

HUMAN NATURAL ANTI- α -GALACTOSYL IgG

II. The Specific Recognition of $\alpha(1 \rightarrow 3)$ -linked Galactose Residues

BY URI GALILI, BRUCE A. MACHER, JOANNE BUEHLER, AND
STEPHEN B. SHOHET

From the MacMillan-Cargill Hematology Research Laboratory; the Cancer Research Institute; the Department of Pharmaceutical Chemistry, and the Department of Laboratory Medicine, the University of California, San Francisco, California 94143

The antibodies that are produced in man as a result of specific vaccination or infectious disease comprise only a small proportion of the bulk of the IgG molecules circulating in the blood. The immunity towards these antigens is attributed mostly to a relatively high number of memory cells and a low titer of synthesized antibodies. In view of kinetic studies of antibody production and the low level of IgG in the serum of germ-free animals, it is safe to assume that a large proportion of circulating IgG molecules are synthesized as a result of immune reactivity to naturally occurring antigens, to which the immune system is constantly exposed. Most of the known high-titer natural antibodies seem to display anticarbohydrate specificity. These include the anti-blood group A and B antibodies, which interact with GalNAc $\alpha 1 \rightarrow 3$ (Fuc $\alpha 1 \rightarrow 2$)Gal and Gal $\alpha 1 \rightarrow 3$ [Fuc $\alpha 1 \rightarrow 2$]Gal (GalNAc, *N*-acetyl galactose; Fuc, fucose)¹, respectively (1, 2). Another natural antibody with anticarbohydrate specificity is the anti-T (Thomsen-Friedenreich) antibody, which binds to Gal $\beta 1 \rightarrow 4$ GalNAc residues (3, 4). It is assumed that these anticarbohydrate antibodies are constantly produced as an immune response to normal gastrointestinal or pulmonary flora that contain bacteria bearing such antigenic epitopes (1, 3).

We have recently (5-7) described a natural IgG antibody that appears to contribute to extravascular lysis of normal and some pathological red cells, since it interacts in situ with normal senescent red cells and more extensively with pathological red cells of thalassemia and sickle cell anemia patients. This antibody, designated anti-Gal, was found in high titer in all normal sera tested, independent of blood type. Furthermore, it represents 1% of the total serum IgG. The reactivity of anti-Gal could be assessed by its interaction with α -galactosyl residues on rabbit red blood cells (RRBC) (8). To evaluate the structure of the RRBC antigen recognized by anti-Gal, we have studied its binding to carbohydrate-

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¹ *Abbreviations used in this paper:* BS, *Bandeiraea simplicifolia*; BSA, bovine serum albumin; CPH, ceramide pentahexoside; CTH, ceramide trihexoside; EA, erythrocyte-antibody; Fuc, fucose; GalNAc, *N*-acetyl galactose; GlcNAc, *N*-acetyl glucose; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PC, phosphatidylcholine; RRBC, rabbit red blood cells.

containing molecules isolated from these cells. We have found that anti-Gal has a binding specificity for glycosphingolipids with an $\alpha 1 \rightarrow 3$ nonreducing terminal galactose, but does not bind to a RRBC glycosphingolipid with a nonreducing terminal $\alpha 1 \rightarrow 4$ galactose. In addition to obtaining a more complete characterization of the binding specificity of this unique natural antibody, these studies may shed light on the nature of the antigenic epitope exposed on human RBC upon normal and pathological senescence.

Materials and Methods

RRBC and Ghosts. Rabbits were bled from the marginal ear vein. The blood was collected in heparinized tubes. The RRBC were washed five times in phosphate-buffered saline (PBS) to remove the plasma and buffy coat. RRBC ghosts were prepared according to the method of Dodge et al. (9). Packed, washed RRBC were resuspended in 80 vol lysing buffer and incubated on ice for 15–30 min. The lysing buffer (pH 7.4) contained 5 mM Tris, 7 mM KCl, and 1 mM diisopropyl fluorophosphate to suppress protease activity. Hemoglobin was removed from the erythrocyte membrane by repeated washings with PBS.

