Distribution of Galα1→3Galβ1→4GlcNAc Residues on Secreted Mammalian Glycoproteins (Thyroglobulin, Fibrinogen, and Immunoglobulin G) As Measured by a Sensitive Solid-Phase Radioimmunoassay†

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ABSTRACT: The study of the expression of Galα1→3Galβ1→4GlcNAc residues on mammalian glycoconjugates is of particular interest since as many as 1% of circulating IgG antibodies in man (the natural anti-Gal antibody) interact specifically with this carbohydrate residue. In recent studies, we have found that Galα1→3Galβ1→4GlcNAc residues are abundant on red cells and nucleated cells of nonprimate mammals, prosimians, and New World monkeys, but their expression is diminished in Old World monkeys, apes, and humans. In the present work, we have analyzed the expression of these residues on secreted mammalian glycoproteins. For this purpose, we have developed a radioimmunoassay (RIA) which enables the quantification of Galα1→3Galβ1→4GlcNAc residues on the secreted glycoproteins. Purified biotinylated anti-Gal was used as the antibody in the RIA, and bovine thyroglobulin enriched for Galα1→3Galβ1→4GlcNAc residues served as a solid-phase antigen. In this study, it is reported for the first time that the evolutionary pattern of Galα1→3Galβ1→4GlcNAc residue distribution in in vivo secreted glycoproteins is similar to that observed in membranes of cell lines and of red cells. Thyroglobulin, fibrinogen, or IgG molecules from nonprimate mammals and from New World monkeys express varying amounts of Galα1→3Galβ1→4GlcNAc residues ranging between 0.01 and 11 residues per molecule, whereas no such residues are present on any of these glycoproteins of human or Old World monkey origin. It is argued that abnormal expression of Galα1→3Galβ1→4GlcNAc residues on human glycoproteins may result in anti-Gal-mediated autoimmune processes.

The asparagine-linked (N-linked) carbohydrate chains of mammalian glycoproteins commonly have the terminal residue of SAα2→3Galβ1→4GlcNAc[1] or SAα2→6Galβ1→4GlcNAc (Kornfeld & Kornfeld, 1985). These residues have been found on bi- and multiantennary chains of secreted N-glycosylated mammalian glycoproteins such as thyroglobulin (Yamamoto et al., 1981; Cummings & Kornfeld, 1982; Dorland et al., 1984), laminin (Arumughan et al., 1986), immunoglobulin (Kornfeld et al., 1971), fibrinogen (Townsend et al., 1982), fetuin (Spiro, 1964; Baenziger & Fiete, 1979; Takasaki & Kobata, 1986), erythropoietin (Takeuchi et al., 1988), and β- and γ-interferon (Kagawa et al., 1988). Another structure, which in recent years has been found to constitute varying proportions of terminal carbohydrate residues on N-glycosylated glycoproteins, is the Galα1→3Galβ1→4GlcNAc oligosaccharide. This carbohydrate structure has been reported to be present on cell-membrane proteins of bovine thyroid cells (Edge & Spiro, 1985), mouse Ehrlich ascites cells (Eckhardt & Goldstein, 1983), mouse lymphoma cells (Cummings & Kornfeld, 1984), and mouse NIH 3T3 fibroblasts (Santer et al., 1989). In addition, it has been found on bovine thyroglobulin (Cummings & Kornfeld, 1982; Dorland et al., 1984; Spiro & Bhoyroo, 1984). Unlike sialylated lactosamine residues (SA-Galβ1→4GlcNAc), α-galactosylated lactosamine residues (i.e., Galα1→3Galβ1→4GlcNAc residues) have not been found on human cells and tissues (Galili et al., 1988; Peters & Goldstein, 1979) or on human thyroglobulin (Spiro & Bhoyroo, 1984). In contrast, man produces large amounts of a natural IgG antibody that interacts specifically with the Galα1→3Galβ1→4GlcNAc residue (Galili et al., 1985, 1987a). This natural antibody, designated "anti-Gal", constitutes approximately 1% of circulating IgG in man (Galili et al., 1984; Davin et al., 1987; Avila et al., 1989) and can be readily isolated from normal sera by affinity chromatography on synthetic Galα1→3Galβ1→4GlcNAc-R silica (Synsorb) adsorbent (Galili et al., 1988).

By using anti-Gal to study the expression of Galα1→3Galβ1→4GlcNAc residues on nucleated cells and red cells of various species, we have recently found a striking evolutionary pattern in the distribution of these carbohydrate residues. They are abundantly expressed on cells of many mammalian species, prosimians, and New World monkeys; however, such structures were not detected on cells of Old World monkeys, apes, and humans (Galili et al., 1987b,c, 1988). Our studies also suggested that this distribution of Galα1→3Galβ1→4GlcNAc residues is the result of differential activity of the enzyme α1→3 galactosyltransferase, which catalyzes the reaction:

$$\text{Galβ1→4GlcNAc-R + UDP-Gal} \rightarrow \text{Galα1→3Galβ1→4GlcNAc-R + UDP}$$

R represents the core mannosylated carbohydrate chain or

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[1] Abbreviations: anti-Gal, natural anti α-galactosyl IgG antibody; BSA, bovine serum albumin; BS lectin, *Bandeiraea simplicifolia* I lectin; Gal, galactose; GlcNAc, *N*-acetylglucosamine; IgG, immunoglobulin G; mAb, monoclonal antibody; PBS, phosphate-buffered saline; RIA, radioimmunoassay; SA, sialic acid; SDS-PAGE, polyacrylamide gel electrophoresis with sodium dodecyl sulfate as detergent; Synsorb 115, Synsorb beads with Galα1→3Galβ1→4GlcNAc conjugates.

