Glycoconjugate Journal (1998) **15:** 93–99

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Characterization of a polyclonal anti-Gal*α*1-3Gal antibody from chicken

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A polyclonal antibody was raised against the Gal*α*1-3Gal carbohydrate epitope, which is expressed by all mammals (except man and the closest primate species) by immunizing hens with rabbit erythrocyte membranes. IgY was isolated from egg yolks, and affinity-purified on a Gal*α*1-3Gal-Synsorb column. Two percent of the initial IgY fraction was recovered. The specificity of the affinity-purified antibody was characterized by: absorption with human, rabbit and pig erythrocytes; by using Synsorb columns; by inhibition with different saccharides; and by immunostaining of glycolipids separated on thin layer chromatograms. A weak reactivity was found toward blood group B or blood group P^k determinant, depending on the assay system used. Such reactivities were abolished after absorption by the appropriate sorbents, yielding a polyclonal anti-Gal*α*1-3Gal antibody with narrow specificity.

***Keywords*: anti-Gal antibody; glycolipids; IgY; xenoantigen**

Introduction

Most of the high titre natural antibodies identified in man are directed against carbohydrate antigens, such as blood group A (GalNAcα1-3(Fucα1-2)Gal*β*-), blood group B (Galα1-3(Fucα1-2)Gal*β*-), or T (Gal*β*1-3GalNAcα1-) determinants. Among them, antibodies termed 'anti-Gal' or 'anti-Galα1-3Gal' are directed against the afucoB epitope (Galα1-3Gal*β*1-4GlcNAc) (Table 1) which is expressed by all mammals except man, apes, and Old World monkeys [1, 2]. A defect in the biosynthesis of this epitope results from the inactivation, by frameshift mutations, of the α-3-galactosyltransferase gene, which is otherwise very similar to that of cow and mouse [3–6]. Consequently, most individuals of a species which do not express the afucoB epitope have naturally occuring anti-Galα1-3Gal antibodies [7]. These antibodies are probably raised by a constant stimulation of the immune system by bacteria residing in the digestive tract [8]. Alternatively, they might be directed against non-human pathogenic viruses that 'borrow' the afucoB epitope from their animal hosts [9].

However, human natural anti-Galα1-3Gal antibodies are poor analytical reagents. The best way to obtain potent anti-Galα1-3Gal antibodies is to immunize individuals of a species not expressing the immunogen. Besides man and monkeys, birds, and especially chickens, are also devoid of Galα1-3Gal expression [2]. Therefore, we chose to raise antibodies in hens by immunizing them with rabbit red blood cell membranes that are known to contain several glycolipids carrying the afucoB epitope at the non-reducing end of carbohydrate chains [10]. As IgY, the major serum antibody of chickens, is highly concentrated in egg yolks, antibodies were isolated from this source, and affinity-purified on Galα1-3Gal Synsorb beads.

Materials and methods

Immunization protocol

Membranes were obtained from rabbit red blood cells according to Dodge *et al.* [11]. Membrane suspension (3 mg protein ml^{-1}) was mixed with an equal volume of complete Freund's adjuvant. One millilitre was subcutaneously inoculated into 22-week-old Warren hens at 2–4 sites. A second 1 ml injection was done subcutaneously 2 weeks after the first. Subsequently, 0.5 ml of membrane suspension without Freund's adjuvant was injected intramuscularly on days 30 and 40. Hens were sacrificed on day 70 after the first injection.

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0282–0080

Table 1. Glycosphingolipids of human and rabbit red blood cells, and of pig kidney cortex. The term afuco-B was used in order to stress the structural similarity with the blood group B determinant as well as the lack of fucose. The generic terms B or afuco-B are followed by the number of glycosyl residues in the molecule

Blood group glycosphingolipids of human erythrocytes	
Gal*α*1-4Gal*β*1-4Glc*β*1-Cer	Gb_3Cer (P^k)
GalNAc*β*1-3Gal*α*1-4Gal*β*1-4Glc*β*1-Cer	Gb_4Cer (P)
Gal*α*1-4Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer	(P_1)
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer Fuc*α*1-2	B-6
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4 GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer Fuc*α*1-2	B-8
Glycosphingolipids of rabbit erythrocytes[a] and pig kidney[b]	
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer	afucoB-5
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer Fuc*α*1-3	Gal*α*3Le^x-6
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4 GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer	afucoB-7
Gal*α*1-3Gal*β*1-4GlcNAc*β*1-6 Gal*β*1-4GlcNAc*β*1-3Gal*β*1-4Glc*β*1-Cer Gal*α*1-3Gal*β*1-4GlcNAc*β*1-3	afucoB-10

[a]See reference [10].
[b]See reference [19].

