Brief Definitive Report

AGE-RELATED GALACTOSYLATION OF THE *N*-LINKED OLIGOSACCHARIDES OF HUMAN SERUM IgG

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Immunological competence is known to vary with age, and such age-related variations are often parabolic in nature. For example, both the level and avidity of the antibody response increase during postnatal development, reach a maximum in adulthood, and then decline during senescence (1). Cellular changes in the T lymphocyte compartment (both helper and suppressor T cell levels) appear to account for most variations in immunological competence (1, 2). Relatively few studies have attempted to correlate these functional changes with variations in the levels and nature of molecules expressed by immunocompetent lymphocytes. A study has therefore been undertaken to evaluate the glycosylation status of IgG during the natural history of human life.

Human serum IgG has been reported to carry at least 30 different *N*-linked oligosaccharides, all of which are of the biantennary complex type, and most of which (>95%) carry *N*-acetylglucosamine in both outer arms (3, 4). Structural differences between these arise primarily from core-substitutions (i.e., fucose and the 'bisecting' GlcNAc), outer-arm galactosylation, and sialylation (Fig. 1 *a*). We have previously reported that the percentage incidence of agalactosyl structures on the serum IgG of a group of patients with active rheumatoid arthritis (mean age 62 yr) was ~51%, placing this group well above the average for a group of normal individuals (3, 5).

We now report that the galactosylation of IgG *N*-linked oligosaccharide changes as a parabolic function of age. This seemingly parallels changes in immunological competence and draws attention to the need to monitor age-dependent variations in the expression of oligosaccharides in addition to those related to differences in cell type (6).

Materials and Methods

Serum was obtained from 151 individuals of both sexes and varying in age from 1–70 yr. None of the individuals >15 yr old were known to have any pathological abnormalities, nor to have had any history of autoimmune disease, although some of the older individuals may have had subclinical levels of age-related diseases (such as osteoarthritis), but none has as yet presented with major symptoms. All children under the age of 15 yr who were studied were undergoing ear, nose, and throat surgery, hernia repairs, or treat-

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ment for β-thalassaemia or epilepsy. Parental permission was obtained before studying any child <18 yr of age.

Total serum IgG was isolated as described previously (3), and the oligosaccharides were released, isolated, and radioactively labeled, also as described previously (7).

To determine the relative percentage incidence of agalactosyl, monogalactosyl, and digalactosyl oligosaccharides, G_0, G_1, and G_2, respectively, an aliquot ($\sim 5 \times 10^5$ cpm) of the unfractionated oligosaccharides isolated from each IgG sample, was incubated for 18 hr at 37°C under a toluene atmosphere with 10 μl of an exoglycosidase mixture containing 50 mU neuraminidase (ex. *Arthrobacter ureafaciens;* Boehringer-Mannheim Biochemicals, Indianapolis, IN), 40 mU α-fucosidase (ex. bovine epididymis; Sigma Chemical Co., St. Louis, MO), 0.85 U β-*N*-acetylhexosaminidase (ex. jack bean; Sigma Chemical Co.), in the presence of 0.1 mg galactonic acid-γ-lactone, in 0.2 M citrate-phosphate (pH 6.0). Reaction was terminated by heating to 100°C for 2 min, and an aliquot (~1%) of the digestion products was subjected to high-voltage paper electrophoresis (3). All radioactivity remained at the origin, indicating complete cleavage of the sialic acid. The remaining digestion products (~99%) were separated by Bio-Gel P4 (−400 mesh) gel-permeation chromatography using a 1.5 × 50 cm column. The regions G_0, G_1, and G_2 indicated in Fig. 1 *b,* were pooled and the relative radioactivity within each was determined.

