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Immunology

Evolutionary relationship between the natural anti-Gal antibody and the Galα1→3Gal epitope in primates*

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ABSTRACT **Anti-Gal is a natural antibody, which constitutes as much as 1% of circulating IgG in humans and displays a distinct specificity for the structure Galα1→3Gal. This glycosidic structure has been found on various tissues of many nonprimate mammals. A comparative study of the occurrence of anti-Gal versus the expression of the Galα1→3Gal epitope was performed in primates, and a distinct evolutionary pattern was observed. Whereas anti-Gal was found to be present in Old World monkeys and apes in titers comparable to those in humans, its corresponding antigenic epitope is abundantly expressed on erythrocytes of New World monkeys. Immunostaining with anti-Gal of glycolipids from New World monkey erythrocytes indicated that the molecules to which anti-Gal binds are similar to those found in rabbit and bovine erythrocytes. These findings indicate that there is an evolutionary reciprocity between New World and Old World primates in the production of the Galα1→3Gal structure and the antibody that recognizes it. The expression of the Galα1→3Gal epitope was evolutionarily conserved in New World monkeys, but it was suppressed in ancestral lineages of Old World primates. The suppression of this epitope was accompanied by the production of anti-Gal. The observed *in vivo* binding of anti-Gal to human normal senescent and some pathologic erythrocytes implies that the Galα1→3Gal epitope is present in man in a cryptic form.**

Anti-Gal is a natural IgG antibody recently found to be present in large amounts in human serum and absent from serum of rodents such as mouse, rat, and guinea pig (1). Anti-Gal constitutes as much as 1% of the circulating IgG in humans, and it displays a distinct specificity for the glycosidic structure Galα1→3Gal (2). This Galα1→3Gal epitope is a unique glycosidic structure, which has been evolutionarily conserved in many mammalian species. It is present on various normal and malignant tissues, and on erythrocytes from such species as mouse (3, 4), rat (5), guinea pig (5), rabbit (6, 7), dog (8), and cow (9–11). The Galα1→3Gal epitope has not been detected, however, in human tissues (9). The distribution of the anti-Gal and the Galα1→3Gal epitope in mammals suggested a possible reciprocal evolutionary pattern in the expression of these two related components. It was therefore of interest to identify the species producing either the antigenic epitope or the antibody in order to determine the evolutionary stage at which Galα1→3Gal synthesis may have been suppressed and the production of anti-Gal initiated. Our findings suggest that this conversion of Galα1→3Gal and anti-Gal expression occurred within the order of primates after the divergence to New World and Old World primates.

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MATERIALS AND METHODS

Erythrocytes and Sera. Erythrocytes and sera were obtained from the heparinized blood of a large number of primate and nonprimate mammals. Primate blood samples were provided by the San Francisco Zoo, National Institutes of Health Primate Center (Bethesda, MD), New York University Primate Center, and the São Paulo (Brazil) Zoo.

Isolation of Anti-Gal from Normal Human Serum. Anti-Gal was purified from human AB serum by affinity chromatography by a modification of the method described (1, 2). The immunoadsorbent used in the present study was the SYNSORB 90 (a gift from Chembiomed, Edmonton, AB Canada) with the glycosidic epitope of Galα1→3Galβ1→4Glc-R, which was found to be more effective than melibiosyl-Sepharose in the binding of anti-Gal. The anti-Gal preparations used in this study were used at a concentration of 100 μg/ml and agglutinated rabbit erythrocytes up to a titer of 1:1280. This titer was chosen since it corresponds to the titer of the antibody found in normal human serum.

