N-Glycan Structures of Pigeon IgG

A MAJOR SERUM GLYCOPROTEIN CONTAINING Galα1–4Gal TERMINI*§

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We had shown previously that all major glycoproteins of pigeon egg white contain Galα1–4Gal epitopes (Suzuki, N., Khoo, K. H., Chen, H. C., Johnson, J. R., and Lee, Y. C. (2001) J. Biol. Chem. 276, 23221–23229). We now report that Galα1–4Gal-bearing glycoproteins are also present in pigeon serum, lymphocytes, and liver, as probed by Western blot with Griffonia simplicifolia-I lectin (specific for terminal α-Gal) and anti-P1, (specific for Galα1–4Galβ1–4GlcNAcβ1– monoclonal antibody. One of the major glycoproteins from pigeon plasma was identified as IgG (also known as IgY), which has Galα1–4Gal in its heavy chains. High pressure liquid chromatography, mass spectrometric (MS), and MS/MS analyses revealed that N-glycans of pigeon serum IgG included (i) high mannose-type (33.3%), (ii) distylated biantennary complex-type (19.2%), and (iii) α-galactosylated complex-type N-glycans (47.5%). Bi- and tri-antennary oligosaccharides with bisecting GlcNAc and α1–6 Fuc on the Asn-linked GlcNAc were abundant among N-glycans possessing terminal Galα1–4Gal sequences. Moreover, MS/MS analysis identified Galα1–4Galβ1–4Galβ1–4GlcNAc branch terminals, which are not found in pigeon egg white glycoproteins. An additional interesting aspect is that about two-thirds of high mannose-type N-glycans from pigeon IgG were monoglucosylated. Comparison of the N-glycan structures with chicken and quail IgG indicated that the presence of high mannosetype oligosaccharides may be a characteristic of these avian IgG.

Pigeon (Columba livia) egg white (PEW) is a rich source of glycoproteins containing Galα1–4Gal (galabiose). All four major PEW glycoproteins, ovotransferrin, two ovalumblins, and ovo mucoid, contain Galα1–4Gal (1), and all of their constituent N-glycans were found to possess Galα1–4Gal at the non-reducing termini of tri-, tetra-, and penta-antennary structures (2). No sialylation is found on the branch that contains the Galα1–4Gal sequence. Because biosynthesis and glycosyl modification of major egg white glycoproteins of chicken are carried out in tubular gland cells of oviduct (3), the most likely site of fabricating Galα1–4Gal linkages on the PEW glycoproteins would be in the corresponding cells of pigeon oviduct.

Galα1–4Gal in glycoproteins is, however, uncommon in nature. Mammals (e.g. human, pig, rat, mouse, and hamster) express Galα1–4Gal mostly on glycolipids (4–8), as in P1 (Galα1–4Galβ1–4GlcNAcβ1–3Galβ1–4Glcβ1–1Cer) and Pk (Galα1–4Galβ1–4Glcβ1–1Cer) antigens, but rarely on glycoproteins. An exception in mammals is the case of glycoproteins with Galα1–4Gal in hydatid cyst fluid and membrane infected by tapeworm Echinococcus granulosus (9–11). In animals other than birds and mammals, the only recorded presence of Galα1–4Gal was in O-glycosides such as those found in an insect cell line from Mamestra brassicae (12) or egg jelly mucins from amphibians, such as Xenopus laevis (13), Rana clamitans (14), and Ambystoma mexicanum (15).

The presence of Galα1–4Gal or substrates similar to P1 antigen on glycoproteins is limited even among other birds that have been studied. For example, P1 antigenic activities is absent in the blood of chicken, gander, turkey, quail, duck, and pheasant (16, 17), and no α-galactoside is found in the glycans of ovomucoid from chicken, quail, and duck (18–22). On the other hand, P1 antigenic activities have been found in the blood and/or eggs of pigeon, turtle dove (Streptopelia resorita), budgerigar, and cockatiel (16, 23, 24). Salivary gland mucin glycoproteins of Chinese swiftlets (genus Collocalia) contain O-linked glycans with Galα1–4Gal sequence (25). The biological significance of Galα1–4Gal in pigeon and some other avian glycoproteins is still unknown. Whether Galα1–4Gal is located specifically on PEW glycoproteins or glycoproteins of other pigeon organs is still unsettled. François-Gérard and co-workers (16, 17) reported the presence of P1 antigenic activity in pigeon blood. However, they did not indicate whether the antigenicity was located on glycolipids or glycoproteins and whether the P1 antigens were produced by pigeon themselves or derived from foreign bodies. To understand further the characteristics of species-specific oligosaccharides, we investigated the presence of glycoproteins with Galα1–4Gal in pigeon serum, lymphocyte, and liver. Here we report that one of the major glycoproteins containing Galα1–4Gal in serum is pigeon IgG, which possesses complex-type N-glycans containing Galα1–4Gal as well as high mannose-type oligosaccharides. The high mannose-type N-glycans are exclusively found on the CH3 domain, and the site specificity is probably a characteristic in avian IgGs. Unlike IgGs in other animals, the complex-type glycans were mainly tri- as well as...
bi-antennary structures. Moreover, N-glycans containing both Galα1–4Galβ1–4GlcNAc and Galα1–4Galβ1–4GlcNAc branches were detected by MS/MS analysis, which were not found in PEW glycoproteins.

EXPERIMENTAL PROCEDURES

Materials—Adult (female) pigeons were from a local racing pigeon breeder in Virginia. Pigeon livers were obtained from NASCO (Fort Atkinson, WI). Human serum, alkaline phosphatase-conjugated goat anti-mouse IgM, tritylphosphine, and 4-vinylpyridine were purchased from Sigma. Glycomidase F² (GAF) was from Prozyme Inc. (San Leandro, CA). Trypsin (sequencing grade) was from Worthington. Alkaline phosphatase-conjugated G2 was purchased from EY Laboratories, Inc (San Mateo, CA). Anti-P, mAb (mouse IgM) was from Gamma Biologicals, Inc. (Houston, TX). 5-Bromo-4-chloro-3-indolyl phosphate/nirotro blue tetrazolium kit for alkaline phosphatase assay was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Ficoll-Paque™ Plus was from Amersham Biosciences. Dulbecco’s phosphate-buffered saline twice. Pellets of

Preparation of Extracts from Pigeon Tissues and Cells—Blood (~5 mlpigeon) drawn from six pigeons were collected in blood collection tubes (13 × 75 mm) containing 0.057 ml of 15% KEDTA solution (BD Biosciences). The lymphocytes were isolated with Ficoll-Paque™ Plus, and washed with Dulbecco’s phosphate-buffered saline twice. Pellets of lymphocytes and recovered plasma were stored at −20 °C until use. The lymphocytes and a portion of pigeon liver were homogenized in 100 mm sodium sacodialyzed (pH 7.0) containing 1% Triton X-100 at 4 °C. The supernatants separated by centrifugation were used as extracts.