Oligosaccharides. Melibiose, α -methyl galactoside, lactose, and β -methyl galactoside were purchased from Sigma Chemical Co. (St. Louis, MO). The oligosaccharides Gal $\alpha 1 \rightarrow 3$ Gal, Gal $\alpha 1 \rightarrow 4$ Gal, and Gal $\alpha 1 \rightarrow 4$ Gal $\beta 1 \rightarrow 4$ GlcNAc (GlcNAc, *N*-acetyl glucose) were kindly provided by Dr. Don Baker (Chem Biomed, Edmonton, Canada, supplied as the O(CH₂)₈CO₂ methylglycosides).

Isolation of Anti-Gal from Normal Serum. The method for purifying this natural antibody from normal AB serum was described previously (5). Briefly, batches of 100 ml of AB heat-inactivated serum or plasma were loaded at 37°C onto a 10 ml melibiose-Sepharose column (Sigma Chemical Co.) bearing terminal α -galactosyl residues. The antibodies retained on the column after extensive washing were eluted by 0.5 M melibiose. The carbohydrate was removed by repeated dialysis against large volumes of PBS, and the anti-Gal IgG molecules were concentrated to a volume of 5 ml using a protein A-Sepharose column (Sigma Chemical Co.). As an optional column, the Synsorb α -gal column (Chem Biomed) was used. To remove any residual anti-T activity, the anti-Gal was absorbed on neuraminidase-treated RBC. The anti-Gal reactivity was determined by the binding of the purified antibody to RRBC, as assessed by agglutination. The anti-Gal preparations used in the present study agglutinated the RRBC up to a titer of 1:512.

Isolation of RRBC Glycosphingolipids. RRBC glycosphingolipids were prepared as follows: A total lipid extract was obtained by extracting ghosts with chloroform/methanol mixtures (2:1, 1:1, 1:2) (vol/vol) at room temperature for 20 min each (10). Neutral glycosphingolipids were separated from gangliosides and phospholipids by DEAE-Sephadex column chromatography (11). Subfractionation of the neutral glycosphingolipids was done by high-performance liquid chromatography (HPLC) using an Iatrobeds column (1 \times 50 cm; 6RS-8010; Iatron Chemical Products, Tokyo, Japan), and a linear gradient of isopropyl alcohol/hexane/water (55:44:1–55:35:10) at 2 ml/min (12). The gradient was completed in 30 min, and the fractionation was continued for 20 min with the 55:35:10 solvent mixture. Fractions containing ceramide pentahexoside (CPH) were pooled and rechromatographed on a small (4.6 mm \times 25 cm) Iatrobead column using the same gradient solvent system. Glycosphingolipids were separated by thin-layer chromatography on Silica Gel 60, high-performance thin-layer chromatography (HPTLC) plates, using chloroform/methanol/water (60:35:8) (vol/vol), and stained with orcinol reagent. Standard glycosphingolipids were either obtained from Supelco (Bellefonte, PA) or prepared from human leukocytes, as previously described (10). The human B glycosphingolipid was kindly provided by Dr. Ten Feizi (Clinical Research Centre, Harrow, United Kingdom).

