fluorescence measurement (excitation 320 nm, emission 400 nm). The results are given as the relative content (%) of each peak, calculated based on the respective peak height.

MALDI-TOFMS Analysis of Pyridylaminated Oligosaccharide—PA oligosaccharide samples, *N*-linked oligosaccharides and peracetylated *O*-linked oligosaccharides, were analyzed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). The mass spectrometer used in this work was a Finnigan Lasermat (Finnigan MAT, Hemel Hempstead, UK). The matrix used was α-cyano-4-hydroxy cinnamic acid (*16*). Components of the *N*-linked oligosaccharide from IgA1 gave positive ion peaks at $m/z=1,724$, 1,870, and 2,093, which are consistent with the molecular masses of the PA biantennary oligosaccharide (calculated 1,719.6), PA fucosyl biantennary oligosaccharide (calculated 1,865.8), and PA triantennary oligosaccharide (calculated 2,085.0), respectively.

Components of the *O*-linked oligosaccharide gave positive ion peaks at $m/z=756$, 1,174, 1,175, and 1,591, which are consistent with the molecular masses of peracetyl PA Galβ1,3GalNAc (calculated 755.7), peracetyl PA mono-sialylated Galβ1,3GalNAc (calculated 1,173.1), peracetylated PA mono-sialylated Galβ1,3GalNAc (calculated 1,173.1), and peracetylated PA di-sialylated Galβ1,3GalNAc (calculated 1,590.5), respectively.

RESULTS AND DISCUSSION

Normal human serum was applied to a jacalin-agarose column and stepwise elution was carried out with 0.1 M Tris-HCl buffer containing 0.02% sodium azide, first with 0.8 M glucose solution and then with 0.8 M galactose solution. As shown in Fig. 1, a fair amount of protein was

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eluted with 0.8 M glucose solution. Immunoelectrophoresis of this fraction showed that the fraction did not contain IgA but contained unknown proteins belonging to the β-globulin fraction (data not shown). Washing of the column with such a high concentration of glucose seemed to be helpful for specific elution of the glycoprotein with galactose from the jacalin column. The column was thoroughly washed with 0.8 M glucose, then IgA1 was subfractionated with a linear gradient to 0.8 M galactose (Fig. 2). Previously, we separated ovalbumin into 4 subfractions on the basis of carbohydrate chain differences (*17*). In the case of this IgA1, the presence of a maximum of 5 *O*-linked oligosaccharides per heavy chain implied the presence of 10 oligosaccharides per molecule which could bind to jacalin. Such a multivalent interaction with lectin should be very strong (*18*). However, the major fraction of IgA1 could be eluted with a low concentration of galactose. This might be explained by a reduced number of sugar chains per molecule or steric hindrance to interaction of the sugar chains with jacalin. This main low-affinity IgA1 subfraction (Fraction number 3-20) was separated from the high-affinity subfraction (Fraction number 21-80). Affinity of the heat aggregated IgA1 toward jacalin was slightly increased compared to that of the control IgA1 (Fig. 2). Since a part of the heat-aggregated IgA1 showed increased the affinity for jacalin, enrichment of the polymerized IgA1 in the high-affinity subfraction from normal human serum obtained here was examined. As expected, the low-affinity IgA1 subfraction was mainly composed of the monomeric form and the high-affinity fraction was rich in polymerized IgA1 (Fig. 3). A comparative study of the sugar chains on these IgA1 subfractions was then carried out. The *O*-linked oligosaccharide released from standard fetuin by gas-phase hydrazinolysis was pyridylaminated and fractionated into four major oligosaccharide peaks, A, B, C, and D, by HPLC as shown in Fig. 4. The structure of each oligosaccharide was estimated from the results of MALDI-TOFMS analysis as described in the "EXPERIMENTAL PROCEDURES" shown in Table I. Previously, the pyridylaminated *O*-linked oligosaccharide from fetuin and IgA1 was fractionated into four major peaks named P1-P4 based on their molecular size and/or hydrophobicity. P1, P2, and P4 in our previous report correspond to peaks D, B, and A, respectively. Because peak C was produced from peak D by its treatment with a neuraminidase specific to α2,3 linked *N*-acetylneuraminic acid, the unidentified peak P3 might correspond to the structure C as indicated in Table I. The relative content of each *O*-linked oligosaccharide fraction was calculated based on each peak height in Fig. 4. Although two-dimensional analysis of the pyridylaminated sugar chain was needed in our previous report, the use of the PALPAK Type