Isolation of Major Glycoproteins from Pigeon Plasma—Fresh pigeon plasma (20 ml) was washed with 1 N HCl (pH 8.0) and equilibrated with 10 mm sodium phosphate (pH 7.0) containing 150 mm NaCl. The flow rates were 0.25 ml/min for the HR 10/30 column and 2 ml/min for the HiLoad 26/60 column. Proteins in the eluate were monitored by A280 and individual peaks were collected manually. GS-I-positive fractions were concentrated with ultrafiltration (YM-10 membrane) and desalted by washing with 100 mm Tris-Cl (pH 8.0) for further purification by ion exchange chromatography. A column of DEAE-Sepharose Fast Flow (HiLoad 26/60 column) was loaded with 1 N HCl (pH 8.0) and equilibrated with 10 mm Tris-Cl (pH 8.0). After the sample injection, the column was washed with 100 mm Tris-Cl (pH 8.0) for 30 min (flow rate, 1 ml/min), and then the concentration of NaCl in the elution was linearly increased up to 0.4 m within 80 min (flow rate, 0.5 ml/min). The major peak was collected and concentrated with a Centriiom YM-10.

GAF Treatment of Glycoproteins—Glycoprotein samples (10 μg each) in 20 μl of 0.4% SDS and 100 mm mercaptoethanol in 10 mm Tris-Cl (pH 8.0), were heat-denatured at 90 °C for 3 min. After the solutions were cooled to room temperature, 1% (v/v) Nonidet P-40, and 1 unit of GAF were added to the solution of glycoproteins. The reaction mixtures were incubated at 37 °C for 16 h to complete de-N-glycosylation and then heated at 100 °C for 5 min to inactivate GAF.

Preparation of Glycopeptides for Peptide Sequencing—To reduce the existing disulfide bonds and to block random reformations of intramolecular disulfide bonds, 16 μmol of tritylphosphine and 0.16 mmol of 4-vinylpyridine were added to 1 mg of pigeon IgG in 0.1 m Tris-Cl (pH 8.5) and incubated at room temperature for 2 h under nitrogen. The reaction mixture was dialyzed against water and lyophilized. One-third of the reduced and alkylated pigeon IgG was suspended with 40 μl of 50 mm NH4HCO3 (pH 8.4) and incubated with 12.5 μg of trypsin (sequencing grade) at 37 °C. After a portion of the supernatant was further treated with GAF (0.5 units/10 μl) at 37 °C, overnight. The peptide mixtures, before and after the GAF treatment, were analyzed with reverse-phase HPLC on a HP C18 column. The TSKgel Ami-200 mesh, free base form, Bio-Rad) resins, washed with water, and then eluted with 50 μl of 25% CH3CN containing 0.05% trifluoroacetic acid.

Exo-glycosidase Digestion—The released N-glycan mixtures were digested sequentially with 500 units of neuraminidase from Arthrobacter ureafaciens (Roche Applied Science) in 25 μl of 50 mm sodium acetate buffer (pH 5.0), 0.5 units of α-galactosidase (from green coffee bean, Calbiochem) in 100 μl of 50 mm citrate-phosphate buffer (pH 6.0), followed by 5 milliunits of β1–4 galactosidase (from Streptococcus pneumoniae, Calbiochem) in 50 μl of 50 mm sodium acetate buffer (pH 6.0). All digestions were carried out at 37 °C for 24 h. Each enzyme digestion was desalted by passing through a mixed bed ion-exchange column packed with a mixture of 1 ml each of Dowex 50W-X8 (50–100 mesh, H+ form) and AG 3-X4 (100–200 mesh, free base form, Bio-Rad) resins, prior to taking an aliquot for permethylation and MS analysis. PA-glycans were desalted instead by passing through a Sep-Pak C18 cartridge (Waters), washed with water, and then eluted with 50% methanol in water.

Chemical Derivatization and GC-MS Linkage Analysis—N-Glycan and PA-derivatized N-glycan samples were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell et al. (27). For GC-MS linkage analysis, partially methyalted alditol acetates were prepared from permethylated derivatives by hydrolysis (2 m trifluoroacetic acid, 121 °C, 2 h), reduction (10 mg/ml NaBH4, 25 °C, 2 h), and acetylation (acetic anhydride, 90 °C, 1 h). GC-MS was carried out using a Hewlett-Packard Gas Chromatograph 6890 connected to a Hewlett-Packard 5973 Mass Selective Detector. Sample was dissolved in hexane prior to splitless injection into a HP-5MS fused silica capillary column (30 m × 0.25 mm inner diameter). The column head pressure was maintained at around 8.2 pounds/square inch to give a constant flow rate of 1 ml/min. The temperature was held at 60 °C for 1 min and increased to 90 °C in 1 min and then to 290 °C in 25 min.

MS Analysis for Glycans and Glycomics—For MALDI-TOF MS glycan profiling, the permethylated derivatives in acetonitrile were mixed 1:1 with 2,5-dihydroxybenzoic acid matrix (10 mg/ml in acetonitrile), spotted on the target plate, air-dried, and recrystallized with ethanol whenever necessary. Data acquisition was performed manually on a benchtop MALDI LR system (Micromass) operated in the reflectron mode. For 2,5-dihydroxybenzoic acid matrix, the laser energy control was set at high and finely adjusted using the % slider.
according to sample amount and spectra quality. Laser shots (5 Hz, 10 shots/spectrum) were accumulated until a satisfactory signal to noise ratio was achieved when combined and smoothed. Glycan mass profiling was also performed on a dedicated Q-TOF Ultima MALDI instrument (Micromass) in which the permethylated samples in acetonitrile were mixed 1:1 with α-cyano-4-hydroxycinnamic acid matrix (in acetonitrile, 0.1% trifluoroacetic acid, 99:1, v/v) for spotting onto the target plate. The nitrogen UV laser (337 nm wavelength) was operated at a repetition rate of 10 Hz under full power (300 μJ/pulse). MS survey data were manually acquired, and the decision to switch over to CID MS/MS acquisition mode for a particular parent ion was made on-the-fly upon examination of the summed spectra. Argon was used as the collision gas with a collision energy manually adjusted (between 50 and 200 V) to achieve optimum degree of fragmentation for the parent ions under investigation.