Analysis of the Expression of Galα1→3Gal Epitopes on Erythrocytes by Hemagglutination Assay with Anti-Gal and *Bandeiraea simplicifolia* IB_4 Lectin. The occurrence of Galα1→3Gal epitopes on erythrocytes of various species was studied by analyzing the interaction of given erythrocytes with anti-Gal. The interaction of this antibody with the erythrocytes was assessed by a hemagglutination test. Dilutions (1:2) of anti-Gal in 50-μl aliquots were mixed with an equal volume of 0.5% erythrocyte suspensions in the wells of a V-shaped microtiter tray. The diluent was phosphate-buffered saline (PBS) (pH 7.4). After a 30-min incubation, the erythrocytes were washed three times in PBS by centrifugation, discarding the supernatant, and resuspension in PBS. The washed erythrocytes were resuspended in rabbit anti-human IgG (Dako, Santa Barbara, CA), and the resulting indirect hemagglutination titer was scored after 1 hr at 24°C. To assess the capacity of a given carbohydrate to inhibit hemagglutination, anti-Gal at a titer of 2 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, the erythrocyte suspension was added, and agglutination was determined as described above. Parallel hemagglutination assays were performed with the lectin *B. simplicifolia* IB_4 (BS lectin) (Vector Laboratories, Burlingame, CA), which also interacts specifically with Galα1→3Gal epitopes (2, 3, 7, 9). The initial concentration of the BS lectin was 1000 μg/ml. Lectin hemagglutination was expressed as

Abbreviations: BS lectin, *Bandeiraea simplicifolia* IB_4 lectin; GSL, glycosphingolipid(s).

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the lowest concentration yielding positive hemagglutination reaction.

Assessment of the Number of Galα1→3Gal Epitopes Expressed per Erythrocyte by Radiolabeled BS Lectin. BS lectin was radiolabeled with ^{125}I using the Bolton–Hunter reagent (New England Nuclear). The specific activity obtained was 10^6 cpm/μg. Aliquots (0.1 ml) of erythrocytes (5.0×10^8 cells per ml) were incubated in serial dilutions of ^{125}I-labeled BS lectin. For assessment of nonspecific binding, the BS lectin was added in a solution of 0.1 M melibiose (α-galactosylglucoside). This carbohydrate prevents the specific binding of the BS lectin to galactosyl residues on erythrocytes. After incubation at 37°C for 60 min, the erythrocyte suspensions were washed five times with PBS and transferred to new tubes for counting in a γ counter. Specific labeling was calculated by subtracting cpm obtained in control suspensions, which included melibiose. The number of Galα1→3Gal residues to which BS lectin bound per erythrocyte was calculated by Scatchard analysis (12).

Isolation of Erythrocyte Glycosphingolipids. Glycosphingolipids (GSL) from erythrocytes of various species were prepared according to a method described in our previous study (2). Briefly, lipids were extracted from erythrocyte ghosts with chloroform/methanol mixtures (2:1, 1:1, and 1:2). Neutral GSL were separated from gangliosides and phospholipids by DEAE-Sephadex chromatography. Subfractionation of the neutral GSL was done by high-performance liquid chromatography (HPLC) using an Iatrobead column (Iatron Chemical, Tokyo) and a linear gradient of isopropyl alcohol/hexane/water (55:44:1 to 55:35:10). GSL 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.

Immunostaining of GSL with Anti-Gal and BS Lectin. Immunostaining of GSL from various erythrocytes was done on aluminum-backed plates of Silica Gel 60 (plates 5538; E. Merck Laboratories, Cincinnati, OH) according to a procedure that is a modification of the method of Magnani *et al.* (13). GSL were chromatographed on HPTLC plates as described above. After drying, the plates were dipped (20 sec) in a solution of 0.05% polyisobutylmethylacrylate (Polyscience, Warrington, PA) in hexane, and air-dried. The plates were soaked as follows: 30 min in PBS with 5% bovine serum albumin, 2 hr in anti-Gal (5 μg/ml), 1 hr in biotinylated goat anti-human IgG (Vector Laboratories), and thereafter in the Vector ABC reagent containing avidin, alkaline phosphatase and substrate (Vector Laboratories). The plates were washed with PBS solution five times between each incubation step. Biotinylated BS lectin (Vector Laboratories) (7.5 μg/ml) was also used to stain GSL separated by TLC. The staining with the BS lectin was performed according to a procedure similar to the immunostaining.

Analysis of Anti-Gal Reactivity in the Serum of Various Primates. The anti-Gal reactivity in primate serum was assessed by indirect hemagglutination of rabbit erythrocytes, which express an abundance of terminal Galα1→3Gal structures (6). The assay was performed as described above. Rabbit anti-human IgG was a suitable secondary antibody for the indirect assay since it was found to interact with the IgG of all primates studied in an Ouchterlony immunodiffusion assay. The α-galactosyl specificity of the anti-Gal in the positive sera was further established by inhibition of the hemagglutination assay in the presence of 0.1 M melibiose.