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polylactosamine chain of N-glycosylated glycoproteins, lactosylceramide, or polylactosaminylceramide. The α1→3 galactosyltransferase is active in microsomal fractions of cells obtained from nonprimate mammals and New World monkeys (Galili et al., 1988). However, the activity of this enzyme is diminished in cells of Old World monkeys, apes, and humans. The study on α1→3 galactosyltransferase activity suggested that this enzyme has been evolutionarily suppressed in Old World primates subsequent to the divergence from New World monkeys. This conclusion was based, however, on studies with cell lines grown in vitro (Galili et al., 1988). It was important, therefore, to determine whether glycoproteins produced in vivo display a similar pattern of Galα1→3Galβ1→4GlcNAc residue distribution. To address this issue, we have studied the expression of Galα1→3Galβ1→4GlcNAc residues on secreted glycoproteins such as thyroglobulin, fibrinogen, and IgG, which were obtained from various species. In addition to the demonstration of these residues by Western blotting analysis and solid-phase binding assay, we have developed a radioimmunoassay (RIA) which enabled the quantification of the Galα1→3Galβ1→4GlcNAc residues on these glycoproteins.

Experimental Procedures

Glycoproteins. Human and mammalian thyroglobulins were purified from thyroid glands by gel filtration (Spiro & Bhoyroo, 1984). Frozen thyroid glands from various mammalian species were obtained from Pel-Freez Biologicals, Rogers, AK, and primate thyroid glands were obtained from the University of California Davis Primate Center. Human thyroid glands from autopsies were received from Dr. R. Winand, University of Liège, Belgium. Fibrinogens from various species and bovine fetuin were purchased from Sigma Chemical Co. Desialylation of fetuin was performed by incubation with 0.05 N H_2SO_4 for 2 h at 80 °C (Spiro, 1964). IgG preparations from different species were obtained by protein A–Sepharose chromatography of sera, elution of the bound IgG with glycine hydrochloride buffer, pH 2.6, and subsequent neutralization and dialysis of the eluates.

Isolation and Biotinylation of Anti-Gal. Isolation of anti-Gal by affinity chromatography has been described previously (Galili et al., 1988). Briefly, 100-mL batches of pooled, heat-inactivated AB plasma were loaded on a column containing 10 mL of the synthetic Galα1→3Galβ1→4GlcNAc residues linked to Synsorb beads (Synsorb 115, Chembiomed, Edmonton, Alberta, Canada). After extensive washing with PBS, antibodies were eluted with cold glycine hydrochloride buffer, pH 2.6, and immediately neutralized with 0.1 N NaOH. The eluate was dialyzed against PBS, pH 7.4, and diluted to a concentration of 100 μg/mL. *N*-Hydroxysuccinimide–biotin (NHS-d-biotin) (Sigma Chemical Co.) dissolved in dimethyl sulfoxide was added to the anti-Gal preparation at a final concentration of 0.2 mg/mL, and the mixture was incubated for 2 h at room temperature and subsequently overnight at 4 °C. After additional dialysis to remove free biotin, the biotinylated anti-Gal was rechromatographed on a Galα1→3Galβ1→4GlcNAc–R Synsorb column. The column was washed extensively with PBS, and the bound anti-Gal was then eluted by incubation with 0.2 M methyl α-galactoside for 4 h at 37 °C. The free carbohydrate was removed from the eluted anti-Gal by four cycles of dialysis against a large volume (4 L) of PBS, and the antibody was diluted to a concentration of 100 μg/mL and stored in aliquots at −70 °C. The extent of anti-Gal biotinylation could not be determined; however, it was substantially biotinylated. As seen in Figure 4, when the Galα1→3Galβ1→4GlcNAc residue is present on the solid-phase antigen, the binding results in a plateau of 40 000 cpm. The same concentration yielded only background counts with asialofetuin (Figure 1), indicating that this antibody is properly biotinylated and that is does not bind to the well-characterized carbohydrate residues on asialofetuin.

Reagents. Bandeiraea simplicifolia I–Sepharose (BS–Sepharose), biotinylated peanut agglutinin (PNA), FITC–avidin, and the ABC reagent for the peroxidase reaction were purchased from Vector Laboratories, Burlingame, CA. ^{125}I-streptavidin (specific activity 30 μCi/μg) was purchased from Amersham Corp., Chicago, IL.