Off-line nanoelectrospray (nano-ESI) using the borosilicate metal-coated glass capillary option was performed on a Q-TOF Ultima API instrument (Micromass) equipped with a nanoflow source, mainly for CID MS/MS analysis of permethylated glycans samples. The capillary voltage was set at about 1.0–1.2 kV. Either protonated or/and siodated doubly or triply charged parent ions may be selected for MS/MS. A collision energy setting of 20–40 eV was usually sufficient for doubly protonated species, but 60–80 eV was needed for singly and doubly siodated parent ions. Permethylated samples were dissolved in 40 μl of 2-aminopyridine solution (1 g of 2-aminopyridine in 580 μl of concentrated HCl (pH 6.8)) and heated at 90 °C for 15 min with heating block. Freshly prepared NaCNBH3 solution (7 mg of 2-aminopyridine in 1 ml of concentrated HCl (pH 6.8)) and heated at 90 °C for 1 h. PA-oligosaccharides were analyzed using gel filtration on a Sephadex G-15 column (1.0 × 40 cm, in 10 mM NH4HCO3), monitored by a fluorescence (excitation, 300 nm; emission, 380 nm), and lyophilized. The mixture of PA-oligosaccharides was first separated by HPLC with TSK gel DEAE-5PW column (7.5 × 75 mm) as described previously (28), and the separated neutral, mono-sialyl, and di-sialyl fractions (monitored by fluorescence, Ex, 320 nm; Em, 400 nm) were collected separately and lyophilized. These fractions were individually dissolved in water and separated on a Shim-Pack CLC-ODS column (6.0 × 150 mm) as described previously (1). Elution was performed at a flow rate of 1.0 ml/min at 55 °C using eluent A (0.05% trifluoroacetic acid) and eluent B (0.5% 1-butanol in eluent A). The column was equilibrated with a mixture of eluents A/B = 90:10 (v/v), and after injection of a sample, the composition of the eluents was changed linearly to A/B = 30:70 in 120 min. Each peak was collected, lyophilized, and analyzed with MALDI-TOF-MS. For determination of N-glycan structures by two-dimensional mapping with HPLC, the isolated PA-oligosaccharides were analyzed using CLC-ODS and TSK gel amide-80 columns (4.6 × 250 mm) as described previously (29). Elution position of each PA-oligosaccharides on CLC-ODS and amide-80 columns was expressed as glucose units (GU), based on the elution position of isomaltos-oligosaccharide series. Reference PA-oligosaccharides from asialo-human IgG, bovine RNase B, and chicken serum IgG were prepared by the same method. Structures of the reference compounds are shown in Fig. 9. PA-derivatized N-glycans B-1, C-1, and D (Fig. 9) were obtained from α-fucosidase and N-acetyl-β-D-hexosaminidase-digested PA-oligosaccharides from human IgG. N-Glycans B-2 and C-2 were prepared from B-1 and C-1, respectively, with β-galactosidase digestion.

RESULTS

Detection of α-Galactoside on Pigeon Glycoproteins by GS-I Lectin and Anti-P1 mAb

Approximately equal amounts of proteins from pigeon serum, liver, lymphocytes, and egg white were subjected to SDS-PAGE, transferred onto a PVDF membrane, and visualized by staining with Coomassie Brilliant Blue (CBB) (Fig. 1A, CBB staining) or probed by blotting with GS-I lectin to detect terminal α-Gal residues. As shown in Fig. 1A (GS-I staining), some proteins from pigeon serum, liver, and lymphocytes were visualized by this staining as were glycoproteins of PEW. Intensities of the GS-I staining did not coincide with those of the CBB staining, suggesting that proteins carry α-Gal residues with varied density. Immunoblotting with anti-P1 mAb (specific for P1, blood type) (Fig. 1A) gave similar staining patterns as shown by the GS-I lectin blotting. Extract from pigeon heart also stained with GS-I and anti-P1 mAb (data not shown). These data indicate that many kinds of glycoproteins in pigeon organs most likely contain terminal Galα1–4Gal as found in PEW (1).

The presence of α-Gal residues on proteins was probed in pigeon, chicken, bovine, and human sera. Although approximately the same amounts of respective proteins in these sera were stained with CBB, only pigeon serum proteins stained strongly by GS-I (Fig. 1B). Because twice as much proteins was used in this experiment (Fig. 1B) as the previous experiment (Fig. 1A), more bands were clearly visualized by the GS-I staining. Bovine is known to produce Galα3Gal or other α-Gal epitopes, was not stained by GS-I, confirming that these serum proteins have no terminal α-galactosyl residues.

Isolation of Pigeon Plasma Glycoproteins

Pigeon plasma proteins (600 μg total) were separated by gel filtration using a Superdex 200 HR 10/30, as shown in Fig. 2A. As molecular size markers, chicken IgG (170 kDa) and BSA (67 kDa) were eluted in separate runs, and their positions were marked. Each of the peaks obtained from the Superdex 200 was collected, and 50 μl of each fraction was analyzed by SDS-PAGE and GS-I lectin blotting under the reducing conditions (Fig. 2, B and C). Proteins in fractions 2–8 were positively stained by GS-I, whereas other fractions (fractions 1 and 9–16)
were negative to GS-I. The fraction 13 was the largest peak detected by A$_{280nm}$, but no protein/peptide bands were visualized with CBB or GS-I staining. One of the glycoproteins in the fraction 6, which was eluted at the same elution position as chicken IgG, showed the strongest intensity by the GS-I staining. In contrast, major proteins in fraction 8, where BSA is expected to elute, were stained with CBB strongly but not at all with GS-I. The similar elution profile was detected when 6 mg of pigeon plasma proteins was separated using a semi-preparative Superdex 200 column (2.6 × 60 cm) (data not shown).

Fraction 6 from Superdex 200 was further purified with a DEAE-Sepharose column eluting with a gradient of NaCl, as shown in Fig. 2D. The major peak on the anion-exchange column was collected, and the purity of the protein was confirmed by SDS-PAGE (Fig. 2D, inset). As in the case of chicken immunoglobulins (33), two distinct bands (67 and 27 kDa) were visualized, suggesting it to be IgG. The protein after the purification with gel filtration and anion-exchange columns was estimated to be ~5 mg from 1 ml of pigeon serum. This value is within the range of reported concentration of IgG in adult pigeon serum (5.92 mg/ml) (34).