Immunostaining of Glycosphingolipids. Immunostaining of glycosphingolipids was done on aluminum-backed plates of Silica Gel 60 (plates 5538; E. Merck Laboratories, Inc.,

Cincinnati, OH). Glycosphingolipids were chromatographed in the solvent mixture chloroform/methanol/water (60:35:8). After drying, the plate was dipped (20 sec) in a solution of 0.05% polyisobutylmethacrylate (Polyscience, Inc., Warrington, PA) in hexane, and air dried. The plate was soaked as follows: 30 min in PBS with 5% bovine serum albumin (BSA), 2 h in the primary antibody (anti-Gal) diluted 1:500 with PBS and 1% BSA, 2 h at room temperature in a secondary antibody (rabbit anti-human IgG; Ortho Immunodiagnostics, Raritan, NJ) diluted 1:500 with PBS and 1% BSA, and for 2 h at room temperature in a solution of ^{125}I -protein A (New England Nuclear, Boston, MA) (10^6 cpm/ml in PBS with 1% BSA). The plate was washed with normal saline solution five times between each incubation step. Finally, it was dried, and an autoradiograph was prepared (Kodak X-O-mat AR2, 24–48 h at -20°C).

Glycosidase Treatment of Glycosphingolipids. Glycosphingolipids were hydrolyzed with α -galactosidase purified from Ficin (13). The reaction mixture contained 50 μg of substrate, 200 μl of 0.05 M sodium citrate, pH 4.5, with 1 $\mu\text{g}/\mu\text{l}$ taurodeoxycholate, and 200 μl of enzyme (0.1 U), and was incubated for 16 h at 37°C . Detergent was removed from the products by DEAE-Sephadex column chromatography using chloroform/methanol/water (30:60:8) (vol/vol) as the eluate. The products were evaluated by thin-layer chromatography and by immunostaining.

Liposome Preparation. Liposomes, with or without glycosphingolipids, were prepared as follows. Phosphatidylcholine (3.3 μmoles) was dried in a tube with (0.08 μmoles) or without glycosphingolipid, resuspended in 1 ml of PBS and sonicated for 30 min. Aliquots were used in the hemagglutination and rosetting assays.

Rosetting Antiglobulin Test. This test was employed in parallel with an agglutination assay to assess the binding of the anti-Gal to the various liposome preparations. The test is based on the high affinity between the Fc portion of RBC-bound IgG molecules and the Fc receptors on the myeloid cell line K562. This interaction leads to the formation of erythrocyte-antibody (EA) rosettes. The proportion of the K562 cells forming EA rosettes is related to the amount of the RBC-bound antibody molecules (14). Washed RRBC (0.1 ml of a 1% solution) were mixed with an equal volume of antibody solution, and incubated for 30 min at 37°C . The RBC were washed twice and resuspended in 0.1 ml of rabbit anti-IgG (Ortho Immunodiagnostics). The suspension was incubated for 30 min at 24°C and the RBC were washed twice. The washed RBC were mixed with 0.1 ml K562 cell suspension in PBS (10^6 cells/ml), spun for 5 min at 200 g, and incubated for 120 min at 4°C . The pellet was resuspended, and the percentage of EA rosettes was determined with a hemocytometer.

Hemagglutination Assay and Inhibition by Carbohydrates. Hemagglutination activity of the isolated anti-Gal was titrated by mixing twofold serial dilutions of the antibody with an equal volume of 0.5% RRBC suspension in the wells of a microtiter tray. The diluent was PBS, pH 7.4. Agglutination was evaluated after the RBC had settled at room temperature for 2 h. Titers were expressed as the greatest dilution of antibody solution that caused complete agglutination.

To assess the capacity of a given carbohydrate to inhibit hemagglutination, the antibody at a titer of two agglutinating units was mixed with various concentrations of the carbohydrate in the titration wells. After a 30 min incubation of the mixture at 37°C , a 0.5% RRBC suspension was added, and agglutination was determined as described above.

*Binding of the *Bandeiraea simplicifolia* (BS) Lectin to RRBC Glycosphingolipids.* The BS lectin is known to bind to various glycoconjugates bearing terminal $\text{Gal}\alpha 1 \rightarrow 3\text{Gal}$ residues, including human B blood type antigen, calf and murine thyroglobulin, and murine laminin (15, 16). The lectin (Vector Laboratories, San Francisco, CA) was radiolabeled with ^{125}I by Bolton-Hunter reagent (New England Nuclear, Boston, MA). The specific activity obtained was 10^6 cpm/ μg . The labeled lectin (1 $\mu\text{g}/\text{ml}$) was used to stain glycosphingolipids separated on aluminum-backed TLC plates. The plates were coated with plastic, soaked with buffer and the labeled lectin, and washed, and an autoradiograph was prepared as described above for the immunostaining procedure.