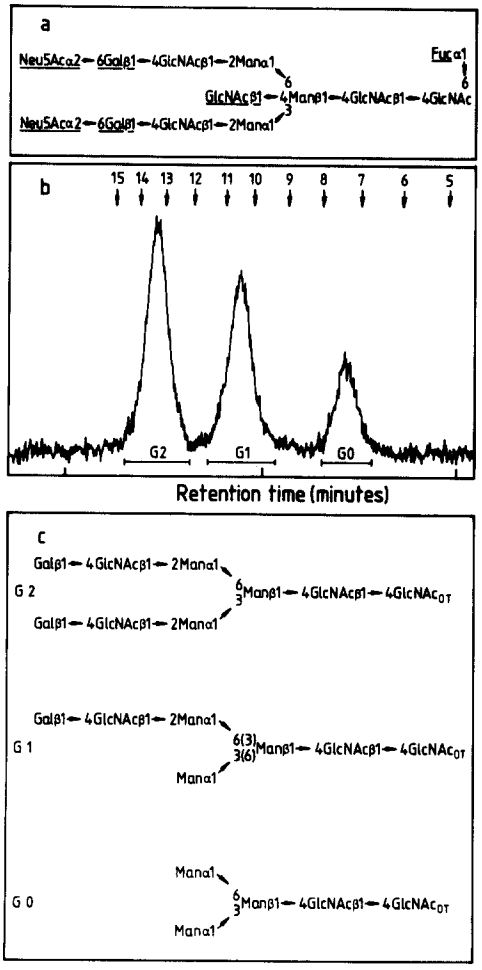

FIGURE 1. (*a*) Composite structure of the *N*-linked oligosaccharides derived from human serum IgG. Structural differences arise from the presence or absence of the residues underlined (3). (*b*) Bio-Gel P4 (−400 mesh) gel-permeation chromatogram of the digestion products G_2, G_1, and G_0 released from the total serum IgG of an individual of age 31 yr (Materials and Methods). The figure shows radioactivity (vertical axis) plotted against retention time. Numerical superscripts refer to the elution volume of glucose oligomers in glucose units, as detected simultaneously by the refractive index monitor (data not shown). (*c*) Primary structures of the digalactosyl G_2, monogalactosyl G_1, and agalactosyl G_0 digestion products obtained from the composite oligosaccharide structure depicted in Fig. 1 *a.* (Materials and Methods). The structure of the products was determined by a mixture of sequential exoglycosidase digestion and methylation analysis, as reported elsewhere (3, 4). Product G_2 is generated from all structures that originally carried two outer-arm galactose residues, G_1 from all structures that originally carried one outer-arm galactose residue, and G_0 from all structures that were originally agalactosyl.

Results

The extent of the outer-arm galactosylation of the *N*-linked oligosaccharides released from each serum IgG sample was determined enzymatically as shown in Fig. 1 *b*. This determination involves the simultaneous use of a mixture of specific exoglycosidases, and leads to the conversion of each IgG-associated oligosaccharide into one of three digestion products in a manner dependent only on its outer-arm galactosylation. Specifically, all digalactosyl structures (i.e., neither outer-arm terminating in *N*-acetylglucosamine or mannose) reduce to or remain as product G_2, all monogalactosyl ones (i.e., one outer arm terminating in *N*-acetylglucosamine or mannose) to product G_1, and all agalactosyl ones (i.e. both outer arms terminating in *N*-acetylglucosamine or mannose) to product G_0 (see Fig. 1 *c*). These three products are all completely resolved by Bio-Gel P4 (−400 mesh) gel-permeation chromatography, and the relative radioactivity within each of the three peaks gives a direct measure of the relative incidence of digalactosyl, monogalactosyl, and agalactosyl oligosaccharides in the original pool of oligosaccharides released from each sample of IgG. The results obtained using this enzymatic method are extremely reproducible (<1% variation for a given IgG sample).

The results for the incidence of agalactosyl and monogalactosyl structures are presented graphically in Fig. 2. It is clear that the relative incidence of agalactosyl structures on total human serum IgG varies continuously with age, but the relative incidence of monogalactosyl moieties is remarkably constant. The incidence of digalactosyl structures varied parabolically and inversely to that of agalactosyl structures. Differences between the sexes in galactosylation of IgG oligosaccharides were not significant (data not shown). The IgG-associated structures from 34 selected individuals varying in age from 18 to 63 yr were analyzed with respect to sialylation, outer-arm *N*-acetylglucosaminylation, and core substitutions (3). None of these parameters varied with age, suggesting that

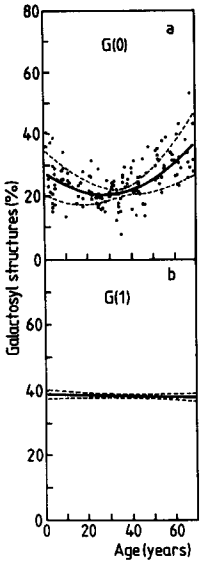

FIGURE 2. (*a*) Relation of the percentage incidence of agalactosyl monosaccharide sequences G_0 to age for IgG from normal subjects. The solid curve depicts the regression function for normal subjects generated by the least squares method and is given by the equation $y = 27.8 - 0.49x + 0.91 \times 10^{-2}x^2$, $n = 151$. The dashed curves represent the 95% confidence bounds of the fit. (*b*) Relation of the percentage incidence of monogalactosyl monosaccharide sequences G_1 to age for IgG from normal subjects. The solid line depicts the regression line of the data, (individual values are not shown). The dashed lines represent the 95% confidence bounds for the least squares fit.

only outer-arm galactosylation is age related. It should be emphasized that in no case was there any evidence for a novel IgG-associated *N*-linked oligosaccharide, but rather a continuous variation in the relative incidence of a constant set of structures. The incidence of structures with both outer arms terminating in *N*-acetylglucosamine first decreases with age (from >30% to ~20% at 25 yr) and then increases continuously to reach a level of ~40% by 70 yr of age. This would correspond to a nearly fourfold increase in those serum IgG molecules whose Fc may be presumed to be totally devoid of galatose between 25 and 70 yr of age. Further, there is limited but significant variation in the incidence of agalactosyl oligosaccharides within an age group.

