

# A UNIQUE NATURAL HUMAN IgG ANTIBODY WITH ANTI- $\alpha$ -GALACTOSYL SPECIFICITY

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It is well accepted at present that the majority of antibodies in human serum are produced as a result of natural immunization. However, the information about the nature and specificity of natural IgG antibodies is relatively scarce. The classical natural antibodies against blood group antigens, which display a well-defined anti-carbohydrate specificity, are mostly of the IgM class and are present in only part of the population, according to the blood type (1, 2). The anti-T or anti-Thomsen Friedenreich antibodies, which interact with  $\beta$ -galactosyl groups usually penultimate to terminal sialic acid residues on various cell membranes, are similarly mostly of the IgM class (3, 4). In a recent study, Guilbert et al. (5) demonstrated, in pooled human sera, low activity of natural IgG antibodies to a variety of evolutionary preserved proteins such as thyroglobulin, actin, or myoglobin. Although some cross-reactivity was noted, these antibodies could be inhibited mainly by their respective antigens. The structure of the epitopes involved could not be determined.

In the present study we report on the identification and isolation of a new natural IgG antibody with a distinct anti- $\alpha$ -galactosyl reactivity. The anti-galactosyl (anti-Gal)<sup>1</sup> antibody was found to be of interest for the following reasons: (a) The anti-Gal is the only natural IgG antibody found to be present in high titers in the serum of every normal individual. (b) The anti-Gal seems to display a physiological role in senescence of human erythrocytes (RBC). The anti-Gal-binding site on the human RBC differs from any known galactose-containing RBC antigen, and possibly is an epitope on membranal glycolipids. (c) Since anti-Gal is constantly produced throughout life, the determination of its titer may serve as a potential tool for the assessment of the humoral immune response in individual patients.

## Materials and Methods

**RBC and Sera.** Whole blood was obtained from normal donors and from  $\beta^+$  thalassemic patients, using sodium citrate or heparin as anticoagulant. Sera were obtained from clotted blood of normal individuals. All blood samples studied were screened for ABO (H) specificities by regular blood banking methods.

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<sup>1</sup> *Abbreviations used in this paper:* Anti-Gal, anti-galactosyl IgG antibody; DAT, direct antiglobulin test; EA, erythrocyte-antibody; Hu, human; PBS, phosphate-buffered saline; Rab, rabbit; RBC, erythrocytes; Staph A, *Staphylococcus aureus*; VCN, *Vibrio cholera* neuraminidase.

*Isolation of Anti-Gal IgG from Normal Serum.* The natural anti-Gal IgG was isolated from heat-inactivated sera of normal individuals of AB blood group by affinity chromatography, using as immunoadsorbent melibiose-Sepharose (Sigma Chemical Co., St. Louis, MO), expressing terminal  $\alpha$ -galactosyl residues. Sera samples were applied in 100-ml batches onto a 10-ml melibiose-Sepharose column at 37°C at a flow rate of 10 ml/h. The unbound serum proteins were eluted with 200 ml phosphate-buffered saline (PBS). The bound antibody was eluted by 20 ml of 0.5 M D-galactose and passed through a 5-ml column of protein A-Sepharose (Sigma Chemical Co.). This second column, which binds the IgG molecules complexed with galactose eluted from the affinity chromatography column, was washed with 500 ml PBS. The IgG was eluted with 7 ml of 0.1 M glycine HCl buffer, pH 2.6 and immediately neutralized with an equal volume of Tris-HCl buffer, pH 8.4. The eluate was dialyzed against two changes of PBS. To remove any possible residual anti-T (Thomsen Friedenreich) antibody activity, the dialyzed eluates were absorbed on *Vibrio cholera* neuraminidase (VCN)-treated human RBC at a 3:1 ratio (3). For this purpose 10 ml of 10% O type RBC were incubated with 0.1 U/ml VCN (Behringwerke, Federal Republic of Germany) for 30 min at 37°C in PBS containing 3 mM CaCl<sub>2</sub>. The absorption was confirmed by demonstrating complete lack of IgG binding to VCN-treated human RBC. The anti-Gal reactivity was assessed by the binding of the antibody to the terminal  $\alpha$ -galactosyl groups on rabbit RBC (RabRBC) glycolipids (6) followed by agglutination or rosetting antiglobulin test with K562 cells (7). The anti- $\alpha$ -galactosyl specificity of the antibody was determined by inhibition experiments with various carbohydrates (Sigma Chemical Co.).