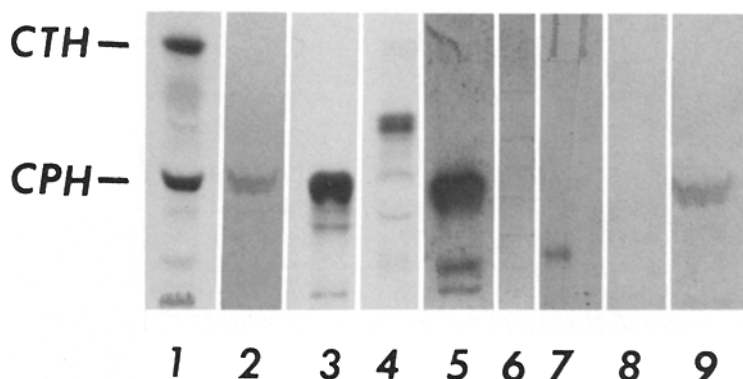


FIGURE 1. Thin-layer chromatogram of glycosphingolipids immunostained with anti-Gal. Total RRBC neutral glycosphingolipids, orcinol stained (lane 1) and immunostained (lane 2); RRBC neutral glycosphingolipids enriched for compounds with more than five sugar residues, orcinol stained (lane 3) and immunostained (lane 5); the enriched neutral glycosphingolipid fraction treated with α -galactosidase, orcinol stained (lane 4) and immunostained (lane 6); human blood group B glycosphingolipid, orcinol stained (lane 7) and immunostained (lane 8); ^{125}I -BS lectin binding to total RRBC neutral glycosphingolipids (lane 9).

Results

Specific Binding of the Natural Anti-Gal to CPH on RRBC. The two major glycosphingolipids in RRBC membranes are ceramide trihexoside (CTH), with a nonreducing terminal $\alpha 1 \rightarrow 4$ galactose, and CPH, with a nonreducing terminal $\alpha 1 \rightarrow 3$ galactose (8). In view of the observed anti- α -galactosyl specificity of the natural anti-Gal binding to RRBC, we studied the possibility that these glycosphingolipids may represent the anti-Gal binding site on the RRBC. Glycosphingolipids were isolated from RRBC and evaluated in several assays for their ability to bind anti-Gal antibody. Fig. 1 shows thin-layer chromatograms of the neutral glycosphingolipids of RRBC, either stained with orcinol (lane 1) to reveal all the glycosphingolipids, or immunostained with anti-Gal (lane 2). A number of glycosphingolipids were found in the RRBC preparation, and the major components chromatographed at the same position as standard glycosphingolipids containing three and five sugar residues. These compounds have been previously characterized (8) as RRBC CTH and CPH (Table I). HPLC fractionation of the total neutral glycosphingolipid fraction revealed a multitude of components, including glycosphingolipids with more than five sugar residues. Some of these probably correspond to complex glycosphingolipids recently characterized (17, 18) as structures containing nonreducing terminal $\alpha 1 \rightarrow 3$ galactose residues. HPLC

TABLE I
Glycosphingolipid Structures

| Structure | Name |
|--|----------------|
| $\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$ | CTH |
| $\text{Gal}\alpha 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$ | CPH |
| $\text{Gal}\alpha 1 \rightarrow 4\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$ | P ₁ |
| $\text{Gal}\alpha 1 \rightarrow 3(\text{Fuc}\alpha 1 \rightarrow 2)\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{GlcNAc}\beta 1 \rightarrow 3\text{Gal}\beta 1 \rightarrow 4\text{Glc}\beta 1 \rightarrow 1\text{Cer}$ | B-antigen |

fractions containing CPH and longer-chain glycosphingolipids were pooled and used in some of the subsequent studies. Fig. 1, lane 3 shows a chromatogram of this pooled preparation.