Anti-Gal Binding to Glycoproteins in Microtiter Wells. Glycoproteins (50 μg/mL) in 0.5 M carbonate buffer, pH 9.5, were incubated overnight in Falcon 3912 microtiter plates (Becton Dickinson, Oxnard, CA) at 4 °C. Each well contained 50 μL of the glycoprotein solution. The wells were then washed with PBS containing 0.05% Tween 20 and subsequently incubated with 2% bovine serum albumin (BSA) for 2 h at 37 °C in order to block nonspecific binding. The plates were then incubated with 2-fold serial dilutions of biotinylated anti-Gal for 1 h at room temperature. At the end of the incubation, the plates were washed with Tween–PBS and incubated for 1 h with ^{125}I-streptavidin (10^5 cpm/50 μL). After the plates were washed again with Tween–PBS, 100 μL of 0.2 N HCl was added to the wells to detach the bound antibodies and the ^{125}I-streptavidin. The amount of ^{125}I-streptavidin was measured in a γ counter.

Immunoblotting (Western) Assay. Fifty-microgram aliquots of various glycoproteins were separated by polyacrylamide gel electrophoresis (SDS–PAGE), blotted onto nitrocellulose paper, and subsequently blocked with 1% BSA. After blocking, the blotted proteins were covered with biotinylated anti-Gal (5 μg/mL) for 1 h at room temperature and washed with Tween–PBS. The bound antibody was detected by a peroxidase reaction using the Vector ABC reagent.

Digestion with Glycosidases. Two hundred micrograms of each glycoprotein in 0.2 mL was incubated for 14 h with 0.4 unit of coffee bean α-galactosidase (Sigma) in sodium acetate buffer, pH 5.0, or with 30 units of bovine testis β-galactosidase in PBS. Similar treatment was performed with glycoproteins bound to microtiter wells.

Chromatography of Glycoproteins on BS–Sepharose. The BS lectin interacts with α-galactosyl residues and in particular with Galα1→3Galβ1→4GlcNAc residues (Wood et al., 1979). The affinity of single α-galactosyl residues for the BS lectin is low (Blake & Goldstein, 1980). Therefore, we postulated that chromatography of glycoproteins on BS–Sepharose followed by extensive washing would result in the subsequent isolation of glycoprotein molecules with increased numbers of Galα1→3Galβ1→4GlcNAc residues as compared with the average number per molecule in the original preparation. Glycoproteins in solution were passed through a 5-mL BS–Sepharose column. After being extensive by washed with PBS, the bound molecules were eluted with 10 mM methyl α-galactoside (Spiro & Bhoyroo, 1984; Shibata et al., 1982) and dialyzed against PBS to remove the free carbohydrate. The concentration of eluted glycoproteins was determined by absorbance at 280 nm.

Quantification of Galα1→3Galβ1→4GlcNAc Residues on Various Glycoproteins by Solid-Phase Radioimmunoassay. Bovine thyroglobulin molecules enriched for Galα1→3Galβ1→4GlcNAc residues were isolated on BS–Sepharose from a commercial preparation of bovine thyroglobulin (Sigma) as described above. This procedure results in the isolation of approximately 10% of the bovine thyroglobulin, which binds with high affinity to BS lectin and is designated BS^+ thyro-

Table I: Interaction of Anti-Gal with Chemically Defined Oligosaccharide Residues on Bovine Glycoproteins with N-Linked or O-Linked Carbohydrate Residues

glycoprotein	oligosaccharide structure	anti-Gal binding
(1) fetuin	SA-Galβ1→4GlcNAc-R	-
(2) fetuin	SA-Galβ1→3GalNAc-Ser(Thr)	-
(3) asialofetuin	Galβ1→4GlcNAc-R	-
(4) asialofetuin	Galβ1→3GalNAc-Ser(Thr)	-
(5) thyroglobulin	Galα1→3Galβ1→4GlcNAc-R	+
(6) thyroglobulin (α-galactosidase)	Galβ1→4GlcNAc-R	-

globulin. This isolated thyroglobulin fraction, which provided the solid-phase Galα1→3Galβ1→4GlcNAc-R antigen, was diluted to a concentration of 50 μg/mL in carbonate buffer, pH 9.5, and used to coat microtiter wells. In preliminary experiments measuring the binding of biotinylated anti-Gal to BS$^+$ bovine thyroglobulin, an antibody concentration of 1 μg/mL was found to be a suitable concentration for performing a highly sensitive solid-phase radioimmunoassay (RIA). For quantification of the number of Galα1→3Galβ1→4GlcNAc residues per glycoprotein molecule, each type of glycoprotein was incubated at different concentrations with biotinylated anti-Gal (1 μg/mL) for 20 h at 4 °C. Subsequently, 50 μL of the anti-Gal–glycoprotein mixture was placed in the microtiter wells, which had been precoated with BS$^+$ bovine thyroglobulin. Under these conditions, glycoproteins lacking the terminal Galα1→3Galβ1→4GlcNAc residues did not inhibit anti-Gal binding to BS$^+$ thyroglobulin coating the wells. However, glycoproteins with the Galα1→3Galβ1→4GlcNAc residues neutralized varying proportions of the biotinylated anti-Gal molecules, thus decreasing anti-Gal binding to BS$^+$ thyroglobulin. The degree of inhibition of anti-Gal binding to BS$^+$ thyroglobulin was assessed by the decrease in the subsequent binding of ^{125}I-streptavidin. To generate a standard curve for comparison, we used the data of Spiro and Bhoyroo (1984), who found an average of 11 Galα1→3Galβ1→4GlcNAc residues per bovine thyroglobulin molecule. Thus the concentration of this glycoprotein inhibiting 50% of anti-Gal binding (25 nM) was used as a standard value for assessing the number of such residues on a variety of glycoproteins (see Figure 3 and Table II).