*Hemagglutination Assay and Inhibition by Carbohydrates.* Hemagglutination activity of the isolated anti-Gal was titrated by mixing twofold serial dilutions of the antibody sample with an equal volume of 0.5% RabRBC suspension in the wells of microtiter tray. The diluent was PBS, pH 7.4. Agglutination was evaluated after the RBC were settled at room temperature for 2 h. Titers were expressed as the greatest dilution of sample 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 an iso-osmotic solution in titration wells. After 30-min incubation of the mixture at 37°C, the 0.5% RabRBC suspension was added and scored for agglutination as described above.

*EA Rosetting Direct Antiglobulin Test (DAT) with K562 Cells.* 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 (8). A quantity of 0.1 ml of 1% suspension of washed RBC was mixed with an equal volume of antibody-containing solution and incubated for 30 min at 37°C. Thereafter, the RBC were washed twice and resuspended in 0.1 ml of rabbit anti-human serum (broad spectrum containing anti-human IgG, IgM, IgA, and anti-complement; Ortho Diagnostics, Raritan, NJ) or, if stated, in specific rabbit anti-human IgG serum (Ortho). The suspension was incubated for 30 min at 24°C. Thereafter, the RBC were washed twice, mixed with 0.1 ml K562 cell suspension in PBS (10<sup>6</sup> cells/ml), spun for 5 min at 200 g, and incubated for 30 min at 4°C. The pellet was resuspended and the percentage of K562 cells binding the IgG-coated RBC and forming EA rosettes was scored in a hemocytometer. This antiglobulin test was found to be 20–40-fold more sensitive than the regular antiglobulin test and highly specific, since RBC lacking bound Ig formed no rosettes with K562 cells (8). Inhibition of the rosetting antiglobulin test by various carbohydrates was performed by their addition to the antibody solution before the incubation with RBC as described above.

*Sensitization of Staphylococcus aureus (Staph A) Bacteria with Anti-Gal.* Staph A strain Cowan 1, containing protein A and prefixed with 0.1% glutaraldehyde, were incubated with the purified anti-Gal at a 5% final concentration of the bacteria, for 24 h at 4°C. The sensitized bacteria were washed and tested for binding to galactosyl residues on RabRBC by mixed hemagglutination. Staph A sensitized with anti-Gal agglutinated 1%

RabRBC at a concentration of bacteria as low as 0.01%. For visualization of anti-Gal binding to various RBC populations, 1% RBC suspensions were mixed with 1% sensitized Staph A, spun for 5 min at 400 g, and incubated for 1 h at room temperature. Thereafter, the pellets were gently resuspended and fixed with 0.1% glutaraldehyde. These samples were processed for scanning electron microscopy according to procedure previously described (9).

**Separation of RBC Age-related Subpopulations.** Senescent and young normal RBC were separated on the basis of age-dependent differences in density, on a discontinuous Percoll gradient (Pharmacia Fine Chemicals, Piscataway, NJ) according to the method of Alderman et al. (10). The subpopulation of senescent RBC with density of  $>1.11$  g/ml (1–2% of total RBC) and the subpopulation of young RBC with density of  $<1.08$  g/ml (1–2% of total RBC) were isolated, washed twice in PBS, and used for further analysis.

**Enzymatic Treatment of HuRBC.** The interaction of the anti-Gal with HuRBC was assessed after treatment with the following enzymes: (a) Pronase (from *Streptomyces griseus*; Sigma Chemical Co.): HuRBC were brought to a 10% suspension in PBS containing 0.1% pronase and incubated for 1 h at 37°C. Thereafter, the RBC were washed twice and adjusted to 10% concentration in PBS for further studies. (b)  $\alpha$ -Galactosidase (from coffee beans; Sigma Chemical Co.): pronase-treated RBC were suspended in 0.1 M citric acid, 0.2 M  $\text{Na}_2\text{HPO}_4$  buffer, pH 5.0 containing 3% glycerol, to a concentration of 10%.  $\alpha$ -Galactosidase was added to a final concentration of 5 U/ml. The suspension was incubated for 3 h at 37°C and washed, thereafter, twice with PBS. Reactivity of the enzyme was confirmed by parallel elimination of the agglutinability of B-type RBC by anti-B antibodies (1, 2). (c)  $\beta$ -Galactosidase (from *E. coli*; Sigma Chemical Co.): the pronase-treated RBC were resuspended in 0.01 M Tris HCl buffer containing 0.01  $\text{MgCl}_2$ , 0.01 M mercaptoethanol, and 0.1 M NaCl, pH 7.5. 500 U of  $\beta$ -galactosidase were added to the 1-ml RBC suspension. The mixed suspension was incubated for 3 h at 37°C and washed twice with PBS. Reactivity of the enzyme was confirmed by the hydrolysis of *o*-nitrophenyl  $\beta$ -D-galactoside to *o*-nitrophenol and galactose.