RESULTS

Binding of the Anti-Gal and BS Lectin to Erythrocytes of Various Species. The expression of Galα1→3Gal epitopes on erythrocytes of various species could be analyzed by hemagglutination studies with affinity-purified human anti-Gal. The erythrocytes of all nonprimate mammals tested were readily agglutinated by anti-Gal. This included erythrocytes of rat, rabbit, cow, pig, and dog (Table 1). A distinct pattern of reactivity was observed in the hemagglutination pattern of primate erythrocytes. No agglutination was detected with the erythrocytes of Old World monkeys, anthropoid apes, and humans. However, erythrocytes of all New World monkey species were readily agglutinated by anti-Gal. Hemagglutination mediated by BS lectin paralleled that observed with anti-Gal. The erythrocytes of all the nonprimate mammals studied and those of New World monkeys were agglutinated by the BS lectin at a concentration of 15 μg/ml or less. Erythrocytes of Old World monkeys, anthropoid apes, and humans were not agglutinated even at a concentration of 1000 μg per ml of the lectin. BS lectin did bind to gibbon and orangutan erythrocytes, but it did so because of the expression of blood group B antigen on their membrane. This antigen, which has the structure Galα1→3(Fucα1→2)Gal, is also known to interact with the BS lectin (14) but not with anti-Gal (2).

Anti-Gal hemagglutination of erythrocytes of all species was mediated via interaction with the α-galactosyl epitopes, since the oligosaccharides containing the α-galactosyl structures α-methylgalactoside and melibiose readily inhibited the hemagglutination (Table 2), whereas the carbohydrates with the β-galactosyl structures β-methylgalactoside and lactose were ineffective in the inhibition of hemagglutination. In a previous study with rabbit erythrocytes, we have shown that a Galα1→3Gal oligosaccharide was more effective than melibiose in inhibiting anti-Gal hemagglutination (2). However, due to lack of a sufficient amount of this oligosaccharide, we could not perform the assay with this carbohydrate molecule and erythrocytes of other species. Table 2 describes representative hemagglutination inhibition studies with erythrocytes of rabbit, cow, dog, and squirrel monkey. Comparable data were obtained with erythrocytes of all species that bound the anti-Gal.

Analysis of the Molecules Binding Anti-Gal on New World Monkey Erythrocytes. Our previous work indicated that anti-Gal and BS lectin bind specifically to GSL molecules with nonreducing terminal Galα1→3Gal structure (2). In rabbit erythrocytes, the major glycolipid with this structure is ceramide pentahexoside (6). Thus, it was of interest to determine whether the observed binding of anti-Gal to erythrocytes of New World monkeys is also mediated via interaction of the antibody with GSL molecules. The erythrocytes of the primates squirrel monkey and cynomolgus monkey were used as sources of New World and Old World monkey glycolipids, respectively. GSL from bovine erythrocytes were used as a positive control for immunostaining with anti-Gal and BS lectin, since bovine erythrocytes are known to have two GSL molecules with terminal Galα1→3Gal structure. One is ceramide pentahexoside, and the other is a ceramide heptahexoside with seven carbohydrate units (10). Immunostaining of bovine GSL separated on TLC showed that both anti-Gal and BS lectin readily bound to these two molecules (Fig. 1*A*). Rabbit erythrocytes have been shown to contain a family of GSL molecules with long complex carbohydrate chains, all having Galα1→3Gal epitopes (7). Bovine erythrocytes, like the rabbit erythrocytes, contain GSL with longer carbohydrate chains that have terminal Galα1→3Gal structures capable of binding both anti-Gal and BS lectin as shown in Fig. 1*A*.