When these glycosphingolipids were immunostained with anti-Gal, three bands were seen on the autoradiogram (Fig. 1, lanes 2 and 5), a major band, which corresponds in mobility to the CPH, and two minor bands, with mobilities corresponding to glycosphingolipids with more than eight sugar residues. No bands were found that corresponded to CTH or any other orcinol-positive components with less than five sugar residues. Other standard glycosphingolipids with terminal galactose residues, including lactosylceramide and neolactotetraosylceramide were also tested by immunostaining, and gave negative results (not shown). Finally, a human blood group B-active glycosphingolipid (Table I) was tested as an antigen for anti-Gal, but this compound also gave negative results (Fig. 1, lane 7, orcinol strain; lane 8, immunostain). Therefore, it appears that a nonreducing terminal $\alpha 1 \rightarrow 3$ galactose is required for anti-Gal binding, and that the presence of a branched fucose on the B-antigen blocks anti-Gal binding. As a control for nonspecific binding in the immunostaining assay, melibiose (0.2 M) was added to the anti-Gal used for immunostaining. No radioactive bands were detected on the autoradiograph of the thin-layer plate under these conditions, indicating that the binding of the antibody to CPH is a specific antibody-antigen reaction.

The removal of the nonreducing terminal galactose residue from the RRBC neutral glycosphingolipids was studied for its effect on anti-Gal binding. Fig. 1, lane 6 shows that, after treatment of CPH with Ficin α -galactosidase, no immunostaining occurred. The corresponding orcinol-stained chromatogram (Fig. 1, lane 4) shows that the CPH had been converted to a compound that cochromatographs with neolactotetraosylceramide. Therefore, conversion of RRBC ceramide pentasaccharide by α -galactosidase to a ceramide tetrasaccharide bearing a terminal β -galactose (see Table I) abolishes anti-Gal binding. Similarly, the anti-Gal binding to longer-chain neutral glycosphingolipids was eliminated by α -galactosidase treatment. These results further substantiate the requirement for a nonreducing α -galactose residue for anti-Gal binding, and indicate that the neutral glycosphingolipids that are recognized by anti-Gal have a similar epitope.

The specificity of anti-Gal binding to RRBC neutral glycosphingolipids was further analyzed by using liposomes containing the purified CPH and CTH. The anti-Gal IgG preparation used in this study agglutinated RRBC up to a titer of 1:400. The addition of phosphatidylcholine (PC) liposomes or PC-CTH liposomes did not reduce the capacity of the anti-Gal to agglutinate RRBC (Table II). However, liposomes of PC-CPH inhibited 75% of the reactivity of the anti-Gal as indicated by a reduction of the titer to 1:100. The specific binding of the anti-Gal to the CPH on the liposomes was further confirmed by the sensitive rosetting antiglobulin test. The agglutination assay gave an anti-Gal titer of 1:400, whereas, by the rosetting antiglobulin tests, the antibodies bound to the RRBC were detected up to a titer of 1:3,200 of the anti-Gal (Fig. 2). No reduction in the proportion of rosettes at any of the anti-Gal dilutions was observed when PC liposomes or PC-CTH liposomes were used. However, the addition of the