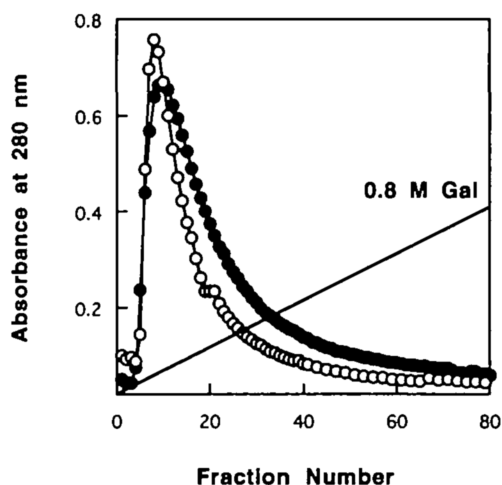

Fig. 2. **Gradient elution of IgA1 from a jacalin column with 0.8 M galactose.** The column was washed with 0.8 M glucose as indicated in Fig. 1, then bound IgA1 was eluted with a linear gradient elution from 0 to 0.8 M galactose as indicated in the figure. Elution profiles of IgA1 from normal human serum (○) and normal human serum treated at 63°C for 2 h (●) are shown. Low-affinity IgA1 subfraction (Fraction number 3-20) and high-affinity subfraction (Fraction number 21-80) from control serum were separately pooled and used for further analysis.

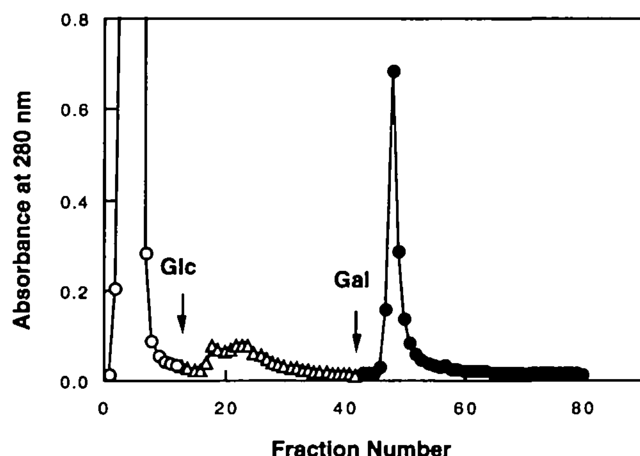

Fig. 1. **Stepwise elution of IgA1 from a jacalin column with glucose and galactose.** Nonspecifically bound materials were eluted from the column with 0.8 M glucose (△) and then IgA1 was eluted with 0.8 M galactose (●) under the conditions described in "EXPERIMENTAL PROCEDURES." Elution with glucose or galactose was started at the point indicated by arrows in the figure.

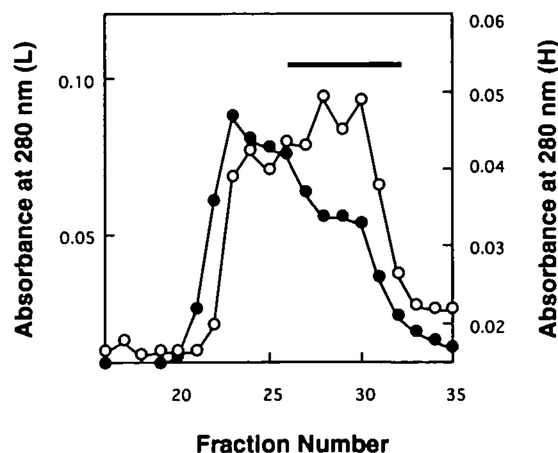

Fig. 3. **Sephacryl S-300 column chromatography of high- and low-affinity IgA1 subfractions.** IgA1 subfractions prepared from control serum (Fig. 2) were fractionated on a Sephacryl S-300 column. (H) and (L) in the figure corresponded to high-affinity (●) and low-affinity (○) subfractions, respectively. The elution position of monomeric IgA1 is indicated by a bar.

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N column in this report simplified the analysis of the glycoform of the sugar chain. A comparison of the *N*-linked oligosaccharide of each subfraction was also carried out (Fig. 5). The elution profiles of the asialo- and sialo-*N*-linked oligosaccharide of the high- and low-affinity IgA1 subfractions were almost the same (Fig. 5). The elution profile of the asialo-*N*-linked oligosaccharide components designated as a, b, and c from both subfractions were very similar to each other. The structure of each peak, a, b, and c, was analyzed using the two-dimensional HPLC mapping method reported by Tomiya *et al.* (*19*). The structure of each PA-oligosaccharide was assigned as PA biantennary oligosaccharide, PA fucosyl biantennary oligosaccharide and PA triantennary oligosaccharide. The MALDI-TOFMS analysis of these three peaks was also carried out. Molecular weights for each peak (m/z=1,724, 1,870, and 2,093) were in good agreement with those of the oligosaccharide