## Results

**Characteristics and Specificity of the Anti-Gal Isolated by Affinity Chromatography.** The normal AB sera contained anti-Gal reactivity at titers of 1:800 to 1:1,600 (Table I), as assessed by the binding to RabRBC in the rosetting antiglobulin test. Due to its IgG nature, the unseparated anti-Gal failed to agglutinate other RabRBC in the presence of the whole serum IgG. After fractionation of 100-ml AB serum samples on a 10-ml melibiose-Sepharose column, the anti-Gal titer decreased in the effluent from 1:800 to 1:200, suggesting that 70–80% of the antibody activity was retained on the column. The specificity of the column absorbing the antibody was demonstrated by the finding that anti-Rh titer in one of the sera tested was not altered after fractionation (Table I). The antibody obtained after specific elution with 0.5 M galactose, passage through the protein A column, and adsorption on VCN-treated RBC, was found to produce 100% rosettes with RabRBC at a titer of 1:500 decreasing to 10% rosettes at the endpoint ranging between 1:1600 and 1:6400. The anti-galactosyl specificity of the antibody is described below (Table II). The antibody preparation directly agglutinated RabRBC at titers ranging between 1:64 and 1:256, depending on the donor of the AB serum. Sepharose column without melibiose did not bind antibody.

The IgG nature of the antibody obtained after the protein A column was further confirmed by the single identical precipitin line obtained in Ouchterlony double immunodiffusion assay against broad spectrum rabbit anti-human Ig and

TABLE I  
*Anti-Gal Reactivity in AB Sera and in Eluates After Affinity Chromatography*

Antibody source	Assay for antibody reactivity		
	Rosetting antiglobulin test with RabRBC	Hemagglutination with RabRBC	Rosetting antiglobulin test with Rh <sup>+</sup> RBC
AB serum	1:800 to 1:1,600*	ND	1:400†
Effluent AB serum from melibiose-Sepharose column	1:200 to 1:400	ND	1:400†
Anti-Gal eluted from melibiose-Sepharose column	1:1,600 to 1:6,400	1:64 to 1:256	No reactivity
Effluent AB serum from Sepharose column	1:800 to 1:1,600	ND	ND

ND, not done.

\* Endpoint of rosetting antiglobulin test defined as the antibody titer yielding >10% rosettes with K562 cells.

† Performed with AB serum from an individual containing anti-Rh antibodies.

TABLE II  
*Inhibition of the Interaction between the Anti-Gal IgG and Rabbit RBC, by Various Carbohydrates*

Test substance	Rosette formation		Inhibition of RabRBC agglutination*
	Percentage of rosettes at 100 mM carbohydrate	Carbohydrate concentration inhibiting 50% rosette formation	
	%	mM	mM
Control	100	—	—
D-Galactose	0	2	6
D-Glucose	85	—	—
D-Fructose	95	—	—
D-Fucose	10	12	25
L-Fucose	100	—	—
D-Mannose	100	—	—
α-Methyl-galactoside	0	0.7	1.5
β-Methyl-galactoside	10	25	50
α-Methyl-glucoside	100	—	—
N-Acetyl-D-galactosamine	95	—	—
N-Acetyl-D-glucosamine	95	—	—
β-Galactosyl-glucoside (lactose)	85	—	—
α-Galactosyl-glucoside (melibiose)	0	0.7	1.5
Sucrose	100	—	—
β-Galactosyl-thiogalactoside	100	—	—
β-Galactosyl-arabinoside	80	—	—
α-Galactosyl-α-galactosyl-α-glucosyl-β-fructose (stachyose)	0	1.5	3

\* The assay was carried out in anti-Gal concentration yielding two agglutination units.

the specific rabbit anti-human IgG serum. A Mancini radial immunodiffusion assay with the isolated antibody indicated that the antibody concentration within the serum ranged from 30 to 70  $\mu\text{g/ml}$ . Isoelectric focusing followed by immune fixation with anti-IgG antibodies showed the isolated anti-Gal to be a polyclonal antibody with pI values ranging from 4.0 to 8.5.