Squirrel monkey GSL contained two molecules that interacted with anti-Gal and BS lectin. One of these molecules cochromatographed with the rabbit and bovine ceramide pentahexoside; the other had a longer carbohydrate chain (Fig. 1*B*). Because GSL with Galα1→3Gal epitopes are less

Table 1. Binding of anti-Gal antibody and BS lectin to erythrocytes of various species

Erythrocyte	Hemagglutination titer by purified anti-Gal	*B. simplicifolia* IB_4	
		Hemagglutination, μg/ml	Number of α-galactosyl sites per cell
Nonprimates			
Rat	1:160	0.25	45,000
Rabbit	1:1280	0.05	150,000
Cow	1:10	15	20,000
Pig	1:160	0.25	60,000
Dog	1:160	0.7	15,000
New World monkeys			
Marmoset (*Callithrix jacchus*)	1:80	1.0	20,000
Tamarin (*Saguinus mystax*)	1:80	1.0	22,000
Squirrel monkey (*Saimiri sciureus*)	1:80	0.5	35,000
Owl monkey (*Aotus trivirgatus*)	1:10	8.0	7,000
Capuchin (*Cebus apella*)	1:20	4.0	6,000
Spider monkey (*Ateles paniscus*)	1:80	2.0	33,000
Howler monkey (*Alouatta caraya*)	1:40	4.0	14,000
Woolly monkey (*Lagothrix lagothricha*)	1:40	4.0	18,000
Old World monkeys			
Rhesus monkey (*Macaca mulatta*)	0	None	<500
Cynomolgus monkey (*M. fascicularis*)	0	None	<500
Celebes monkey (*M. maurus*)	0	None	<500
Pigtailed Macaque (*M. nemestrina*)	0	None	<500
Stump-tailed Macaque (*M. arctoides*)	0	None	<500
Diana monkey (*Cercopithecus diana*)	0	None	<500
Baboon (*Papio papio*)	0	None	<500
Anthropoid apes			
Chimpanzee	0	None	<500
Gibbon	0	1.0	ND
Orangutan	0	15	ND
Gorilla	0	None	<500
Human			
Type O	0	None	<500
Type B	0	60	ND

Data are presented from a representative sample out of two to six individuals of each species. ND, not determined. None, no agglutination.

abundant in squirrel monkey erythrocytes than those of either rabbit or bovine erythrocytes, their reactivity with the antibody and lectin could only be shown after HPLC enrichment of GSL with carbohydrate chains of more than four units. In contrast to GSL from the erythrocytes of squirrel monkey, GSL from the Old World monkey, cynomolgus showed no anti-Gal staining even after HPLC fractionation (Fig. 1*C*). Similarly, there was no staining of the cynomolgus monkey glycolipids with the BS lectin. Therefore, the results obtained by immunostaining, demonstrating GSL with Galα1→3Gal residues in erythrocytes of New World but not

Table 2. Inhibition of anti-Gal-mediated agglutination of erythrocytes from various species by α-galactosyl carbohydrates

Carbohydrate	Origin of erythrocytes			
	Rabbit	Cow	Dog	Squirrel monkey
Galactose	3.0	6.0	6.0	3.0
α-Methyl galactoside	0.7	1.5	3.0	0.3
β-Methyl galactoside	50	50	50	25
Melibiose (α-galactosyl glucoside)	0.7	1.5	3.0	0.3
Lactose (β-galactosyl glucoside)	>50	>50	>50	>50
Glucose	>50	>50	>50	>50
Fucose	>50	>50	>50	>50
Mannose	>50	>50	>50	>50

Results are expressed as lowest concentration (mM) of carbohydrate inhibiting hemagglutination.