Identification of the Pigeon Serum Glycoprotein by Partial Peptide Sequencing

N-terminal amino acid sequences of the light (L) and heavy (H) chains were homologous to the corresponding variable regions of immunoglobulins of chicken, duck, and human (Table I). Because avian IgG, IgA, and IgM share the same N-terminal variable domains (35), internal peptide sequencing on the Fc region would be required for the determination of the immunoglobulin classes (36–38). The predominant serum immunoglobulin in birds is IgG, also known as IgY, because of its unique structure. It is functionally equivalent to mammalian IgG but has one additional constant region domain in its heavy (H) chains (Fig. 3) (33, 39). Throughout this paper, we refer to the predominant serum immunoglobulin in birds as serum IgG for convenience. The amino acid sequences of chicken and duck immunoglobulin H chains (H chains) suggest that two potential N-linked glycosylation sites are indeed well conserved between chicken and duck, located in the Fc region (Fig. 3) (36, 40). In contrast, positions of potential N-glycosylation sites on Fc regions of avian IgA and IgM are different from those of IgG. Therefore, isolation and peptide sequencing of N-glycosyl peptides were performed in order to determine the immunoglobulin class based on sequence homology. The C18-HPLC elution profiles of tryptic peptides of pigeon IgG, before and after GAF treatment, indicated that “Peak A” shifted its position from 46.4 to 50.1 min after GAF treatment (Fig. S1). As shown in Table I, the glycopeptide sequence of the pooled peak A is homologous to that of a CH3 domain of chicken or duck IgG. The sequence alignment showed that conservation of the potential N-glycosylation sites is extended to pigeon, chicken, and duck but not to chicken IgM and IgA. Based on these sequence data, the isolated protein was identified as pigeon serum IgG. The CH3 domains of chicken and duck IgG resemble the CH2 domain of mammalian IgG (36, 39, 40). This appears to be the case for pigeon IgG also, because the peptide sequence containing the N-glycosylation site was similar to the corresponding reference molecules (chicken IgG and BSA) are shown. Individual peaks from the column were collected and analyzed on SDS-PAGE, using CBB staining (B) and GS-I lectin blotting (C). D, purification of the major glycoprotein from pigeon plasma with DEAE-Sepharose. Desalted fraction (fr.) 6 from the Superdex 200 column was applied to a DEAE-Sepharose column (1 ml) and eluted with a linear gradient of NaCl in 10 mM Tris-HCl (pH 8.0). The major peak (fr. 3) was collected and analyzed by SDS-PAGE (inset).
Sequence identity search was performed using NCBI data base. N-Linked glycosylation sites are indicated by bold face type. The N-glycosylation site on pigeon IgG was assigned by the presence of Asp, which occurred after GAF digestion.

### Table I

| Protein          | Sequence                  | NCBI accession no.\(^{a}\) | Identity\(^{b}\) (%) |
|------------------|---------------------------|----------------------------|----------------------|
| **Light chain (\(\alpha\)), N-terminal sequence** |                           |                            |                      |
| Pigeon           | ALTQPSSVSTTLGGTVQIT        | BABA2724                   | 15/19 (79)           |
| Chicken          | ALTQPAYSAASLGGTQKT        | CA975588                   | 13/19 (68)           |
| Duck             | ALTQPASKVPNVPGTQIT        | CA934183                   | 12/19 (63)           |
| Human            | LTQPPSVAASLGAASVIT       |                            |                      |
| **Heavy chain, N-terminal sequence** |                           |                            |                      |
| Pigeon           | AIELVESGGGLVSPGGLRL       | AAA84886                   | 13/20 (65)           |
| Chicken          | AVTLDSSGGLQTPGGLSLL       | CA463222                   | 16/20 (80)           |
| Duck             | AATLDESGGGLVSPGGLTTL     | P01776                     | 15/20 (75)           |
| Human            | EVQLLGSYSGGLPQGGLRL      |                            |                      |
| **Heavy chain (\(\gamma\)), N-glycosylation site** |                           |                            |                      |
| Pigeon           | AQPNGFTFTATSEVPVAT        | CAA30161                   | 10/18 (56)           |
| Chicken (CH3)    | EHHNFYSAVSAVPSTQQ        | CA446222                   | 9/18 (50)            |
| Duck (CH3)       | EHHNFYFTASLASSSTSTQQ     | CAC20454                   | 5/18 (28)            |
| Human (CH2)\(^{c}\) | EQQNTRYRVVSVEVTLIQ        | CA962762                   | 5/18 (28)            |
| Chicken IgM (CH3)\(^{d}\) | LQSNGLYTVGDVGATVCAS     | AAB23141                   | 5/18 (28)            |
| Chicken IgA (CH3)\(^{d}\) | RQADGRTYVRSFLRCAE        |                            |                      |

\(^{a}\) Primary accession numbers for the data base.

\(^{b}\) Identity of the peptide segment (%) were calculated as (number of identical amino acid residues) \(\times 100\) / (number of total amino acid residues).

\(^{c}\) CH2 domains of mammalian IgG is equivalent to CH3 domains of avian IgG.

\(^{d}\) The positions of indicated sequences correspond to that in CH3 domain of chicken IgG.

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### MS and MS/MS Analysis of N-Glycans from Pigeon IgG

The N-glycans released by GAF from pigeon IgG were first profiled by MALDI-TOF MS analysis of the permethylated derivatives. Substantial heterogeneity was evident (Fig. 4A), but the glycans can be conveniently categorized into the following: (i) high mannose-type, (ii) di-sialylated biantennary complex-type, and (iii) \(\alpha\)-galactosylated complex-type structures.

**High Mannose-type N-Glycans**—The two most abundant peaks in Fig. 4A correspond to Hex\(_3\)HexNAc\(_2\) (m/z 2397) and Hex\(_2\)HexNAc\(_2\) (m/z 2601), which also afforded oxonium-type fragment ions at m/z 2097 and 2301, respectively, via cleavage at the chiitoiose core. Smaller amounts of Hex\(_2\)HexNAc\(_2\) (m/z 1579) and Hex\(_2\)HexNAc\(_2\) (m/z 2192) were also present. Upon \(\alpha\)-mannosidase treatment, only these components were digested, giving two major signals at m/z 1579 (Hex\(_2\)HexNAc\(_2\)) and 1783 (Hex\(_2\)HexNAc\(_2\)) in addition to a signal corresponding to Hex\(_2\)HexNAc\(_2\) (data not shown). The same digestion when applied to high mannose structures derived from RNase B yielded only the expected product, i.e. Man\(_7\)GlcNAc\(_2\). The data are therefore consistent with the presence of a Glc residue at one of the non-reducing termini of a Man\(_7\)GlcNAc\(_2\) that would block the digestion of the Man residues (later to be shown to be on the \(\alpha\)-3-arm), allowing a removal of only 4 or 5 Man residues from the \(\alpha\)6-arm. The presence of a monoglucosylated Man\(_7\)GlcNAc\(_2\) along with Man\(_7\)GlcNAc\(_2\) as the major glycoforms has been reported for chicken and quail IgG (41–43) and may be a characteristic of avian IgG. Further characterization based on two-dimensional HPLC mapping of the PA-derivatives confirmed this structure (see below) and has further resolved Hex\(_3\)HexNAc\(_2\) into Man\(_7\)GlcNAc\(_2\) (n=6, major component) and Glc\(_5\)Man\(_7\)GlcNAc\(_2\) (n=7, minor component), along with Glc\(_6\)Man\(_7\)GlcNAc\(_2\) (n=8), as the predominant high mannose-type structures. Interestingly, only (\(\pm\)Glc\(_1\)Man\(_7\)GlcNAc\(_2\)) and (\(\pm\)Glc\(_1\)Man\(_7\)GlcNAc\(_2\)) structures were found on the glycopeptide isolated from the CH3 domain (Fig. 4C).