Results

Specific Interaction of Anti-Gal with Galα1→3Galβ1→4GlcNAc Residues on N-Glycosylated Glycoproteins. Anti-Gal was previously shown to interact specifically with Galα1→3Galβ1→4GlcNAc residues on glycosphingolipids or with such synthetic oligosaccharides. It did not interact with Galα1→4Galβ1→4GlcNAc residues (P_1 antigen), β-galactosyl residues, or other carbohydrate residues on either glycosphingolipids or synthetic oligosaccharides (Galili et al., 1985, 1987a). To determine the capacity of anti-Gal to serve as a reagent for detection of Galα1→3Galβ1→4GlcNAc residues on N-glycosylated glycoproteins, we examined its interaction with secreted glycoproteins that have chemically defined carbohydrate residues (Table I). Bovine fetuin has three N-linked and three O-linked carbohydrate chains. Both types of oligosaccharides (structures 1 and 2 in Table I) have terminal sialic acid residues (Spiro, 1964; Baenziger & Fiete, 1979; Takesaki & Kobata, 1986). The results in Figure 1 demonstrate that anti-Gal did not interact with any of these carbohydrate residues on fetuin when the binding assay was carried out in microtiter wells. Removal of the terminal sialic acid from fetuin results in the exposure of penultimate Galβ1→4GlcNAc and Galβ1→3GalNAc residues, respectively (Table I, structures 3 and 4). Anti-Gal also did not interact with these residues, as indicated by the complete absence of binding to asialofetuin (Figure 1). As a positive control for binding of asialofetuin in the microtiter wells, we used the lectin peanut agglutinin, which interacts specifically with Galβ1→3GalNAc residues (Pereira et al., 1976). Such residues are exposed on the O-linked carbohydrate chains of fetuin subsequent to the removal of the terminal sialic acid residue (Table I, structure 4) and readily interact with the peanut agglutinin (Figure 1).

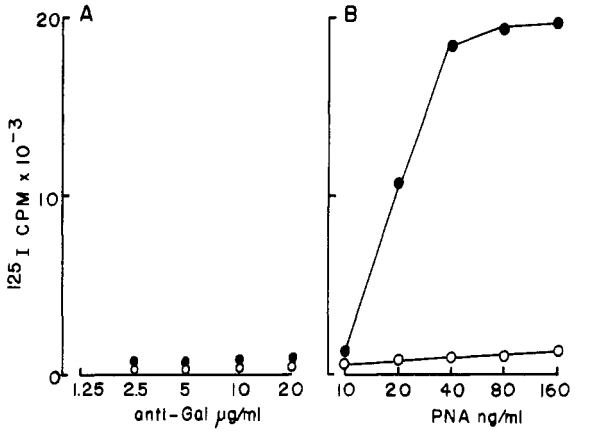

FIGURE 1: Binding of anti-Gal (A) and peanut agglutinin (PNA) (B) to bovine fetuin (○) and bovine asialofetuin (●) in microtiter wells.

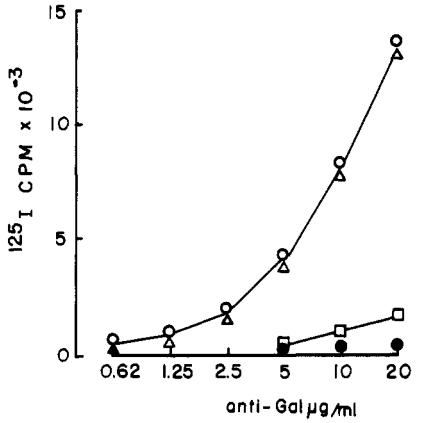

FIGURE 2: Binding of anti-Gal to thyroglobulin in microtiter wells: (○) bovine thyroglobulin; (□) bovine thyroglobulin treated with α-galactosidase; (△) bovine thyroglobulin treated with β-galactosidase; (●) human thyroglobulin.

An N-glycosylated glycoprotein in which part of the terminal carbohydrate residues were biochemically characterized as Galα1→3Galβ1→4GlcNAc is bovine thyroglobulin (Cummings & Kornfeld, 1982; Dorland et al., 1984; Spiro & Bhoyroo, 1984). Moreover, Spiro and Bhoyroo (1984) reported that bovine thyroglobulin has an average of 11 such residues per molecule, whereas no such structures are present on human thyroglobulin. In accordance, we have found that anti-Gal bound to bovine thyroglobulin but not to human thyroglobulin (Figure 2). Removal of the terminal α-galactosyl residues from bovine thyroglobulin by α-galactosidase (Table I, structure 6) eliminated its capacity to bind anti-Gal. As expected, treatment of bovine thyroglobulin with β-galactosidase had no effect on anti-Gal binding (Figure 2).