Purification of IgY

Hens began laying eggs at 25 weeks of age. Eggs were collected for 40 days from two hens immunized with rabbit red blood cell membranes. IgYs were prepared from egg yolks by polyethylene glycol 6000 fractionation according to Polson *et al.* [12]. The final pellets of precipitated IgY were dissolved in a volume of PBS half that of the original yolks. Polyacrylamide gel electrophoresis indicated that at least 85% of the proteins obtained were pure IgY.

Affinity purification of IgY

IgY solutions isolated from egg yolk were applied to a 1 ml column of Synsorb 14 (Chembiomed) carrying the Galα1-3Galβ disaccharide. Retained antibody was eluted successively with 1% and 2% NH_4OH (2 ml each), and immediately neutralized with 0.5 M KH_2PO_4. Protein concentrations were assayed by the method of Lowry *et al.* [13].

Absorption and inhibition experiments

For absorption tests, affinity-purified antibody was serially diluted in 1% bovine serum albumin in phosphate buffered saline (PBS/BSA) (100 µl) and incubated with packed red blood cells (100 µl) for 30 min at room temperature. Supernatants were assayed for anti-Galα1-3Gal activity by enzyme linked immunosorbent assay (ELISA). Alternatively, antibody diluted in PBS/BSA (1.5 ml) was applied to different Synsorb beads packed in minicolumns (0.2 ml) made of 1 ml plastic syringes. The first 0.5 ml were discarded. The subsequent eluants were assayed for anti-Galα1-3Gal activity. For inhibition experiments, antibody diluted in PBS/BSA was incubated for 1 h in the presence of various concentrations of sugars. Galα1-3Gal-terminated di- and trisaccharides were obtained from Dextra Laboratories (Reading, UK). Residual activities were measured by ELISA.

Specific ELISA assay

In order to quantitate the immunological activity of antibody against Galα1-3Gal epitopes, ELISA was performed using purified neutral glycosphingolipids from rabbit erythrocyte membranes [14]. Glycolipids were dissolved in methanol:water (80:20, (v/v); 20 µg/ml), and the solution was aliquoted onto Dynatech Immulon-1 microtitration plates (50 µl/well). The solvent was allowed to evaporate for 1 h at room temperature. A PBS/BSA solution was added for 1 h in order to block non-specific sites. Antibody solutions at the appropriate dilution were added to wells. After incubation, antibody solutions were discarded, and wells were washed five times with PBS. Peroxidase-conjugated rabbit anti-chicken IgY antibody (Sigma) diluted 1:10 000 was added and incubated for 30 min. Wells were washed five times with PBS. Enzyme activity was detected with ABTS (Boehringer Mannheim) as substrate. Intensity of the colour reaction was measured at 405 nm after incubation for 30–60 min at 37 °C.

Glycosphingolipid purification

Lipids were extracted from lyophilized red blood cell membranes according to previously published procedures [15]. Glycosphingolipids were purified as acetylated derivatives

on Florisil columns according to Saito and Hakomori [16]. Deacetylated and dialysed total glycolipid fractions were submitted to DEAE–Sephadex chromatography in order to separate neutral and acidic glycolipids [17]. Neutral glycolipids were analysed by high performance thin layer chromatography (HPTLC) on silica gel-coated aluminium-backed plates (Merck).