The specific interaction of the anti-Gal with  $\alpha$ -galactosyl residues on the RabRBC was shown by the rosetting antiglobulin test as well as by hemagglutination (Table II). Galactose at a concentration of 100 mM completely inhibited binding of the anti-Gal to the RabRBC and formation of rosettes, whereas 50% rosette inhibition was observed by a galactose concentration as low as 2 mM. Agglutination of the RabRBC by the antibody was noted at a galactose concentration not higher than 6 mM. The  $\alpha$ -galactosyl-containing carbohydrates melibiose, stachyose, and  $\alpha$ -methyl-galactoside inhibited the anti-Gal reactivity more potently than did galactose. In contrast,  $\beta$ -galactosyl-containing disaccharides did not affect the binding even at 100 mM. Accordingly,  $\beta$ -methyl-galactoside was 30-fold less effective than  $\alpha$ -methyl-galactoside in inhibiting anti-Gal reactivity.

Other carbohydrates tested, with the exception of D-fucose, failed to inhibit the binding of the anti-Gal to RabRBC even at the concentration of 100 mM. The capacity of D-fucose to partially inhibit the anti-Gal reactivity is probably due to the identical arrangement of hydrogen and hydroxyl groups at positions C-2, C-3, and C-4 as in D-galactose.

**Binding of Anti-Gal to Human RBC.** Since anti-Gal is present in high titers in normal sera, it is not surprising that the antibody does not bind to freshly isolated human RBC (Table III). Proteolytic treatment of human RBC by 0.1% pronase for 60 min at 37°C resulted in extensive binding of the anti-Gal, as indicated by the high proportion of rosettes obtained after incubation of treated RBC with the antibody. This interaction was readily inhibited by galactose and  $\alpha$ -galactosyl-containing carbohydrates, but not by  $\beta$ -galactosyl-containing carbohydrates, fructose, glucose, or mannose (not shown). Furthermore, incubation of the pronase-

TABLE III  
*Anti-Gal Binding by Various Human RBC Populations*

RBC source	Enzymatic treatment	Percentage of rosettes
		%
1. Normal RBC	None	3 $\pm$ 0.6*
2. Normal RBC	Pronase	100
3. Normal RBC	Pronase followed by $\alpha$ -galactosidase	7 $\pm$ 1.3
4. Normal RBC	Pronase followed by $\beta$ -galactosidase	100
5. Normal young RBC <sup>†</sup> (density <1.08 g/ml)	None	5 $\pm$ 1.5
6. Normal senescent RBC <sup>†</sup> (density >1.11 g/ml)	None	56 $\pm$ 7.6
7. Thalassemic RBC	None	72 $\pm$ 8.3

\* Mean  $\pm$  SE of results obtained with the RBC of 8–10 individuals of 0 blood group, after incubation with purified anti-Gal isolated from AB serum.

<sup>†</sup> Rosetting antiglobulin test performed with VCN-treated K562 cells.