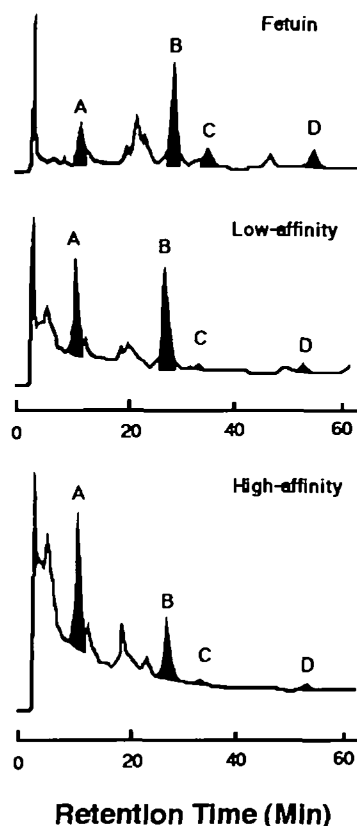

Fig. 4. **HPLC profiles of PA oligosaccharide prepared from fetuin and low- and high-affinity IgA1 subfractions.** PA oligosaccharide was fractionated with PALPAK Type N column. The component in each hatched peak A, B, C, and D, in the figure was purified and analyzed by MALDI-TOFMS as described in "EXPERIMENTAL PROCEDURES."

TABLE I. **Relative content of PA *O*-linked oligosaccharide components from low- and high-affinity IgA1 subfractions.** Peaks A, B, C, and D correspond to those from fetuin in Fig. 4. "Low" and "High" in the figure indicate low- and high-affinity IgA1 subfractions toward jacalin.

Peak	Structure	Relative content (%)	
		Low	High
A	Galβ1,3GalNAc-PA	28	49
B	NeuAcα2,3Galβ1,3GalNAc-PA	54	39
C	NeuAcα2,6 (on GalNAc) Galβ1,3GalNAc-PA	8	6
D	NeuAcα2,6 (on Gal) NeuAcα2,3Galβ1,3GalNAc-PA	10	6

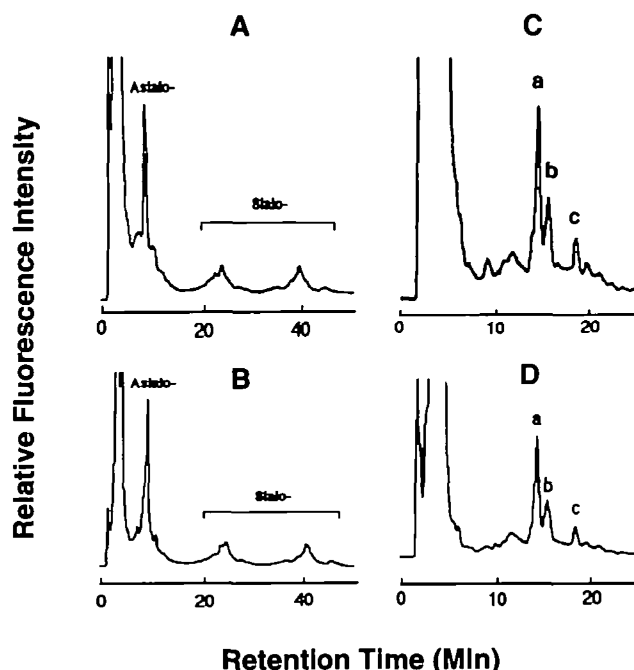

Fig. 5. **HPLC profile of PA *N*-linked oligosaccharide from low- and high-affinity IgA1 subfractions.** PA *N*-linked oligosaccharide from low- and high-affinity subfractions was analyzed by HPLC. Figures A and B corresponded to the elution profiles of PA oligosaccharide from low- and high-affinity subfractions on a PALPAK Type N column. Figures C and D corresponded to the elution profiles of asialo-PA oligosaccharide from low- and high-affinity subfraction on a TSK-GEL AMIDE-80 column. Peaks a, b, and c were separated and further analyzed by MALDI-TOFMS as described in "EXPERIMENTAL PROCEDURES."

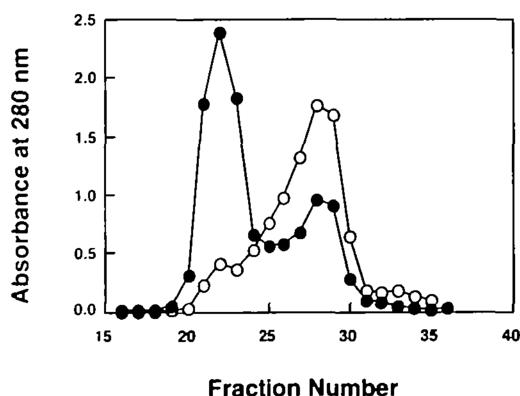

Fig. 6. **Sephacryl S-300 column chromatography of IgA1 and heat-treated IgA1.** IgA1 (○) and heat-treated IgA1 (●) were fractionated on a Sephacryl S-300 column (1.5×62 cm) equilibrated with 50 mM ammonium bicarbonate solution. The heat-labile IgA1 subfraction (Number 20-24) and heat-stable subfraction (Number 26-30) were pooled and lyophilized. Each subfraction was used for further analysis.