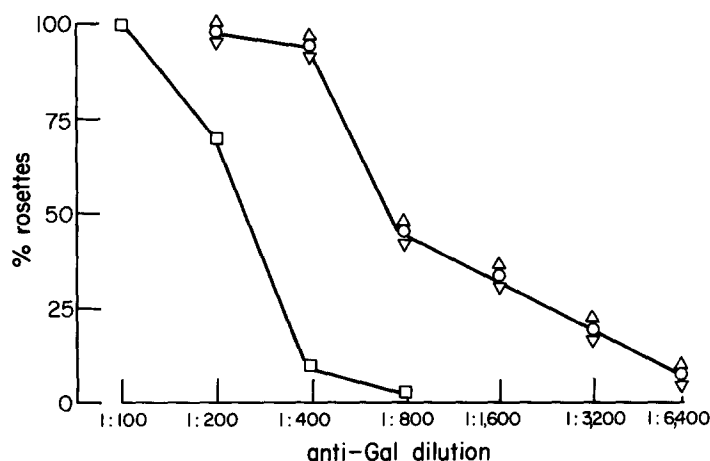


FIGURE 2. Binding of anti-Gal to liposome containing glycosphingolipids, as determined by inhibition of rosetting antiglobulin test. Rosette formation following incubation of anti-Gal with: PBS control (Δ); PC liposomes (∇); PC CTH liposomes (\circ); PC CPH liposomes (\square).

PC-CPH liposomes decreased the reactivity of the anti-Gal in this assay to a titer of 1:400 (90% inhibition of reactivity).

Inhibition of Anti-Gal Binding to RRBC by Various Galactosyl-containing Oligosaccharides. In accord with our previous studies (5), the agglutination of RRBC by anti-Gal is readily inhibited by α -methyl galactoside and melibiose, but not by β -methyl galactoside or lactose (Table III). The saccharide Gal α 1 \rightarrow 3Gal inhibited the agglutination at a lower concentration (0.3 mM) than melibiose (Gal α 1 \rightarrow 6Glc). Saccharides containing the Gal α 1 \rightarrow 4Gal structure failed to inhibit the observed hemagglutination when they were added at a concentration 33-fold greater than that of the Gal α 1 \rightarrow 3Gal disaccharide.

Specific Binding of the BS Lectin to CPH. In view of the reported (15, 16) Gal α 1 \rightarrow 3 specificity of the BS lectin we wished to compare its binding to RRBC glycosphingolipids with that of anti-Gal. As shown in Fig. 1, lane 9, 125 I-BS lectin appears to bind to the same component in the RRBC glycosphingolipid mixture as anti-Gal. However, in hemagglutination inhibition studies using oligosaccharides of known structures, differences were found between the BS lectin and anti-Gal. In addition to the oligosaccharides that inhibited anti-Gal-mediated hemagglutination of RRBC (Table II), BS lectin-mediated hemagglutination was effectively inhibited by GalNAc, and Gal α 1 \rightarrow 4Gal (not shown).

TABLE II

Inhibition of Anti-Gal-mediated Agglutination of RRBC by Liposomes Containing Glycolipids

| Liposome | Anti-Gal dilution | | | | |
|--|-------------------|-------|-------|-------|-------|
| | 1:50 | 1:100 | 1:200 | 1:400 | 1:800 |
| No liposomes | + | + | + | + | - |
| Liposome alone | + | + | + | + | - |
| Liposome + CTH Gal α 1 \rightarrow 4Gal | + | + | + | + | - |
| Liposome + CPH Gal α 1 \rightarrow 3Gal | + | + | - | - | - |

* +, agglutination; -, no agglutination.

TABLE III
Inhibition of Anti-Gal-mediated Agglutination of RRBC by Various Galactosyl-containing Oligosaccharides

| Carbohydrate | Carbohydrate concentration | | | | | | |
|---|----------------------------|---|-----|------|-----|-----|------|
| | 10 | 5 | 2.5 | 1.25 | 0.6 | 0.3 | 0.15 |
| | <i>mM</i> | | | | | | |
| α -Methyl Gal | —* | — | — | ± | + | + | + |
| β -Methyl Gal | + | + | + | + | + | + | + |
| Gal α 1 \rightarrow 6Glc (melibiose) | — | — | — | — | + | + | + |
| Gal β 1 \rightarrow 4Glc (lactose) | + | + | + | + | + | + | + |
| Gal α 1 \rightarrow 3Gal | — | — | — | — | — | — | + |
| Gal α 1 \rightarrow 4Gal | + | + | + | + | + | + | + |
| Gal α 1 \rightarrow 4Gal β 1 \rightarrow 4GlcNAc (P ₁ substance) | + | + | + | + | + | + | + |

* +, agglutination; —, no agglutination.