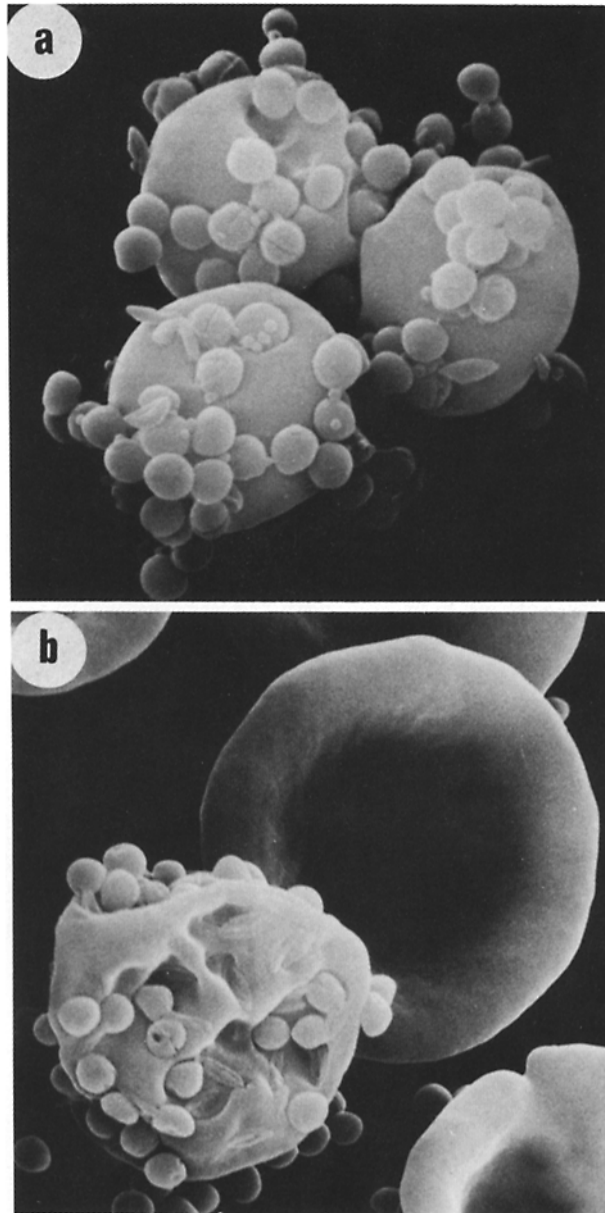


FIGURE 1. Demonstration in scanning electron microscope of the binding of *Staphylococcus aureus*-sensitized with anti-Gal IgG to various RBC. (a) Rabbit RBC (RabRBC).  $\times 6,000$ . (b) Thalassemic RBC.  $\times 6,600$ . Note the surface deformation of this pathologically aged RBC. (c) Human normal senescent RBC, with density of 1.11 g/ml, obtained from Percoll density gradient.  $\times 9,500$ .

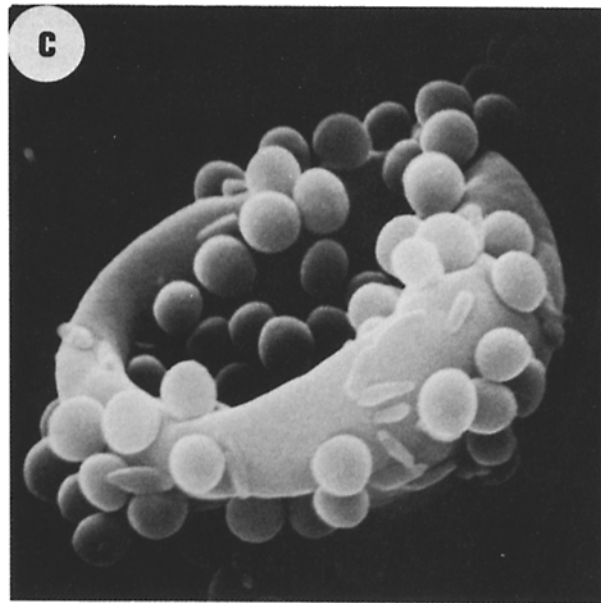


FIGURE 1c

treated RBC with  $\alpha$ -galactosidase eliminated the capacity of these RBC to interact with the anti-Gal, whereas incubation with  $\beta$ -galactosidase did not affect the capacity to bind the antibody. As expected, the anti-Gal was devoid of antibodies binding to the  $\beta$ -galactosyl units penultimate to terminal sialic acid residues, since the isolated antibody was adsorbed on VCN-treated RBC (see Materials and Methods). Accordingly, no antibody binding was detected after incubation of VCN-treated RBC with the anti-Gal.

In addition to binding to RabRBC- and pronase-treated human RBC, the anti-Gal bound to thalassemic RBC, which are known to be prematurely aged RBC (11). The binding, as assessed by the rosetting antiglobulin test (Table III), could be visualized in scanning electron microscopy using Staph A-sensitized with anti-Gal (Fig. 1b). In a previous study we have reported that thalassemic RBC bind in situ IgG antibodies with anti-galactosyl specificity (12). Thus, the thalassemic RBC were incubated with 0.1 M galactose for 60 min at 37°C, for the elution of the autologous antibodies. After this incubation, thalassemic RBC formed 5–15% rosettes with K562 cells, whereas the binding of the natural anti-Gal to these RBC resulted in 55–100% rosette formation. In accordance, the Staph A sensitized with the anti-Gal readily bound to these deformed pathological RBC (Fig. 1b). Nonsensitized Staph A did not bind to thalassemic RBC. In extension of this finding, experiments have shown that there is specific binding of anti-Gal to normal senescent, but not young, RBC (Table III, Fig. 1c). To detect the relatively small amount of anti-Gal molecules bound to normal senescent RBC, the K562 cells were pretreated with 0.04 U/ml of VCN. The VCN cleaved the sialic acid units from the K562 cells and diminished the zeta potential between the myeloid cell and the RBC. Thus, the affinity of the Fc portion of the RBC-bound IgG molecule for the Fc receptor on the K562 cell was increased. While

half of K562 cells formed rosettes with the senescent RBC following their incubation with anti-Gal, only a few rosettes were detected with the young RBC.