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TABLE II. **Relative content of PA *O*-linked oligosaccharide components from heat-labile and heat-stable IgA1 subfractions.** A, B, C, and D corresponded to those from fetuin in Fig. 4. "Labile" and "Stable" in the table indicate the heat-labile and heat-stable IgA1 subfractions in Fig. 6, respectively.

Peak	Structure	Relative content (%)	
		Labile	Stable
A	Galβ1,3GalNAc-PA	27	18
B	NeuAcα2,3Galβ1,3GalNAc-PA	51	67
C	NeuAcα2,6 Galβ1,3GalNAc-PA	10	5
D	NeuAcα2,6 NeuAcα2,3Galβ1,3GalNAc-PA	12	9

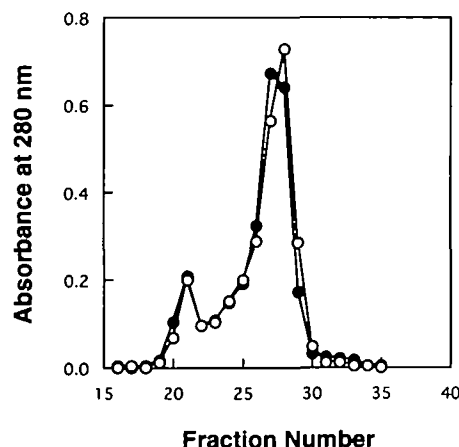

Fig. 7. **Sephacryl S-300 column chromatography of heat-stable IgA1 subfraction and retreated heat-stable IgA1 subfractions.** The elution profile of the heat-stable IgA1 subfraction (○) overlapped the elution profile of the retreated heat-stable IgA1 (●) under the conditions described in Fig. 6.

structures. On the other hand, the glycoform of the *O*-linked oligosaccharide was significantly different between the low-affinity and high-affinity subfractions, as shown in Table I. The relative content of asialo-Galβ1,3GalNAc was rich in high-affinity subfraction.

In order to examine the relation between the aggregation of IgA1 and its incomplete glycosylation, artificially aggregated IgA1 was prepared by heating the IgA1 in PBS at 63°C for 2 h. IgA1 was clearly separated into a heat-labile IgA1 (Fraction number 20-24) and a heat-stable material (Fraction number 26-30) by Sephacryl S-300 (Fig. 6). The heat-stable IgA1 was isolated and treated again under the same conditions. As shown in Fig. 7, the elution profile of the retreated IgA1 subfraction was very similar to that of the isolated heat-stable IgA1 sample. This heat-stability seemed to be correlated with the glycoform of the hinge *O*-linked oligosaccharide. The heat-stable IgA1 contained a much larger amount of the monosialylated IgA1 (Table II). A clear contrast of the glycoform of the *O*-linked oligosaccharide was observed between high-affinity IgA1 subfraction and heat-stable IgA1 subfraction.

Thus, it was found that the glycoform of the *O*-linked oligosaccharide was heterogeneous even for IgA1 from healthy individuals. The IgA1 subfractions having mutually different glycoforms of the *O*-linked oligosaccharide were obtained for the first time in this study. The results suggest a strong relationship between the stability of the IgA1 molecule and the glycoform of the *O*-linked oligosaccharide. Regarding the role of *O*-linked oligosaccharide, a contribution to the structural integrity of mucin-type glycoprotein has been reported (*20*).

In our previous report, the relative content of Galβ1,3GalNAc was increased in the serum IgA1 from IgA nephropathy patients compared with that from healthy controls (*11*). In addition, a comparison of the jacalin-binding ability of IgA1 using the inhibition method with galactose indicated a significantly higher value for IgA1 from IgA nephropathy patients than that from healthy controls (*10*). If the glycoform of IgA1 were related to the stability of the IgA1 molecule and/or its self-aggregation, this result would suggest an increase in unstable IgA1 and an increase in polymerized IgA1 in IgA nephropathy patients. Higher content of the polymerized IgA1 in immune complex of patients' sera has already been reported (*21-23*). However, it was not clear whether all of the high-molecular-weight IgA1 came from the immune complex. There is a possibility that the incomplete structure of the hinge *O*-linked oligosaccharide is one factor favoring the production of such polymerized IgA1.

We are now examining the relationship between self-aggregation *via* specific IgA1-IgA1 interaction and the glycoform of the *O*-linked oligosaccharide.

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