Discussion

Most of the antigenic structures recognized by the known natural antibodies in human serum have not been characterized. Low titers of IgG antibodies to various evolutionarily conserved proteins, including actin, myosin, thyroglobulin, myoglobin, and band 3 of the red cell membrane have been found in normal sera (19, 20). These antibodies display various degrees of crossreactivity, bind to epitopes of unknown structures, and are present in quantities of <1 μ g/ml of serum. In contrast to other natural antibodies, anti-Gal occurs in high titers and quantities (50–100 μ g/ml) in every individual tested, and displays a distinct anti- α -galactosyl specificity (5).

In this study, we have shown that anti-Gal not only recognizes an α -linked galactose nonreducing terminal residue, but also binds specifically to α -galactose residues linked 1 \rightarrow 3 and not 1 \rightarrow 4 to a penultimate galactose. Immunostaining and hemagglutination studies with the RRBC membrane glycosphingolipids have indicated that the anti-Gal does not interact with Gal α 1 \rightarrow 4Gal residue that is characteristic of CTH, but binds specifically to Gal α 1 \rightarrow 3Gal, which is the terminal disaccharide of CPH. This binding was eliminated by removal of the terminal galactosyl group by α -galactosidase. The specific interaction of the anti-Gal with Gal α 1 \rightarrow 3Gal structures was further demonstrated by the use of a Gal α 1 \rightarrow 3Gal oligosaccharide, which inhibited the agglutination of RRBC by the antibody. The lack of interaction between anti-Gal and B-antigen, either on red cells (5) or in its purified form, as demonstrated herein, implies that the α 1 \rightarrow 2 fucose linked to the penultimate galactose prevents anti-Gal binding. The RRBC CPH has long been considered (8, 21) to be serologically related to the B antigen in the human ABO system, since both molecules bear terminal Gal α 1 \rightarrow 3Gal β residues. However, Betteridge and Watkins (21) have recently shown that the enzyme that catalyzes the transfer of the terminal α -galactosyl residue to form the rabbit CPH molecule is different from the enzyme transferring galactose to produce the B antigen molecule present in rabbit stomach mucosa (21). Based on these enzyme studies and observations that RRBC fail to completely absorb the anti-B-agglutinins from many human group A sera, these authors recom-

mended the reevaluation of the concept that CPH is a B-related substance (21). The lack of binding of anti-Gal to the B-antigen, as demonstrated herein, supports this recommendation.

The Gal α 1 \rightarrow 3 specificity of the natural anti-Gal seems to partially parallel that of the lectin BS, which was previously found by Eckhardt and Goldstein (15) to bind to glycoconjugates bearing Gal α 1 \rightarrow 3 residues on murine Ehrlich ascites cells and basement membrane in various tissues of the mouse, and by Spiro and Bhoyroo (16) to interact with Gal α 1 \rightarrow 3 residues on calf and murine thyroglobulin molecules. Both the antibody and the lectin bind to CPH and not to CTH from RRBC. However, unlike anti-Gal, BS lectin readily binds to the B blood group antigen (15). Thus, the BS lectin displays a broader specificity than anti-Gal. It should be noted that, whereas BS lectin agglutinates RRBC at a concentration as low as 0.02 μ g/ml, 3,000 times as much (60 μ g/ml) of the lectin is needed to agglutinate human B-type red cells (our unpublished observation). This suggests that the fucosylation of the penultimate galactosyl, which completely prevents the interaction with the anti-Gal, greatly reduces, but does not completely eliminate the binding capacity of the BS lectin to the glycosphingolipid.