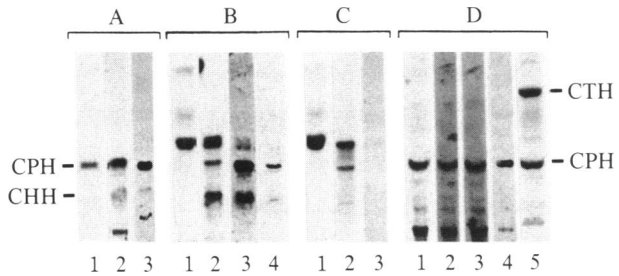

FIG. 1. Thin-layer chromatogram of erythrocyte glycolipids immunostained with anti-Gal or BS lectin. (*A*) Cow glycolipids stained with orcinol (lane 1), human anti-Gal (lane 2), BS lectin (lane 3). (*B*) Squirrel monkey glycolipids stained with orcinol (lane 1), orcinol (HPLC-enriched longer-chain glycolipids) (lane 2), glycolipids as in lane 2 stained with human anti-Gal (lane 3), glycolipids as in lane 2 stained with BS lectin (lane 4). (*C*) Cynomolgus monkey glycolipids stained with orcinol (lane 1), orcinol (HPLC-enriched longer-chain glycolipids) (lane 2), glycolipids as in lane 2 stained with human anti-Gal (a similar negative staining was observed with BS lectin) (lane 3). (*D*) Rabbit glycolipids stained with human anti-Gal (lane 1), rhesus monkey anti-Gal (lane 2), chimpanzee anti-Gal (lane 3), BS lectin (lane 4), orcinol (lane 5). CTH, ceramide trihexoside, Galα1→4Galβ1→4Glc1→1Cer; CPH, ceramide pentahexoside, Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc1→1Cer; CHH, ceramide heptahexoside, Galα1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4Glc1→1Cer.

Old World monkeys, are in accordance with the hemagglutination data obtained with anti-Gal and BS lectin (Table 1).

Anti-Gal Reactivity in Primate Serum. The presence of anti-Gal antibodies in the serum of primates was studied along with the evaluation of anti-Gal binding sites on the erythrocytes. Anti-Gal reactivity was assessed by indirect hemagglutination of rabbit erythrocytes. Whereas serum of all Old World monkeys and anthropoid apes contained IgG antibodies that agglutinated rabbit erythrocytes in titers comparable to those of human serum, the serum of New World monkeys lacked such antibodies (Table 3). The observed antibody reactivity in serum of Old World monkeys and anthropoid apes was the result of anti-Gal reactivity, since melibiose (α-galactosylglucoside) reduced the hemagglutination titer (Table 3). Anti-Gal was isolated from sera of Old World monkeys and anthropoid apes by affinity chromatography, according to the procedures used for isolation of human anti-Gal. Immunostaining of rabbit GSL demonstrated that anti-Gal antibodies from chimpanzee and rhesus monkey bound to rabbit ceramide pentahexoside and longer-chain glycolipids in a pattern similar to that obtained with human anti-Gal (Fig. 1*D*). The same binding pattern was also observed with anti-Gal of cynomolgus monkey, gibbon, orangutan, and gorilla.

DISCUSSION

Previous studies on the distribution of anti-Gal (1) and its corresponding Galα1→3Gal epitope (3–11) in mammals suggested to us that there is an evolutionary reciprocity in the expression of this antibody and antigenic structure. The results of our current study indicate that the Galα1→3Gal epitope has been evolutionarily conserved not only in many nonprimate mammals, but also in New World monkeys. This was demonstrated by the distinct anti-Gal hemagglutination of erythrocytes from all New World monkey species studied. Furthermore, immunostaining of GSL on TLC plates showed that among the Galα1→3Gal-bearing molecules that bind anti-Gal, a similar ceramide pentahexoside is detectable in rabbit, bovine, and New World monkey erythrocytes. In accord with the expression of these glycosidic epitopes on their erythrocytes, New World monkeys lack anti-Gal reactivity in their serum, probably as a result of immunologic tolerance. Studies of Old World monkeys and anthropoid apes revealed an inverse pattern. In contrast to New World monkeys, these primates lacked detectable Galα1→3Gal epitope on their erythrocytes, but synthesize anti-Gal in amounts comparable to that of humans. The presence of anti-Gal in the sera of Old World monkeys and anthropoid apes was demonstrated by the potent hemagglutination of rabbit erythrocytes and the specific inhibition of this reaction by melibiose. Furthermore, the anti-Gal isolated by affinity chromatography from sera of Old World monkeys and anthropoid apes bound to the rabbit GSL in a pattern similar to that obtained with human anti-Gal.