These data on the specific binding of anti-Gal to Galα1→3Galβ1→4GlcNAc residues on bovine thyroglobulin, as well as our former observations on anti-Gal specificity (Galili et al., 1985, 1987a), implied that this antibody is a suitable reagent for detecting Galα1→3Galβ1→4GlcNAc residues on N-glycosylated glycoproteins.

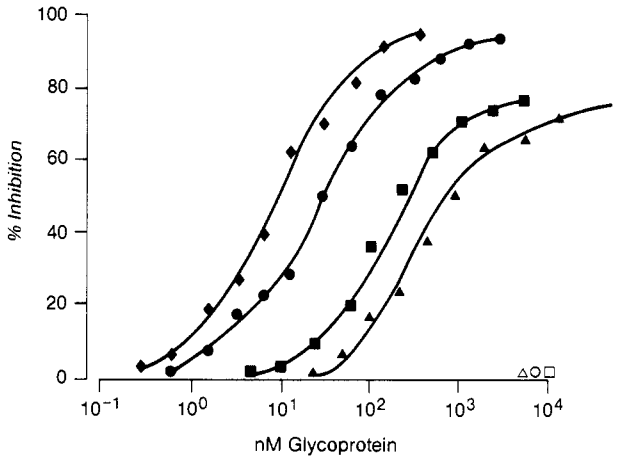

FIGURE 3: Solid-phase RIA based on inhibition of anti-Gal (1 μg/mL) binding to bovine BS$^+$ thyroglobulin by preincubation of the antibody with various glycoproteins: (●) bovine thyroglobulin; (◆) BS$^+$ bovine thyroglobulin; (■) goat fibrinogen; (▲) mouse monoclonal IgG; (△) bovine IgG; (○) human thyroglobulin; (□) human fibrinogen.

Quantification of Galα1→3Galβ1→4GlcNAc Residues on Thyroglobulin of Various Species. In order to determine the number of Galα1→3Galβ1→4GlcNAc residues on various secreted glycoproteins, we developed a modified solid-phase RIA in which bovine thyroglobulin enriched for Galα1→3Galβ1→4GlcNAc residues (BS$^+$ thyroglobulin) was used as a solid-phase antigen bound to microtiter wells. Inhibition of anti-Gal binding to the BS$^+$ bovine thyroglobulin subsequent to coincubation of the antibody with various glycoproteins in solution was dependent on the presence of Galα1→3Galβ1→4GlcNAc residues on the glycoproteins tested.

Bovine thyroglobulin at a concentration of 25 nM yielded 50% inhibition of anti-Gal binding (Figure 3, Table II). The bovine BS$^+$ thyroglobulin concentration causing 50% inhibition was only 9 nM (Figure 3, Table II). By using Spiro and Bhoyroo's (1984) data of an average of 11 Galα1→3Galβ1→4GlcNAc residues per bovine thyroglobulin molecule as a standard, the number of such residues on the BS$^+$ bovine thyroglobulin could be estimated to be 30 per molecule. The number of these residues on porcine thyroglobulin was estimated to be six per molecule (Table II). This is similar to the value reported by Spiro and Bhoyroo, who measured the release of terminal α-galactosyl residues by α-galactosidase. Furthermore, the inhibition curve with porcine thyroglobulin paralleled that of bovine thyroglobulin. Thus the thyroglobulin concentration for a given inhibition was approximately double with porcine as compared with bovine thyroglobulin (not shown). The Galα1→3Galβ1→4GlcNAc residue was also found to be expressed in varying amounts on thyroglobulin molecules of other mammals, including mouse, rat, hamster, rabbit, and dog (Table II). When primate thyroglobulins were studied, this residue was readily detected on squirrel monkey (a New World monkey) thyroglobulin in amounts comparable to those found in various nonprimate mammals. This residue was not found, however, on rhesus monkey (an Old World monkey) thyroglobulin or on human thyroglobulin (Figure 3, Table II), as indicated by the fact that thyroglobulin molecules from both species, even at the high concentration of 15 μM, had no inhibitory effect on the binding of anti-Gal to the solid-phase BS$^+$ bovine thyroglobulin.

Studies on Mammalian Fibrinogens. Townsend et al. (1982) have shown that human fibrinogen molecules have four N-linked carbohydrate chains with the terminal SA-Galβ1→4GlcNAc residue. The pattern of anti-Gal binding to fibrinogen molecules from various species suggested that

Table II: Quantification of Galα1→3Galβ1→4GlcNAc Residues on Various Glycoproteins As Measured in Solid-Phase RIA

glycoprotein[a]	species	50% inhibition (nM)	Galα1→3Galβ1→4-GlcNAc residues per molecule (approximation)
thyroglobulin	bovine	25	11[b]
	BS$^+$ bovine	9	30
	mouse	800	0.25
	rat	420	0.5
	guinea pig	450	0.5
	hamster	1500	0.14
	rabbit	130	2
	pig	45	6
	squirrel monkey[c]	300	1
	rhesus monkey[d]	*e*	*f*
	human	*e*	*f*
fibrinogen	bovine	88	3
	porcine	83	3
	sheep	150	2
	horse	4000	0.06
	cat	41	7
	goat	200	1
	rabbit	3500	0.08
	dog	860	0.3
	baboon[d]	*e*	*f*
	human	*e*	*f*
IgG	mouse mAb	505	0.5
	bovine	*g*	*f*
	porcine	*g*	*f*
	sheep	10000	0.03
	horse	*h*	*f*
	rabbit	16000	0.02
	dog	8000	0.03
	aotus[c]	33000	0.01
	squirrel monkey[c]	22000	0.013