*Anti-Gal in Normal Sera of Donors of Various Age Groups.* The titer of anti-Gal was assessed in the sera of 300 individuals of varying ages. In >95% of the normal adult population, anti-Gal titers ranged between 1:800 and 1:1,600 irrespective of the blood group (Fig. 2). The anti-Gal, like other IgG antibodies, cross the placenta and were detected in cord blood in titers only slightly lower than those found in the maternal blood (Fig. 2). The anti-Gal titer decreases to its lowest level at the age of 3–6 mo, correlating with the decrease in the total IgG level at this age. The antibody titer was found to increase gradually thereafter, reaching the adult level by the age of 2–4 yr. The anti-Gal titer in 20 elderly individuals (70–90 yr), was found to be within the same range as that of young adults. The binding of nonpurified anti-Gal to RabRBC in titer yielding 100% rosettes was inhibited by D-galactose- and  $\alpha$ -galactosyl-containing carbohydrates, to the same extent as the inhibition observed using the purified antibody. Other carbohydrates, including  $\beta$ -galactosyl-containing disaccharides, failed to affect this interaction (not shown).

*Anti-Gal Reactivity in Immunodeficient Individuals.* The ubiquitous presence of anti-Gal in high titers throughout life implies a constant antigenic stimulation. It was thus assumed that the titration of anti-Gal may serve as a useful method for the assessment of humoral immunodeficiency disorders. The serum of an infant with Bruton type agammaglobulinemia contained only 20% of the anti-Gal reactivity observed in the serum of an age-matched healthy individual (Table IV). A similar difference was observed in the serum IgG concentration of the two infants. After administration of a  $\gamma$ -globulin preparation to the immunodeficient patient, a 10-fold increase in the anti-Gal titer was observed. Reduced activity of the anti-Gal was similarly found in acquired immunodeficiencies. The

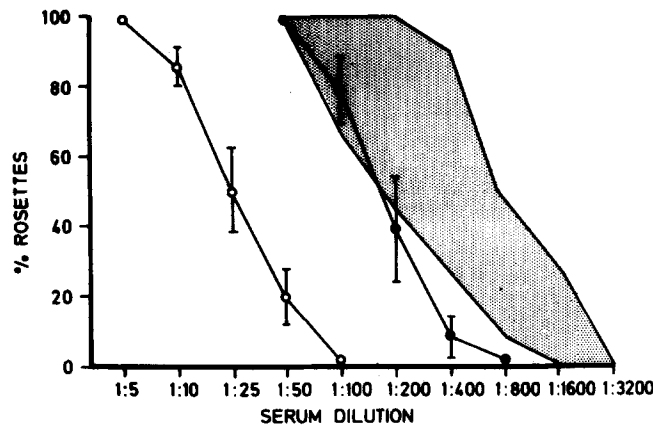


FIGURE 2. Anti-Gal reactivity in normal sera of various age groups, as assayed by the interaction with RabRBC in the rosetting antiglobulin test. Dotted area, anti-Gal reactivity in 290 out of 300 normal sera of all blood types. This group of serum donors included 20 elder individuals (70–90 yr old) and 30 youngsters (3–10 yr old). Closed circles, cord blood sera of 30 individuals (mean  $\pm$  SE). Opened circles, sera of the 3–6-mo-old children (20 individuals, mean  $\pm$  SE).