**Di-sialylated Biantennary N-Glycans**—The sialylated components were first identified by the m/z values of the molecular ion signals and subsequently confirmed by digestion with neuraminidase (Fig. 4B). A prominent disialylated component afforded a major molecular ion at m/z 2071 corresponding to a biantennary complex-type structure that was absent before desialylation (Fig. 4A). The biantennary branching pattern was
established by MS/MS analysis (Fig. S3). In particular, the ion at m/z 1143 from MALDI-MS/MS (Fig. S3B) unambiguously defined the presence of two antennae extending from the trimannosyl core, whereas the ion pair at m/z 432 and 464 from nano-ESI-MS/MS (Fig. S3A) indicated an N-acetyllactosamine (LacNAc) unit and not a type 1 chain (Galβ1–3GlcNAc) (27).

Removal of two Hex units by β4-galactosidase from S. pneumoniae resulted in a shift of the corresponding molecular ion signal at m/z 2071 (Fig. 5A) to m/z 1661 (Fig. 5B) which confirmed this assignment. Finally, methylation analysis of the desialylated samples clearly demonstrated a disappearance of 6-linked Gal (data not shown) as compared with the GC-MS profile of the sample not treated by neuraminidase (Fig. S4). The molecular ion at m/z 2794 in Fig. 4A could thus be assigned as α2–6-disiallylated biantennary complex-type N-glycan which shifted to give a molecular ion of m/z 2071 after neuraminidase digestion (Fig. 5A). No other sialylated component of high abundance was apparent from the analysis of the total mixtures, although monosialylated counterparts of several other complex-type structures could be detected as minor peaks, the detailed analysis of which was made possible after HPLC fractionation (see below).

α-Galactosylated N-Glycans—Consistent with the earlier detection by antibody and lectin, the majority of the non-sialylated complex-type structures were shown to carry terminal α-Gal by two criteria. First, the m/z of most of the molecular ions detected shifted to lower values after digestion with α-galactosidase (Fig. 5A). Second, methylation analysis indicated a high abundance of terminal Gal and 4-linked Gal (Fig. S4), supporting the presence of Galβ1–4Gal epitope. The mass spectrum (Fig. 4A) indicated a high degree of heterogeneity within the α-Gal-containing N-glycans, but their m/z signals could be assigned as having ±(Fuc1)Hexα2–6HexNAc4 and ±(Fuc1)Hexα2–3HexNAc4 on the trimannosyl core (Manα,GlcNAc). After sequential α-galactosidase and β4-galactosidase digestion, the major products observed were (GlcNAc)Manα,GlcNAc ± core fucosylation (m/z 2153 and 2327) and (GlcNAc)Manα,GlcNAc2 ± core fucosylation (m/z 1907 and 2082), as shown in Fig. 5B. MS/MS analysis showed that Fuc was indeed conjugated to the inner GlcNAc of the chitobiose core, whereas all non-reducing terminal HexNAcs occur as single HexNAc linked.
to the trimannosyl core (data not shown). No HexNAc-HexNAc type termini were detected. The other major molecular ion signal at \( m/z \) 1661, (GlcNAc\( _{2}\)Man\( _{3}\)GlcNAc\( _{3}\)), corresponded to the digested product of the sialylated biantennary structure, whereas the high mannose structures at \( m/z \) 2397 and 2601 remain unchanged.

When the major molecular ion signals from samples not treated with the exoglycosidase (Fig. 4A) were subjected to MALDI CID MS/MS analysis, additional unique structural features were unveiled. Fig. 6 shows spectra of MS/MS analysis for the Hex\( _{4}\)HexNAc\( _{3}\)CF series, where CF denotes fucosylated trimannosyl core. Interpretation of the MS/MS data for the Hex\( _{4}\)HexNAc\( _{3}\)CF series was summarized in Fig. 7. As expected, the presence of Gal-Gal-GlcNAc terminal epitope was indicated by the sodiated b ion at \( m/z \) 690 which could be detected in all of the glycans analyzed. For the larger components, however, another b ion at \( m/z \) 894, corresponding to sodiated Hex\( _{2}\)HexNAc\( _{4}\), was also very prominent. In the case of Hex\( _{4}\)HexNAc\( _{3}\)CF, sequential loss of the Hex\(_{2}\)HexNAc or Hex\( _{3}\)HexNAc terminal units concomitant with emergence of free OH group(s) yielded the complementary set of y ions (e.g. at \( m/z \) 3292, 2420, 1548, 3088, and etc., see Fig. 7E). MS/MS data also indicated that the majority of the Hex\( _{2}\)HexNAc\( _{4}\)CF components is triantennary carrying a bisecting GlcNAc that is readily lost from the parent ion under the MS/MS conditions employed. Two ions at \( m/z \) 1548 (corresponds to the fucosylated trimannosyl core with a bisecting GlcNAc and three free OH groups resulting from the loss of all three antennary sequences, Fig. 7E, left) and \( m/z \) 1289 (corresponds to a further loss of the bisecting GlcNAc giving a total of four free OH groups, Fig. 7E, right), which were present in all Hex\( _{4}\)HexNAc\( _{4}\)CF series structures, supported the presence of bisecting GlcNAc. Thus, the structure of Hex\( _{4}\)HexNAc\( _{4}\)CF is most likely a triantennary bisected and fucosylated trimannosyl core, extended with one Hex-Hex-HexNAc (Hex\(_{2}\)HexNAc) and two Hex-Hex-Hex-HexNAc (Hex\(_{3}\)HexNAc) sequences (Fig. 7E).