Immunodetection of glycolipids on thin layer plates

HPTLC chromatograms were plasticized by dipping plates in a 0.3% solution of polyisobutylmethacrylate (Plexigum P 28, Röhm) in hexane-diethylether (1:1) for 1 min [18]. After drying overnight at room temperature, plates were sprayed with PBS/BSA and immersed in the same solution for 1 h. Plates were removed from the solution and drained. They were overlaid with a solution of hen IgY in PBS/BSA. After 1 h at room temperature, the antibody solution was discarded and plates were washed five times with PBS. Plates were overlaid with a solution of peroxidase-conjugated anti-chicken IgY antibody diluted 1:500 in PBS/BSA. After 1 h incubation and five washes, glycolipids were revealed by chemiluminescence with the ECL Western blotting system (Amersham), and short exposure to a blue light-sensitive autoradiography film (Hyperfilm ECL, Amersham). Glycolipids were visualized chemically after immunostaining by spraying the plate with a 5% phenol solution in 1 M sulphuric acid.

Results

Kinetics of immunization

Anti-Galα1-3Gal activity of IgY extracted from egg yolks (IgY extracts) was assayed by ELISA using glycolipids of rabbit red blood cell membranes as ligand adsorbed on microtitration plates [14]. Anti-Galα1-3Gal antibody titre was already high in eggs collected 3 weeks after the first injection of rabbit red blood cell membranes, continued increasing till the fourth injection, and then decreased slowly (Figure 1). Anti-Galα1-3Gal titre in non-immunized hens was less than 3% of the maximum titre obtained after immunization.

Affinity purification of anti-Galα1-3Gal IgY

IgY extracts were applied to a Galα1-3Gal Synsorb column in order to select antibodies specific for this epitope among anti-rabbit red cell antibodies. As the column was overloaded at the first passage, the flow-through fraction was applied again after elution of the retained antibodies. Several rounds of fixation and elution were performed until the flow-through did not contain any anti-Galα1-3Gal activity as measured by ELISA. Retained and eluted fractions were pooled. They contained 1.9–2.6% of the IgY extract proteins which were applied on the column (four experiments). All further experiments were done with IgY purified on Galα1-3Gal Synsorb and are referred to as affinity-purified IgY.

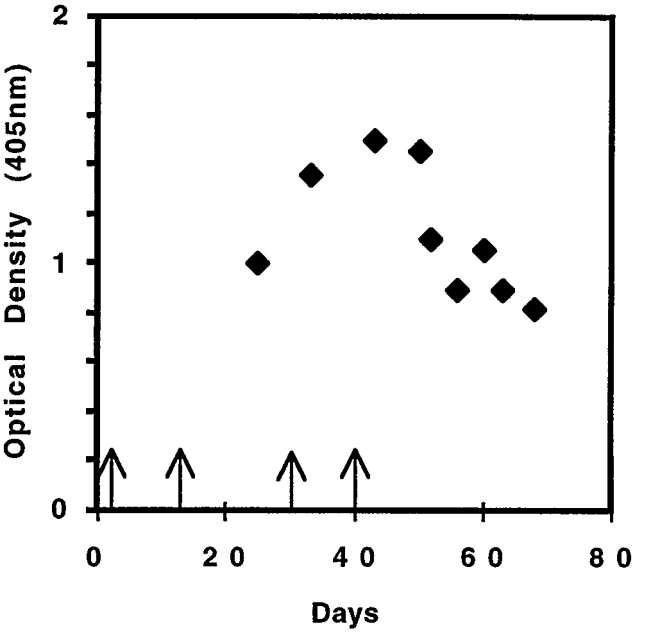

Figure 1. Kinetics of appearance of anti-Galα1-3Gal activity of IgY isolated from egg yolk. Days on which antigen was injected are indicated by arrows. Activity is indicated as OD_{405} measured by ELISA on rabbit erythrocyte glycolipids with antibody solutions at constant protein concentration (10 μg/ml). IgY of non-immunized hens had an OD $\leqslant$ 0.05