TABLE IV  
Anti-Gal Titers in the Sera of Immunodeficient Patients

Disease	Patients			Age-matched controls	
	Age	Anti-Gal titer	IgG <i>mg/ml</i>	Anti-Gal titer	IgG <i>mg/ml</i>
1. Bruton type agammaglobulinemia*	4 mo	1:10	0.4	1:50	2.2
After administration of 10 ml $\gamma$ -globulin preparation		1:100	3.8		
2. Multiple myeloma <sup>‡</sup>	65 yr	1:10	45.0	1:800	12.0
3. Chronic lymphocytic leukemia <sup>‡</sup> ( $10^5$ lymphocytes/mm <sup>3</sup> blood)	70 yr	1:25	0.7	1:800	12.0

\* Diagnosis was made due to the absence of B lymphocytes in the blood and the complete lack of IgM and IgA antibodies in the serum.

<sup>‡</sup> A representative case from a group of five patients where similar results were obtained.

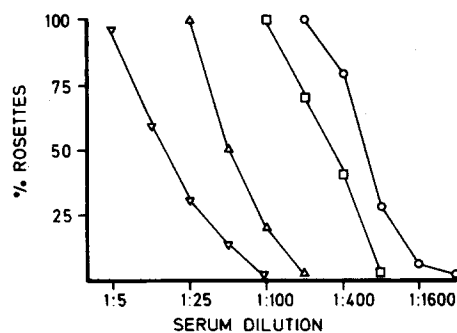


FIGURE 3. Anti-Gal reactivity in the serum of a 6-yr-old patient receiving 40 mg/d prednisolone as immunosuppressive regimen. Anti-Gal reactivity at (○) day zero; (□) 30 d of treatment; (Δ) 60 d, and (▽) 75 d.

sera of the multiple myeloma patients tested, contained four- to fivefold increase in the serum IgG concentration, however, anti-Gal titers were 40–80-fold lower than that in normal sera. This is due to the abnormal clone of IgG comprising most of the serum immunoglobulin entity. Advanced chronic lymphocytic leukemia is another type of secondary immunodeficiency state where most of the antibody-producing lymphoid tissue is replaced by the malignant lymphocytes. Accordingly, anti-Gal titer was found to be very low.

Active immune suppression caused by glucocorticoid treatment was similarly reflected in the titer of anti-Gal, as seen in the serum of a 6-yr-old patient suffering from hemophagocytic lymphohistiocytosis, receiving a daily dose of 40 mg prednisolone for the suppression of antibody production. It should be noted that a decrease of anti-Gal reactivity to 1:400 titer was observed only after 30 d of treatment, whereas additional 30 d of administration resulted in the further decrease of anti-Gal titer to 1:100 (Fig. 3). Prednisolone administration for 75 d

resulted in a decrease of the anti-Gal titer to 1:50. This titer did not alter upon continuation of the immunosuppressive treatment for additional 45 d.

### Discussion

The natural anti-Gal IgG that is described in the present study seems to be the same antibody previously described by us to be present in situ on thalassemic RBC (12). In that study we isolated the antibody through binding to RabRBC and elution by galactose. In the present study, we demonstrate the isolation of the antibody by affinity chromatography with a chemically defined antigen and show the distinct anti- $\alpha$ -galactosyl specificity of the antibody. The anti-Gal differs from all human natural antibodies with known anti-galactosyl specificity. The anti-blood group B antibody, which also displays an anti- $\alpha$ -galactosyl specificity, is present only in A type and O type individuals and is mostly of the IgM class (2). The anti-Gal is present in sera of all blood groups and is mainly an IgG antibody, as indicated by the almost similar titers observed in maternal and fetal blood. IgM antibodies do not cross the placenta. The anti-Gal differs from the anti-T antibody, which is mostly of the IgM class and interacts specifically with  $\beta$ -D-Gal(1  $\rightarrow$  4)GlcNAc residues naturally present on cortical thymocytes or exposed on other cell types after VCN treatment for removal of terminal sialic acid units (3, 13, 14). It should be stressed that, unlike anti-T antibodies, which are found in the serum of all mammals tested (3), the anti-Gal reactivity as assessed by its binding to RabRBC could be demonstrated only in human or baboon serum, but not in the serum of mice, rats, guinea pigs, and rabbits. From the structural components known to be present on RabRBC, it is most likely that the anti-Gal binds to the glycosphingolipid Gal $\alpha$ (1  $\rightarrow$  3)Gal $\beta$ (1  $\rightarrow$  3)GlcNAc $\beta$ (1  $\rightarrow$  3)Gal $\beta$ (1  $\rightarrow$  4)Glc-O-ceramide, which is found to be present on these RBC (6). The assumption that anti-Gal binds to glycolipids on RabRBC, while not yet proven, is supported by the finding that pronase-treated RabRBC bind the anti-Gal four- to eightfold more than do nontreated RabRBC (not shown). It is possible that the IgM moiety of the anti-Gal, which was not investigated in the present study, may reflect the heterophilic antibodies to RabRBC described long ago by Schiff (15) to be present in normal human sera.