Similarly, Hex\( _{2}\)HexNAc\( _{4}\)CF was determined to be the triantennary structure with bisected and fucosylated trimannosyl core carrying two Hex\( _{2}\)HexNAc and one Hex\(_{3}\)HexNAc sequence (Fig. 7D). The majority of Hex\( _{2}\)HexNAc\( _{4}\)CF carries three Hex\(_{2}\)HexNAc units (Fig. 7C), but the presence of \( m/z \) 894, albeit of low abundance, indicated possible isomers with one antenna each of Hex\(_{2}\)HexNAc, Hex\(_{3}\)HexNAc, and Hex\(_{4}\)HexNAc. Likewise Hex\(_{2}\)HexNAc\( _{2}\)CF (Fig. 7B) showed the presence of Hex\(_{2}\)HexNAc\( _{2}\) (\( m/z \) 894) and Hex\(_{4}\)HexNAc\( _{2}\) (\( m/z \) 486). Hex\(_{2}\)HexNAc\( _{2}\)CF showed very little \( m/z \) 894, but \( m/z \) 282 (terminal HexNAc) was found instead, along with \( m/z \) 486 and 690 (Fig. 7A). A similar pattern was found for the Hex\(_{2}\)\(_{2}\)-HexNAc\( _{2}\)CF series which were predominantly bisected biantennary structures. MS/MS analysis suggested that the main isomer of Hex\(_{2}\)HexNAc\( _{2}\)CF contained two Hex\(_{2}\)HexNAc antennas, whereas Hex\(_{2}\)HexNAc\( _{2}\)CF mostly carries a Hex\(_{2}\)•
HexNAc and a Hex$_3$HexNAc antenna (data not shown). The predominance of bisected complex-type structures was in harmony with the methylation analysis (Fig. S4). The presence of unsubstituted GlcNAc and a triply substituted (3,4,6-linked) Man supported this conclusion. Only 4-linked GlcNAc was found (indicative of the type 2 chain, Gal$\beta$1–4GlcNAc$\beta$1–4GlcNAc), and the amount of 3-linked GlcNAc residue was insignificant. The Gal residue linked to GlcNAc was susceptible to $\beta$4-galactosidase. The linkage analysis data suggest that Hex$_3$HexNAc and Hex$_4$HexNAc terminal units may be Gal$\beta$1–4Gal$\beta$1–4GlcNAc, because no other residue, e.g. branched Gal residue, was found. The results of digestion using $\alpha$- and $\beta$4-galactosidases (Fig. 6) suggest that they are most likely to be Gal$\alpha$1–4Gal$\beta$1–4GlcNAc$\beta$1–4Gal$\beta$1–4GlcNAc, as labeled on the figure. Assignment of the fragment ions are illustrated by diagrams in Fig. 7. Some fragment ions could not be labeled or assigned, because of complication arising from isomeric structures.

Fractionation and Structural Analysis of PA-derivatized $\text{N}$-Glycans from Pigeon IgG

The presence of Gal$_3$GlcNAc antenna was unexpected and has resulted in greater heterogeneity, because each glycan as assigned above may consist of smaller amounts of isomers with different combinations of fully galactosylated to non-galactosylated antennae. This was found to be the case when the glycans from pigeon IgG were derivatized with 2-aminopyridine and fractionated first into neutral, monosialylated, and disialylated fractions with a DEAE column (Fig. 8A) and then further fractionated on an ODS column (Fig. 8B), followed by MS and MS/MS analyses of the collected major fractions (Tables II and III and Supplemental Material Figs. S1 and S2). Molar ratios of pigeon IgG N-glycans calculated from the peak areas of PA-oligosaccharides were neutral 64.8% (high mannose-type, 33.3%, others, 31.5%), monosialylated 16.0%, and disialylated 19.2%. N-Glycan structures of the three categories were determined as follows.

PA-derivatized High Mannose-type N-Glycans—Mass spectrometric analysis revealed that fractions of neutral N-glycans eluted at around 4–12 min on the ODS column (n=5 to n=10) were Hex$_5$, 8–11HexNAc$_5$-PA, suggesting that these are high mannose-type oligosaccharides. Their elution positions on the ODS column also confirm this assignment (29). The fractionated high mannose-type N-glycans (n=5, n=6, n=7, n=8, and n=9) were further examined by the two-dimensional HPLC mapping (29). The elution position of the PA-N-glycans on both ODS and
amidine-80 columns, before and after α-mannosidase digestion, were compared with those of the reference compounds derived from bovine RNase B and chicken serum IgG (Fig. 9). Because the elution positions on the two columns as well as mass values for the N-glycans of n-5, n-6, n-7, n-8, and n-9 were indistinguishable with those of Man6GlcNAc2-PA, Man6GlcNAc2-PA, Glc3Man9GlcNAc2-PA, Glc3Man9GlcNAc2-PA, and Man7GlcNAc2-PA, respectively (data not shown), their structures were determined as shown in Table II.

**PA-derivatized Disialylated Biantennary N-Glycans**—The two disialylated N-glycans (ds-6 and ds-8) were analyzed by MALDI-MS before and after permethylation to define their molecular compositions and by MALDI MS/MS and HPLC for structural assignment (Table III). N-Glycan ds-8 clearly corresponded to the disialyl biantennary structures detected in the MS analysis of the total mixtures for which further MS/MS analysis of the disialylated structures (Fig. S3) have unambiguously established its biantennary structures. In addition, after α-neuraminidase digestion, the elution positions of ds-8 on HPLC columns were indistinguishable from human IgG N-glycan D (Fig. S5B), confirming its structure as non-bisected biantennary complex-type without core fucosylation. MS and MS/MS analysis of N-glycans ds-6 (Fig. S5A and Table III) led to identification of a unique NeuAc-HexNAc-HexNAc antenna on ds-6 in which the Hex to HexNAc substitution constitutes the sole difference between ds-6 and ds-8. This was supported by the fact that the elution of ds-6 (GUAmide 6.9) on the amide-80 column was 0.3–0.4 GUAmide earlier than that of ds-8 (GUAmide 7.2) (Fig. S5B), which is attributable to the difference in unit contribution values between Hex and HexNAc (29, 44). Likewise, the GUAmide of both ds-6 and ds-8 was reduced by about 0.3–0.4 GU after desialylation, indicative of their α2–6 but not α2–3 sialylation (45, 46). When the desialylated ds-6 was further treated with N-acetyl-β-D-hexosaminidase, the elution positions on ODS and amide-80 columns (GUODS 10.3; GUAmide 5.6) were indistinguishable from the reference N-glycan B-1 but different from C-1 (GUODS 7.7; GUAmide 5.7). This suggests that desialylated ds-6 released two HexNAcs from the Manα1–3 branch. Further digestion with β-D-galactosidase resulted in a product eluting at the same position as N-glycan B-2 (GUODS 9.5; GUAmide 4.7) but different from C-2 (GUODS 7.1; GUAmide 4.6), thus further confirming that the NeuAc-HexNAc-HexNAc antenna of ds-6 is extending from the Manα1–3 branch. NeuAc-HexNAc-HexNAc unit is most likely corresponding to NeuAc2–6GalNAcβ1–4GlcNAc, although no further evidence can be obtained at present because of lack of the sufficient sample quantity.

**PA-derivatized α-Galactosylated Neutral N-Glycans**—The ODS-fractionated neutral complex-type structures (eluted over 20–70 min), identified by MS and MS/MS, are listed in Table S1. Each of the fractions (Fig. S8) was first screened by MALDI-TOF MS, and the assigned composition was further confirmed by subsequent analysis of the permethylated derivatives. MALDI MS/MS was performed where the sample quan-
Conclusions that can be drawn from more detailed MS/MS analysis of the individually purified components are in general consistent with the analyses of the total mixtures (Fig. 4A), except that ODS fractionation of the PA-derivatives separated isomeric series and also led to detection of a more complete range of minor components.