Previous studies (5-7) have suggested that anti-Gal may play a role in the removal of normal senescent and pathological human red cells from the circulation. Only 1% of normal human red cells were found to bind anti-Gal. These cells had the highest density, and are thought to be the senescent red cells. Moreover, much greater proportions of thalassemic and sickle cells interact with anti-Gal. On the basis of these observations, it was postulated that *in situ* anti-Gal binding to human red cells involves the *de novo* exposure of a glycoconjugate(s) bearing a terminal α -linked galactose. Only three glycoconjugates with a nonreducing terminal α -linked galactose are known to be present on human red cells, CTH, P₁, and B antigen. The results obtained herein suggest that neither CTH nor P₁, which are abundant on human red cells, serve as the binding site for anti-Gal, since both have Gal α 1 \rightarrow 4Gal terminal disaccharides (22). Furthermore, anti-Gal does not bind to the B antigen.

The distribution of the Gal α 1 \rightarrow 3Gal structure and of the anti-Gal natural antibodies in various mammals is of special interest. The anti-Gal antibodies were found in baboons, in addition to man, but not in mice, rats, guinea pigs, and, evidently, rabbits (5). However, whereas Gal α 1 \rightarrow 3Gal structures were found to be abundant in mouse, rabbit, and calf glycoconjugates, no such structures, except for the fucosylated B antigen, have been found thus far in human tissues using the BS lectin (23). Hence, it may be possible that, with the evolutionary need for a permanent production of anti-Gal in primates and man, perhaps toward some gastrointestinal or pulmonary bacteria, a concomitant suppression of the synthesis of Gal α 1 \rightarrow 3Gal glycoconjugates occurred. This possibility is supported by studies that have shown that bacterial lipopolysaccharides from *Salmonella* and *E. coli* contain nonreducing terminal α 1 \rightarrow 3 galactose (24, 25). The recently reported findings (5-7, 26) on anti-Gal binding to α -galactosyl residues exposed on normal human senescent red cells, thalassemic, and sickle red cells implies that the suppression of Gal α 1 \rightarrow 3Gal glycoconjugate synthesis in man is not absolute. The characterization of these molecules in human red

cells is currently under study. Finally, in view of the high amounts of the anti-Gal in human serum, the identification of such glycoconjugates on other tissues might imply a possible role for this unique natural antibody in the development of various autoimmune states.

Summary

A natural IgG antibody (anti-Gal) with α -galactosyl binding specificity has been found in large amounts (0.5–1.0% of serum IgG) in all individuals tested. It has been purified by affinity chromatography on a column of melibiose-Sepharose. In addition to its affinity for normal and pathological senescent human red cells, the antibody readily interacts with rabbit red blood cell (RRBC) glycolipids with α -galactosyl terminal residues. Two types (glycosidic linkages of 1 \rightarrow 3 vs. 1 \rightarrow 4) of rabbit red cells glycolipids with terminal α -galactosyl residues were tested for antibody binding. The antibody specifically bound to glycolipids with Gal α 1 \rightarrow 3 terminal residues, and treatment of these glycolipids with α -galactosidase abolished binding. Hemagglutination inhibition studies with oligosaccharides of known structure also showed that the antibody binds specifically to glycoconjugates with an α 1 \rightarrow 3 terminal galactose residue. Anti-Gal did not bind to a human B-active glycolipid, indicating that fucose-linked α 1 \rightarrow 2 to the penultimate galactose prevents anti-Gal binding. The anti-Gal specificity for RRBC glycolipids also paralleled that of the α -galactosyl-specific *Bandeiraea simplicifolia* lectin. The possible reasons for the occurrence of this unique antibody in human serum are discussed.

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