Discussion

Both polypeptide structure and cell type have been shown to influence the *N*-glycosylation of secreted and cell surface glycoproteins (6). The results presented here indicate that within a cellular population (i.e., B lymphocytes), galactosylation of the outer arms of the *N*-linked oligosaccharides of a secreted glycoprotein (i.e. IgG) varies continuously with age. This variation differs from previously reported age-dependent molecular parameters (8, 9), such as glucose mediated crosslinking of proteins (9), in so far as it appears to be a continuous and natural process applied to a serum protein, of relatively short half-life, in a manner independent of the level of that protein.

The molecular basis for this variation is not yet known, but it could be due to a naturally age-related expression of β-galactosyltransferase (or β-galactosidase) activity within all B lymphocytes (e.g., epigenetic error at the DNA level [10] or extrinsic factors [8]). Alternatively, certain clones of B lymphocytes, differing with respect to their *N*-glycosylation capacity (glycotype), may dominate at different developmental stages of the immune system. The latter is consistent with the increased frequency of idiopathic paraproteinemias during aging (11). The variations observed within an age group could arise from different genetic endowments, or they could be a response to extrinsic factors. Further studies are needed to define the molecular basis of these variations.

The high incidence of agalactosyl IgG in the serum of young children may indicate a particular *N*-glycosylation capacity of B lymphocytes in children. It is especially interesting that in children with juvenile rheumatoid arthritis, the high incidence of agalactosyl structures found during the early years after birth is maintained and does not return to normal until periods of sustained remission (5). It has recently been reported that human B lymphocytes that secrete rheumatoid factor (largely IgM, but also IgG) are of the Leu-1$^+$ (OKT 15; CD5) phenotype, and that they are particularly prevalent both in patients with rheumatoid arthritis and in cord blood (12, 13). The results presented here and previously raise the possibility that Leu 1+ B-lymphocytes may also be characterised by a particular N-glycosylation capacity. Paradoxically the increased frequency of autoantibodies and rheumatoid factor are characteristic of surviving older humans (14).

Finally, there is increasing evidence that the precise *N*-linked oligosaccharides carried by immunoglobulins can significantly affect its functional properties (4, 15). If the results presented here are generally valid, and *N*-glycosylation of

other glycoproteins does vary with the age of the individual, then biological functions influenced by carbohydrates must presumably also be affected. For example, the activity of the insulin receptor is influenced by the oligosaccharides it carries (16). The migration of lymphocytes is also dependent on cell surface *N*-linked oligosaccharides, suggesting that changes in *N*-glycosylation may be responsible for the unique property of the inflamed synovium in patients with rheumatoid arthritis due to direct extravasation of T lymphocytes (17). Age related variations in oligosaccharide-mediated functions may, in some cases, be the basis for the age-association of certain diseases. This raises the possibility that one lesion in rheumatoid arthritis may be an accelerated aging process within the B lymphocyte compartment of affected individuals. Studies are therefore underway to follow variations with age in the *N*-glycosylation of other glycoproteins, and in particular the biological consequences of the varying exposure of nonreducing terminal *N*-acetylglucosamine residues with aging (18).

Summary

In a study of 151 normal, healthy individuals of both sexes varying in age from 1–70 yr, it was found that the relative incidence of agalactosyl (with both outer arms terminating in *N*-acetylglucosamine) *N*-linked oligosaccharides on total serum IgG decreased from birth to a minimum (at 25 yr of age) and then increased with age. The relative incidence of digalactosyl structures varied inversely to this, and the relative incidence of monogalactosyl structures was constant. Galactosylation of the *N*-linked oligosaccharides of the human serum IgG of normal individuals is therefore an age-related molecular parameter. Several reports have suggested that rheumatoid arthritis is associated with a decreased galactosylation of serum IgG (3–5). The normal variation in galactosylation with age as described here allows a true assessment of disease-associated changes in this parameter, and raises the possibility that one of the lesions in rheumatoid arthritis is an accelerated aging of the immune system. In addition, heterogeneity within age groups may be due to intrinsic differences in genetic endowment, or may reflect the impact of extrinsic factors (8).

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