Table 3. Anti-Gal reactivity in sera of various primates as assayed by agglutination of rabbit erythrocytes

Serum source	Hemagglutination titer: Serum alone	Hemagglutination titer: Serum + 0.1 M melibiose
New World monkeys		
Marmoset	<1:20	ND
Tamarin	<1:20	ND
Squirrel monkey	<1:20	ND
Owl monkey	<1:20	ND
Capuchin	<1:20	ND
Spider monkey	<1:20	ND
Howler monkey	<1:20	ND
Woolly monkey	<1:20	ND
Old World monkeys		
Rhesus monkey	1:5120	1:2560
Cynomolgus monkey	1:1280	1:40
Pigtailed Macaque	1:5120	1:1280
Diana monkey	1:5120	1:1280
Baboon	1:2560	1:640
Apes		
Orangutan	1:640	1:160
Gibbon	1:320	1:160
Chimpanzee	1:2560	1:160
Gorilla	1:640	1:80
Human	1:1280	1:320

Data were obtained from a representative sample out of two to five serum samples of each species. A titer of <1:20 indicates that within a given species, the hemagglutination titer ranged between no reactivity and a titer of 1:10. ND, not done.

These comparative studies in primates may suggest that less than 35 million years ago, after the continents of Africa and South America separated, an evolutionary factor (e.g., a deleterious infectious agent) endemic to the Old World provided selective pressure for the evolution of ancestral primates capable of producing high titers of anti-Gal. Such an evolutionary process in the Old World would likely have resulted in the extinction of lineages of ancestral primates that failed to suppress expression of the Galα1→3Gal epitopes, since the presence of both antigen and antibody would have generated severe autoimmune disorders. The nature of such a hypothetical evolutionary factor is currently unknown. However, the occurrence of infectious agents endemic to the Old World, which affected the evolution of the immune system in primates, is well-documented. A distinct example is the Epstein–Barr virus, which arose from Herpes virus on the African continent after the continental separation. Thus primates of the Old World, but not of the New World, evolved natural immunity to Epstein–Barr virus infection (15).

It should be recognized that it may not be possible to substantiate this hypothesis on the contribution of environmental factors to the evolution of anti-Gal synthesis. However, the contemporaneous production of anti-Gal in humans, anthropoid apes, and Old World monkeys probably represents an ongoing immune response to Galα1→3Gal structures found on various gastrointestinal bacteria, including *Salmonella, Klebsiella*, and *Escherichia coli* (16–18). Our studies have indeed demonstrated a specific *in vitro* interaction of purified anti-Gal with these bacterial strains (unpublished data).

Of special interest in regard to the present study is the finding that on glycolipids or glycoproteins of nucleated cells, erythrocytes, and thyroglobulin of mouse (3, 4), rat (5), rabbit (6, 7), and cow (9–11), the Galα1→3Gal epitope is always expressed as the terminal residue on a polylactosamine core structure. The polylactosamine glycosidic structure is also abundant on human cells as unbranched structure of the i antigen or as the bi- or multiantennary branched structure of the I antigen (19). The I and i antigens are also the biosynthetic precursors of the ABH antigens (20), and *in vitro* they were found to serve as acceptors of bovine α1→3 galactosyltransferase for the synthesis of Galα1→3Gal epitope (21). This may imply that the production of Galα1→3Gal epitopes in man and Old World primates has been evolutionarily diminished via the suppression of the gene encoding the α1→3 galactosyltransferase. We hope to obtain a DNA probe for the gene encoding this enzyme, which will enable us to perform a detailed study on the mode of regulation of α1→3-galactosyltransferase in various species. Such a probe would allow us to test the above hypothesis.

We have recently found that a small number of glycoconjugates capable of interacting with anti-Gal are apparently present on human erythrocytes in a cryptic form (1). The structure of these cryptic epitopes has not been characterized as yet. In view of the narrow specificity of anti-Gal, it is probable that these epitopes also have a Galα1→3Gal structure. If so, their small number and cryptic form must be insufficient to induce immune tolerance in humans, as is the case with New World monkeys.

The anti-Gal binding epitope on human erythrocytes may become exposed either as these cells age (1, 22) or as they are damaged in the circulation in hematologic disorders such as β-thalassemia (23) and sickle cell anemia (24). We are currently attempting to characterize these cryptic molecules. Demonstration of Galα1→3Gal structures on these glycoconjugates would imply that this epitope, which is abundant in nonprimate mammals and New World monkeys, is present in a small quantity and a cryptic form in humans, and its interaction with anti-Gal may suggest some pathophysiologic role for this natural antibody.

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