[a] Glycoprotein preparations were pooled from two individuals or more, except for human thyroglobulin, which was isolated from two individual assayed separately. [b] This value was obtained from the study of Spiro and Bhoyroo (1984) and was used as a standard value for the RIA. [c] New World monkey. [d] Old World monkey. [e] No inhibition at 15 μM. [f] No measurable Galα1→3Galβ1→4GlcNAc. [g] No inhibition at 17 μM. [h] No inhibition at 20 μM.

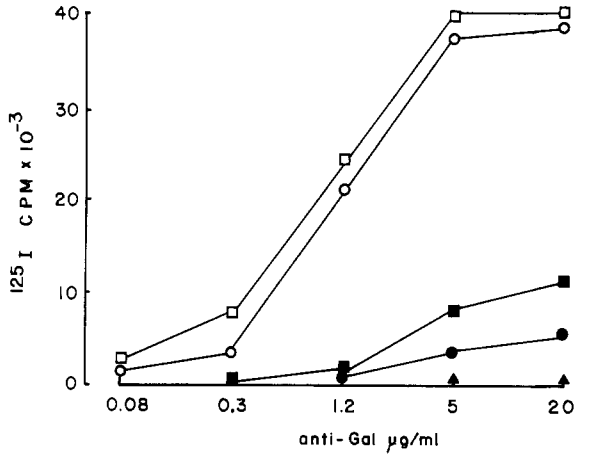

FIGURE 4: Binding of anti-Gal to bovine (■), porcine (●), and human (▲) fibrinogen in microtiter wells. Open symbols represent fibrinogens fractionated on BS-Sepharose.

the Galα1→3Galβ1→4GlcNAc residue is present on bovine and porcine fibrinogen (Figure 4). Affinity chromatography of bovine and porcine fibrinogen on BS-Sepharose resulted in the isolation of approximately 1% of the fibrinogen molecules with a high affinity for BS lectin. These fibrinogen molecules expressed more Galα1→3Galβ1→4GlcNAc residues than the average number, as seen in the increased interaction with anti-Gal (Figure 4). This interaction could also be demonstrated by SDS-PAGE followed by Western blotting

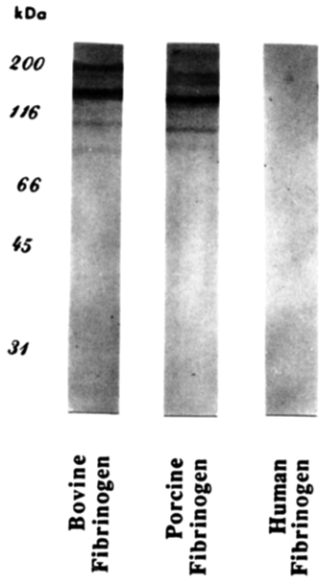

FIGURE 5: Western blot and anti-Gal immunostaining of fibrinogens obtained from different species. The bovine and porcine fibrinogens were isolated on BS–Sepharose prior to the SDS–PAGE procedure.

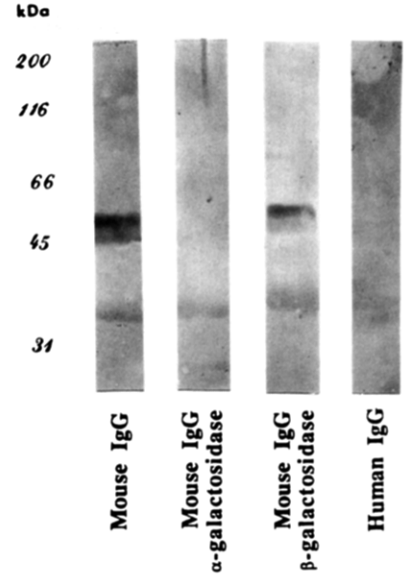

FIGURE 6: Western blot of mouse and human IgG and subsequent immunostaining with anti-Gal. The mouse IgG was also pretreated with α- or β-galactosidase.

and immunostaining with anti-Gal (Figure 5). No anti-Gal binding was observed with human fibrinogen (Figures 4 and 5). From the RIA, the average number of Galα1→3Galβ1→4GlcNAc residues per molecule of fibrinogen could be determined. The porcine and bovine fibrinogens were found to have approximately three Galα1→3Galβ1→4GlcNAc residues per molecule (Table II, Figure 3). Assays with fibrinogen from other mammalian species indicated variable expression of the carbohydrate structure in numbers ranging from seven residues per molecule of cat fibrinogen to an average of one residue per seventeen molecules of horse fibrinogen (Table II). In accordance with the direct binding assay (Figure 4), no interaction between anti-Gal and human or baboon fibrinogen was observed in the RIA even at a glycoprotein concentration of 15 μM (Table II, Figure 3).