α-Galactosidase digestion of Hex$_2$HexNAc$_2$-PA from n-37 resulted in a loss of three α-Gal residues, and subsequent β4-galactosidase digestion converted it to HexNAc$_2$-PA (Fig. S7). Together with the MS/MS results (Table S1), the structure of n-37 is most likely (Galα1-4Galβ1-4Galβ1-4GlcNAc)-Galα1-4Galβ1-4GlcNAc-$\text{NCF-PA}$, where NCF denotes fuco-sylated trimannosyl core with bisecting GlcNAc. Similarly, sequential α/β-galactosidase digestions of Hex$_2$HexNAc$_2$-PA from n-38 released two α-Gal and four β-Gal residues and thus implicated a likely structure of (Galβ1-4Galβ1-4GlcNAc)-(Galα1-4Galβ1-4GlcNAc)$_2$-NCF-PA. These results indicated that for n-38, one of the Hex$_2$HexNAc antennae was not α-Gal capped. The non-reducing termini of pigeon IgG N-glycans may therefore comprise Galβ1-4Galβ1-4GlcNAc, Galβ1-4GlcNAc, and GlcNAc in sub-galactosylated structures.

**PA-derivatized α-Galactosylated Monosialylated N-Glycans**—Although only a single disialylated biantennary structure was prominent, the presence of other minor sialylated structures was noted from MS analysis of the total mixtures (Fig. 4A). Indeed, after PA derivatization and fractionation on the ODS column, about 60 peaks could be detected for the monosialylated fraction. A select few of the monosialylated components were screened by MALDI-MS before and after permethylation to define their molecular compositions and, wherever possible, analyzed by MALDI MS/MS for structural assignment (Table S2). MS/MS analysis of the major monosialylated glycans indicated that all sialylated antennae are NeuAc-Hex-HexNAc with no additional Gal inserted (Fig. S8 and Table S2 and Fig. 10). However, the other antennae can be Hex$_2$HexNAc or Hex$_3$HexNAc, likely to be identical to the α-galactosylated antenna found in the neutral glycans. It thus appears that the predominant bisected bi- and triantennary complex-type structures with core α6-fucosylation are highly heterogeneous due primarily to a combination of different capping moieties on the Galβ1-4GlcNAc antennae, i.e. Galα1-4-, Galα1-4Galα1-4-, and NeuAcα2-6-. In the case of Galα1-4Galβ1-4Galβ1-4GlcNAc termi, an additional β-Gal may be added to the LacNAc unit without the concomitant α-Gal capping. Although we have not analyzed all of the isomeric structures resolved by HPLC, the general conclusion may apply to the overall glycosylation heterogeneity of pigeon serum IgG.

**DISCUSSION**

The diversity of carbohydrate structures is manifest in nature at many different levels, but expression of different oligosaccharide structures among different species is an important subject of inquiry in glycobiology. Highly complex oligosaccharides are speculated to be generated by necessity for survival among hosts and microbes through the symbiotic-commensal-pathogenic continuum (47, 48). However, only a few case of species-specific oligosaccharide moiety had been investigated systematically. One of the better known examples is the Galα1-3Gal epitope, which is widely distributed in all mammals except human, apes, and Old World monkeys. This moiety has been reported to be absent from fish, amphibian, reptile, and bird fibroblasts but present on both cell surfaces and secreted glycoproteins of the noncattarhine mammals (31, 32, 49). N-Glycans possessing Galα1-3Gal, found in mammalian glycoproteins, have some structural analogy to those possessing Galα1-4Gal, found in PEW glycoproteins (1), because both α3-Gal and α4-Gal are located on non-reducing termini of Lac-
NAc. With the example of Galα/H9251–3Gal in mind, we first examined the presence of Galα/H9251–4Gal in pigeon serum, lymphocyte, and liver and then isolated one of the major serum glycoproteins possessing Galα/H9251–4Gal, which was identified as IgG.

Moreover, analysis of oligosaccharide structures of pigeon IgG unveiled several unique glycosylation properties not found in other avian IgGs or in mammalian IgGs. First, little or no -galactosylated oligosaccharides have been detected in normal serum IgG of non-primate mammals such as dog, cow, sheep, horse, rat, mouse, rabbit, and cat (42, 50), even though these animals have functional -1,3-galactosyltransferase (GalT). A well conserved N-glycosylation site in mammalian IgG is located at Asn-297 (Eu numbering) on CH2 domains (51), and oligosaccharide chains can be seen occluding the cavity at the center of the Fc (52). The N-glycosylation at Asn-297 with biantennary complex-type oligosaccharides is indispensable, because it influences mammalian IgG in its thermal stability, interaction with Fc receptors, association with C1q, and induction of antigen-dependent cellular cytotoxicity (53–55). A well conserved N-glycosylation site in mammalian IgG is located at Asn-297 (Eu numbering) on CH2 domains (51), and oligosaccharide chains can be seen occluding the cavity at the center of the Fc (52). The N-glycosylation at Asn-297 with biantennary complex-type oligosaccharides is indispensable, because it influences mammalian IgG in its thermal stability, interaction with Fc receptors, association with C1q, and induction of antigen-dependent cellular cytotoxicity (53–55).

Second, about one-third of the total N-glycans from pigeon serum IgG was high mannose-type oligosaccharides, whereas normal mammalian serum IgG contain exclusively biantennary complex-type N-glycans (42). It should be pointed out that 61.7% of the high mannose-type glycans in pigeon IgG are mono-glucosylated as found in chicken (serum, egg yolk) (41) and quail (egg yolk) IgGs (43). Specifically, we demonstrated that pigeon IgG-CH3 glycopeptides contain only high mannose-type oligosaccharides. Because the cognate site specificity was also found in chicken serum IgG3, this site-specific N-glycosylation pattern might have resulted from the steric hindrance imposed by the unique conformational structures of avian IgG. The steric hindrance on avian IgG-CH3 may be more severe than that on mammalian IgG-CH2 and allow only limited process on the N-glycans.

Because N-glycans located on CH3 domain are exclusively high mannose-type, complex-type N-glycans were consequently located at other glycosylation sites on avian IgG. Interestingly, structural analysis of N-glycans revealed that the complex-type

| Fraction | Relative Quantity (%) | GU on ODS | GU on Amide | m/z Detected | Reference N-Glycans | Deduced Structures |
|----------|----------------------|-----------|-------------|--------------|-------------------|-------------------|
| n-5      | 0.71                 | 4.8       | 8.8         | 1822.60      | Man8              |                   |
| n-6      | 8.40                 | 5.2       | 9.5         | 1984.86      | Man9              |                   |
| n-7      | 1.71                 | 5.8       | 9.5         | 1984.81      | GlcMan8           |                   |
| n-8      | 16.7                 | 6.2       | 10.1        | 2147.06      | GlcMan9           |                   |
| n-9      | 2.35                 | 7.2       | 6.0         | 1984.86      | Man5              |                   |

\[a\] Relative quantity was calculated based on CLC-ODS elution profiles.

\[b\] Mass/charge (m/z) of neutral oligosaccharides (non-permethylated) were detected as [M + Na]+.

\[c\] PA-derivatized reference N-glycans whose elution positions on both CLC-ODS and Amide-80 columns and [M + Na]+ coincide with the pigeon IgG PA-oligosaccharides were indicated on the table. Structures of the reference compounds were shown in Fig. 9.

\[d\] Monosaccharides were denoted as follows: M, mannose; Glc, glucose; GN, N-acetylglucosamine.