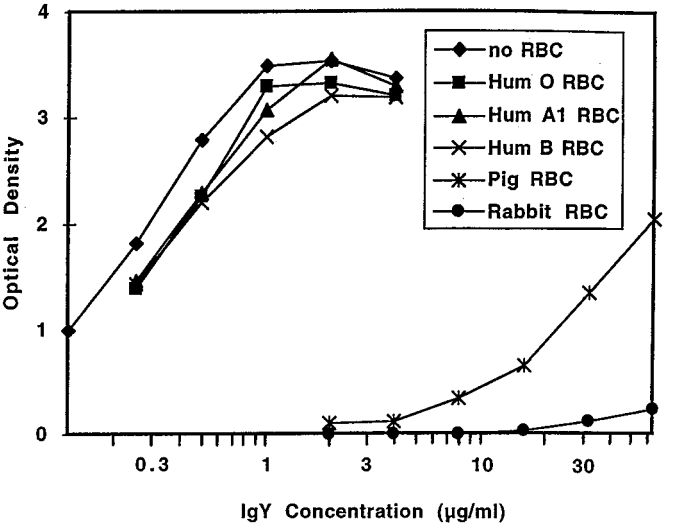

Figure 2. Activity of affinity-purified IgY after incubation with erythrocytes. Antibody solutions at the indicated concentrations were incubated with an equal volume of packed human erythrocytes of blood groups A1, B, and O, and also with pig and rabbit erythrocytes. Anti-Galα1-3Gal activity was measured in the supernatants by ELISA. RBC, red blood cells erythrocytes.

Absorption of affinity-purified IgY by erythrocytes

Affinity-purified IgY was better absorbed by rabbit than by pig erythrocytes, and bound weekly to human erythrocytes (Figure 2). Half maximal absorbance in the ELISA

was reached at 0.25 μg ml^{-1} of unabsorbed antibody, 0.35 μg ml^{-1} of antibody preincubated with human erythrocytes, 50 μg ml^{-1} of IgY preincubated with pig erythrocytes, and at more than 500 μg ml^{-1} after preincubation with rabbit erythrocytes. The concentrations for half maximal absorbance between native antibodies and antibodies preincubated with human erythrocytes were small and similar for A, B, or O group erythrocytes.

Absorption of affinity-purified IgY on Synsorb columns

When a diluted solution of affinity-purified IgY was applied to different Synsorb columns (Figure 3), it was maximally absorbed by Galα1-3Galβ1-4GlcNAcβ- and Galα1-3Galβ-Synsorb, the former being the most efficient (Figure 3, lane 2). Synsorbs carrying Galα1-4Galβ were also able to retain some of the antibody (Figure 3, lanes 4–6). Two consecutive absorptions by Galα1-4Galβ1-4GlcNAcβ- Synsorb (Figure 3, lanes 6–8) were sufficient to remove the portion of Galα1-3Gal antibodies which cross-reacted with Galα1-4Galβ1-4GlcNAc. The N-acetyllactosamine-Synsorb did not retain any antibody (Figure 3, lane 3).

Inhibition by soluble mono-, di- and trisaccharides

Affinity-purified IgY were maximally inhibited by the trisaccharide Galα1-3Galβ1-4GlcNAc (Figure 4). This was expected since this determinant is found in the immunogen (Table 1). IgY were inhibited slightly less efficiently by the trisaccharide Galα1-3Galβ1-4Gal and the disaccharide Galα1-3Gal. Melibiose, Galα1-6Glc, was a weak inhibitor, and free galactose was even weaker.