Galα1→3Galβ1→4GlcNAc Residues on IgG Molecules. The IgG molecules of both mouse (Mizouchi et al., 1987) and man (Kornfeld et al., 1971) were reported to have two N-linked carbohydrate chains with terminal SA-Galβ1→4GlcNAc residues. In view of our recent studies (Galili et al., 1988) on the expression of Galα1→3Galβ→4GlcNAc residues on mouse myeloma cells and the activity of the enzyme α1→3 galactosyltransferase in these cells, we hypothesized that the secretory product, i.e., IgG molecules synthesized by mouse myeloma cells, may also have Galα1→3Galβ1→4GlcNAc residues. To study this possibility, we used the mouse monoclonal IgG antibody designated Gal-13 (Galili et al., 1987c) as a representative mouse monoclonal IgG. As seen in Figure 6, anti-Gal bound to the heavy chain of mouse IgG. This binding was not affected by treatment of the IgG molecules with β-galactosidase. However, preexposure to α-galactosidase completely eliminated the binding. As expected, anti-Gal did not bind to human IgG (Figure 6).

The mouse monoclonal IgG antibody assayed by the RIA was found to have an average of one Galα1→3Galβ1→4GlcNAc residue per two IgG molecules (Table II). No such residues were detected on bovine or porcine IgG. However, small numbers of Galα1→3Galβ1→4GlcNAc residues were detected on IgG molecules from sheep, rabbit, dog, and New World monkey. Similar studies could not be performed with IgG from Old World monkey, ape, or man, since these species have large amounts of autologous anti-Gal (Galili et al., 1987b), which may compete with the labeled anti-Gal.

Discussion

The use of antibodies, mostly of the monoclonal type, for identifying carbohydrate structures on cells and on isolated glycoconjugates, has increased in recent years (Feizi, 1985). Although this approach is qualitative rather than quantitative, it does provide a sensitive assay for the detection of a unique carbohydrate structure when such a structure is a minor component on cells or is within a heterogeneous population of oligosaccharides linked to a given protein.

The natural human antibody anti-Gal, used in the present study for the identification of Galα1→3Galβ1→4GlcNAc residues, although not a monoclonal antibody, is highly specific for this carbohydrate structure on mammalian glycoproteins. Anti-Gal was shown previously to interact with melibiose (Galα1→6Glc) (Galili et al., 1984, 1985) and may hypothetically bind to Galα1→2 residues; however, no Galα1→2 or Galα1→6 residues have been detected on mammalian glycoproteins.

The biochemical quantification of the number of Galα1→3Galβ1→4GlcNAc residues on bovine thyroglobulin (Spiro & Bhoyroo, 1984) enabled us to use this molecule as a convenient standard for establishing the RIA for the quantification of this residue. In addition to their finding of 11 residues of Galα1→3Galβ1→4GlcNAc residues on bovine thyroglobulin, Spiro and Bhoyroo (1984) reported that porcine thyroglobulin molecules have half as many terminal α-galactosyl residues as bovine thyroglobulin. In accordance, the RIA measurements indicated that porcine thyroglobulin has six Galα1→3Galβ1→4GlcNAc residues, further demonstrating the accuracy of the RIA quantification. The assay described in the present study may serve as a general method for assessing the number of a given carbohydrate residue on various glycoproteins by using the appropriate antibody and corresponding solid-phase antigen.

The pattern of anti-Gal binding to thyroglobulin from various mammals suggested a distinct species distribution of Galα1→3Galβ1→4GlcNAc residues. These residues were detected on thyroglobulin molecules from many nonprimate mammals. It is of interest to note that, whereas in fibroblasts from various nonprimate mammals the number of Galα1→3Galβ1→4GlcNAc cell-membrane residues was invariably

high (Galili et al., 1988), the number of these residues greatly varied in thyroglobulin molecules of different species. It was found to be abundant on bovine and porcine thyroglobulin but scarce on thyroglobulin of mouse or rat. Among primates, the Galα1→3Galβ1→4GlcNAc residue was demonstrated on New World monkey thyroglobulin but not on Old World monkey and human thyroglobulin.