The specific binding of anti-Gal to normal and pathologically senescent RBC may imply a physiological role for this antibody in the aging of human RBC. In situ binding of autologous IgG to normal senescent RBC has been reported (10, 16).

The observed selective in vitro binding of the isolated natural anti-Gal to senescent but not young RBC possibly reflects an in vivo mechanism for the labeling of the aging RBC to be recognized by the macrophages of the reticuloendothelial system.

The binding site of anti-Gal on human RBC is still under study. The extensive binding of the anti-Gal to pronase-treated RBC suggests that the antibody interacts with a glycolipid rather than a glycoprotein. The elimination of the antibody binding as a result of treatment with  $\alpha$ -galactosidase further implies the  $\alpha$ -galactosyl structure on the binding epitope. The only known glycolipid to be present on all human RBC and to bear terminal  $\alpha$ -galactosyl residues is the trihexose ceramide molecule Gal $\alpha$ (1  $\rightarrow$  4)Gal $\beta$ (1  $\rightarrow$  4)Glc-O-ceramide (17), which

is related to P<sup>k</sup> antigen (18). The possibility that trihexose ceramide is the antigenic determinant, which in course of senescence becomes accessible to anti-Gal binding through the removal of membranal proteins (including those bearing sialic acid), is currently under investigation.

The anti-Gal was found to be present in sera of individuals above the age of 4 yr in a remarkably high titer ranging between 1:800 and 1:1600. A marked decrease in the anti-Gal titer was found only in infants of 3–6 mo. This is compatible with the total decrease of maternal IgG and the initiation of self IgG synthesis. The unaltered production of the antibody throughout life implies a constant antigenic stimulation which, as in the anti-blood group antibodies, may originate in the intestinal flora (1). *E. coli* as well as shigella were reported to bear  $\alpha$ -galactosyl groups on the cell wall (19) and thus may be the source of such antigenic stimulation. Mixed agglutination experiments between the anti-Gal-sensitized Staph A and various intestinal bacterial strains may help to establish this issue.

The observed invariable high titer of the anti-Gal may have a diagnostic significance in providing information on the general function of the humoral immune system. This is demonstrated by the finding that primary and secondary humoral immune deficiencies, as well as active immune suppression, are reflected in the anti-Gal titer of the individual patient. The assessment of immune reactivity by determination of the anti-Gal titer has the advantage over the commonly used titration of anti-blood group antibodies, since it is present in all individuals. In addition, blood group antibodies decrease with age (20).

Further investigation of the natural anti-Gal antibody may not only provide clues to the mechanism of senescence of RBC, but may also prove that this antibody takes part in the nonspecific amplification of local immune responses, since the trihexose ceramides are lipids present in membranes of a large amount of cells including fibroblasts (17), epithelial and kidney cells (21, 22). These cells, which normally are not exposed to serum antibodies due to physiological barriers, may be found to bind the antibody following local inflammatory reactions resulting from primary specific immune reactions.

Additional study of this unique human natural IgG antibody may thus prove to be of immunological theoretical as well as practical interest.

### Summary

A new natural anti- $\alpha$ -galactosyl IgG antibody (anti-Gal) was found to be present in high titer in the serum of every normal individual studied. The antibody was isolated by affinity chromatography on a melibiose-Sepharose column. The reactivity of the antibody was assessed by its interaction with  $\alpha$ -galactosyl residues on rabbit erythrocytes (RabRBC). The specificity was determined by inhibition experiments with various carbohydrates. The anti-Gal interacts with  $\alpha$ -galactosyl residues, possibly on glycolipids of human RBC (HuRBC), after removal of membrane proteins by treatment with pronase. In addition, the anti-Gal bind specifically to normal and pathologically senescent HuRBC, suggesting a physiological role for this natural antibody in the aging of RBC. The ubiquitous presence of anti-Gal in high titers throughout life implies a constant antigenic stimulation. In addition to the theoretical interest in the antibody, the study of

the anti-Gal reactivity seems to bear immunodiagnostic significance. Decrease in the antibody titer was found to reflect humoral immunodeficiency disorders.

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