3 N. Suzuki and Y. C. Lee, unpublished data.
N-glycans in IgG were very different between pigeon and chicken. This might be explained that the sites for complex-type N-glycans on these avian IgG are more exposed and accessible by "processing" enzymes, which may form more branched, longer N-glycans. The presence of triantennary N-glycans is unique in pigeon IgG, as it has not been seen in chicken and quail IgGs (41–43).

Third, we found a novel N-glycan structure containing Galα1–4Galβ1–4Galβ1–4GlcNAc branches on pigeon IgG. The presence of the structures could not be shown by lectin and immunostainings, because of the coexistence of Galα1–4Galβ1–4GlcNAc, but MALDI CID MS/MS analysis identified these two branches. The result was surprising, because N-glycans of major PEW glycoproteins (2) as well as known N-glycan structures of chicken (41) and quail egg yolk IgG (43) do not contain Galα1–4Gal sequence at all. The absence of Galα1–4Gal on PEW glycoproteins may be because the putative β1,4-galactosyltransferase (Galβ1,4-GalT) responsible for the synthesis of Galβ1–4Gal is inactive in pigeon oviduct but is active in IgG-producing cells. Whether the same α1,4-GalT in pigeon mediates α1,4-galactosylation onto both Galβ1–4GlcNAc and Galβ1–4Galβ1–4GlcNAc remains to be established. The only other occurrence of the Galα1–4Galβ1–4Galβ1–4GlcNAc sequence is in O-linked glycans of salivary gland mucin glycoproteins of Chinese swiftlet (genus Collocalia) (25). Although Galα1–4Galβ1–4Galβ1–4GlcNAc sequence has been found in only two avian species so far, Galβ1–4Galβ1–4GlcNAc sequence is known to occur at either internal or non-reducing terminal position of N-glycans in several fish eggs (57–60). The Galβ1–4Galβ1–4GlcNAc sequence is also found on a glycolipid isolated from ostrich liver (61). Galβ1–4Galβ1–4GlcNAc sequence has not been found in mammals on either glycoproteins or glycolipids, and high titer of natural antibody against terminal Galβ1–4Gal was detected in human sera (61). Thus, the expression of Galβ1–4Gal also appears to have emerged in a species-specific fashion.

The ability to produce Galα1–4Galβ1–4Galβ1–4GlcNAc and Galα1–4Galβ1–4GlcNAc sequence on N-glycans of pigeon IgG also affords greater microheterogeneity. In addition to the variations of α1,6-Fuc, α1,2,Fuc, α1,4-Galactosylation LacNAc branches seems to compete with α1,2,6-sialylation in pigeon IgG, because neutral forms are dominant in α1,4-galactosylated N-glycans (neutral/monosialyl = 2:1). On the other hand, about 20% of disialylated biantennary N-glycans were also detected, and they do not possess core α1,6-Fuc, bisecting GlcNAc, or α1,4-
One may speculate that some N-glycans on variable region are preferred acceptors for α-2,6-sialyltransferase but not for α-1,4-GalT.

Currently, no clear function has been assigned to Galα1–4Gal. The α-galactosyl residues are expressed in some species of birds (e.g. pigeon and swiftlet) but absent in chicken even though they belong to the same Class Aves, suggesting that such an epitope is apparently not uniformly required by all animals. Nevertheless, glycoproteins with Galα1–4Gal are widely distributed or circulating in the body of the pigeon. The enigmatic presence of Galα1–4Gal, Galβ1–4Gal, and Galα1–4Galβ1–4Gal may have some important function such as protection against specific carbohydrate-binding proteins expressed on infectious microbes. Alternatively, because Galα1–4Gal, Galβ1–4Gal, or Galα1–4Galβ1–4Gal on non-reducing termini are strongly antigenic in animals that do not express the antigens by themselves, they may work for species-specific barrier against certain viral infections, as speculated for Galα1–3Gal (47, 62, 63). How these Galα1–4Gal structures were acquired or lost during the course of diversification of birds can be investigated through structural analysis of the genes encoding Gal:1,4-GalT and α-1,3-GalT.

Finally, it should be mentioned that biomolecules produced by birds inhabiting close to human habitat influence our lives. For example, pigeon fanciers’ lung (PFL), one of extrinsic allergic alveolitis, is caused by the repeated inhalation of pigeon droppings and feather bloom (64). The pathogenesis of PFL is

| Fraction (disialyl) | Relative Quantity (%) | GU on ODS | GU on Amide | Reference N-Glycans | Key Fragment Ion (m/z) |
|--------------------|----------------------|-----------|-------------|---------------------|-----------------------|
| ds-6               | 2.93                 | 10.8      | 6.9         | B-1                 | 392, 398, 643, 847, 866, 888, 1111 |
|                    |                      |           |             | +2HexNAc +2NeuAc    |                       |
|                    |                      |           |             | D                   | (see Fig. S3)         |
| ds-8               | 14.0                 | 11.1      | 7.2         |                     |                       |

* Relative quantity was calculated based on CLC-ODS elution profiles, as noted in Table II.

* PA-derivatized reference N-glycans whose elution positions on both CLC-ODS and amide-80 columns and [M + Na]+ coincide with the pigeon IgG PA-oligosaccharides were indicated on the table. Structures of the reference compounds are shown in Fig. 9.

* Key fragment ions detected in MALDI MS/MS analysis of the selected parents, the production mechanism of which is shown in Fig. S5.

* Monosaccharides were denoted as follows: M, mannose; GN, N-acetylglucosamine; G, galactose; HN, N-acetylhexosamine; NA, N-acetyleneuraminic acid.

Gal. One may speculate that some N-glycans on variable region are preferred acceptors for α-2,6-sialyltransferase but not for α-1,4-GalT.
related to hypersensitivity reactions to pigeon antigens, one of which had been identified as pigeon intestinal mucus oligosaccharides (65). Although N-glycans on pigeon IgG has not been determined as key antigens for FPL, they deserve future scrutiny. Wild pigeons can be carriers of pathogenic bacteria. Indeed Shiga toxin-producing Escherichia coli strains had been detected in fecal samples from some pigeons (66). Because Gal–4Gal is known to be a minimum structure recognized by several bacterial adhesins and enterotoxins including Shiga toxin (1), it may be worthwhile to assess the relation of Galα1–4Gal production in wild birds and their potentials as carriers of pathogenic bacteria.

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