This pattern of distribution of Galα1→3Galβ1→4GlcNAc residues in mammals is apparent not only with thyroglobulin but also in other secreted glycoproteins that thus far have been assumed to have only SA-Galβ1→4GlcNAc residues, i.e., fibrinogen and IgG molecules. The occurrence of the Galα1→3Galβ1→4GlcNAc residue on mouse monoclonal IgG antibodies has been recently confirmed by Krotkiewski et al. (1989), who demonstrated this residue on the mouse monoclonal antibody OKT3 by the use of ^{1}H NMR and fast atom bombardment mass spectrometry. These findings suggest that the general pattern of distribution of Galα1→3Galβ1→4GlcNAc residues observed on the red cell and nucleated cell membrane glycoconjugates of various mammalian species (Galili et al., 1987a,b, 1988) is also applicable to secreted N-glycosylated glycoproteins. It should be stressed that the data obtained in the radioimmunoassay are an approximation of the number of carbohydrate residues per molecule. It is conceivable that in suspension, if Galα1→3Galβ1→4GlcNAc residues are distant from each other on a glycoprotein molecule, the interaction of anti-Gal with this residue would be in a 1:1 ratio, whereas if clustered, one anti-Gal IgG molecule would probably bind two residues. Nevertheless, this assay provides information on whether a given glycoprotein carries many, few, or no such residues. This approach may be useful in assessing the expression of Galα1→3Galβ1→4GlcNAc residues on recombinant glycoproteins produced for therapeutic purposes. If such glycoproteins express the Galα1→3Galβ1→4GlcNAc residues, their turnover in the circulation may be accelerated as a result of in vivo interaction with anti-Gal (Kagawa et al., 1988).

The capacity of synthesizing the Galα1→3Galβ1→4GlcNAc residues on secreted glycoproteins seems to reflect a differential activity of the enzyme α1→3 galactosyltransferase in the Golgi apparatus. The activity of this enzyme was demonstrated in cells of mouse (Blake & Goldstein, 1981; Elices et al., 1986; Cummings & Mattox, 1988), rabbit (Basu & Basu, 1973; Betteridge & Watkins, 1983), and bovine (Blanken & Van den Eijnden, 1985) origin. In a recent study (Galili et al., 1988), we have shown that the activity of this enzyme can be demonstrated in microsomal fractions obtained from mouse, bovine, and marmoset (New World monkey) cells. Our present study indicates that, in addition to the synthesis of Galα1→Galβ1→4GlcNAc residues on membrane glycoconjugates (Galili et al., 1987b, 1988), this enzyme links terminal α-galactosyl residues to the *N*-acetyllactosaminyl (i.e., Galβ1→4GlcNAc) moiety of different types of secreted glycoproteins in nonprimate mammals and New World monkeys. It is not clear as yet whether the low level of α-galactosylation of thyroglobulin in species such as mouse, rat, and hamster is the result of low α1→3 galactosyltransferase activity in the Golgi apparatus of thyroid cells of these species or of high activity of sialyltransferases that compete successfully for the common Galβ1→4GlcNAc–R acceptor. Measurement of the two types of enzymes in microsomal fractions obtained from thyroid cells would enable the elucidation of this question.

The most striking pattern to emerge from the present study is the absence of Galα1→3Galβ1→4GlcNAc residues on Old World monkey and human secreted glycoproteins. Since the enzymatic repertoire for synthesizing Galβ1→4GlcNAc-R residues on N-glycosylated glycoproteins is present and active in humans (Kornfeld & Kornfeld, 1985), the lack of Galα1→3Galβ1→4GlcNAc residues on human N-glycosylated glycoproteins seems to be the result of diminished activity of the enzyme α1→3 galactosyltransferase. We have argued that the gene for this enzyme is present in the human genome but that it has been largely suppressed during the evolution of ancestral Old World primates (Galili et al., 1988). In recent studies, the cDNA of α1→3 galactosyltransferase has been cloned by Lowe et al. from a mouse cDNA library (Lowe et al., 1989; Larsen et al., 1989) and by Joziasse et al. (1989) from a bovine cDNA library. Southern blot analysis using these cDNA probes revealed homologous sequences in human DNA, suggesting that the α1→3 galactosyltransferase gene has been evolutionarily conserved in humans (Joziasse et al., 1989; Lowe, personal communication). This hypothesis has been recently supported by two additional studies: (1) Kagawa et al. (1988) have shown that a human lung carcinoma cell line can synthesize and secrete recombinant β-interferon containing carbohydrate chains with Galα1→3Galβ1→4GlcNAc residues, and (2) Castronovo et al. (1989) have reported that human mammary carcinoma cell lines and the malignant cells from primary lesions of patients with mammary carcinoma readily bind both anti-Gal and BS lectin. These two studies suggest that the α1→3 galactosyltransferase gene, which appears to be suppressed in man, may undergo deregulation, resulting in the increased synthesis of Galα1→3Galβ1→4GlcNAc residues on human glycoconjugates. Deregulation of human α1→3 galactosyltransferase in vivo may be of pathologic consequence, since it could result in the initiation of an autoimmune process mediated by anti-Gal binding to the de novo synthesized Galα1→3Galβ1→4GlcNAc-R glycoconjugates (Galili, 1989). The use of the sensitive RIA assay described in the present study may enable the assessment of the possible contribution of Galα1→3Galβ1→4GlcNAc residues on human glycoproteins to the pathology of autoimmune diseases.

Added in Proof

At the request of the authors, Dr. Robert G. Spiro of Harvard Medical School analyzed the occurrence of the Galα1→3Galβ1→4GlcNAc-R structure on various glycoproteins using the hydrazine/nitrous acid/NaB^3H_4 procedure (Spiro & Bhoyroo, 1988). Whereas this structure was detectable by this method on thyroglobulin molecules of various species, it was not identified on any of the fibrinogens analyzed in the present work. The discrepancy between the results of the RIA and the chemical method is currently under study.

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