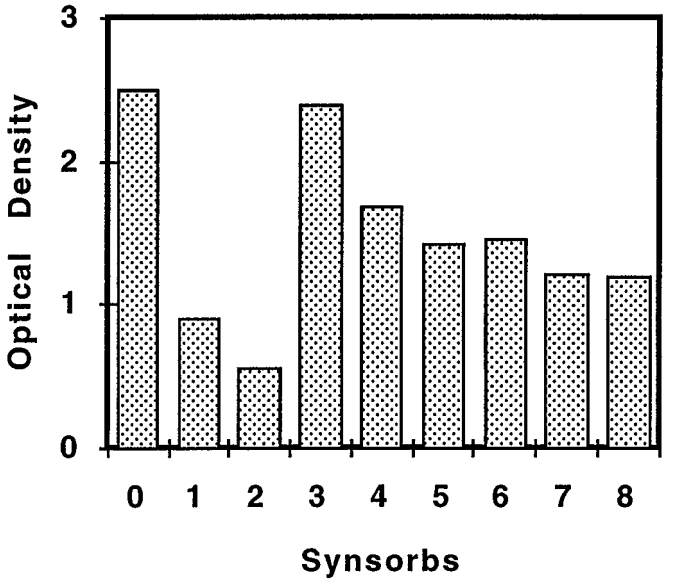

Figure 3. Activity of affinity-purified IgY after absorption by Synsorb columns. Antibody solution (250 ng/ml, 1.5 ml) was loaded onto 0.2 ml columns of: (1) Gal*α*1-3Gal*β*-; (2) Gal*α*1-3Gal*β*1-4GlcNAc*β*-; (3) Gal*β*1-4GlcNAc*β*-; (4) Gal*α*1-4Gal*β*1-4Glc*β*-; (5) Gal*α*1-4Gal*β*-; (6) Gal*α*1-4Gal*β*1-4GlcNAc*β*-Synsorb. The flow through fraction of Gal*α*1-4Gal*β*1-4GlcNAc*β*-Synsorb (No. 6) was applied a second (No. 7) and a third time (No. 8) to the same column. The activity of flow-through fractions was measured by ELISA. 0; activity of the initial solution of affinity-purified antibodies.

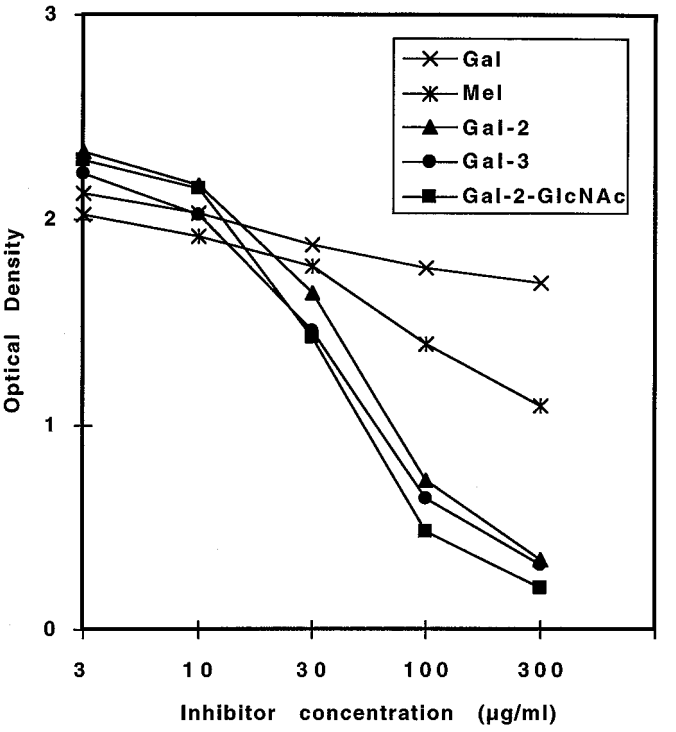

Figure 4. Activity of affinity-purified IgY in the presence of soluble saccharides. IgY (175 ng/ml) was incubated with galactose (Gal), melibiose (Mel), Gal*α*1-3Gal (Gal-2), Gal*α*1-3Gal*β*1-4Gal (Gal-3), and Gal*α*1-3Gal*β*1-4GlcNAc (Gal-2-GlcNAc) at the indicated concentrations. Residual activity was measured by ELISA.

Specificity of affinity-purified IgY antibodies for Galα1-3Gal-terminated glycolipids

Affinity-purified IgY was overlaid on chromatograms of glycolipids of rabbit and human erythrocytes, and porcine kidney cortex (Figure 5, panel A). Several glycolipids of rabbit erythrocytes were labelled, some of which have been structurally identified by Egge *et al.* [10] as the Galα1-3Gal-terminated penta-, hepta-, and decaglycosylceramides of the neolactoseries (Table 1, afucoB-5, 7, 10). IgY labelled several glycolipids of pig kidney: the afucoB glycolipids detected in rabbit erythrocytes, and, in addition, glycolipids that have been demonstrated to carry the Galα3Le^x determinant [19]. IgY was able to label blood group B-active glycolipids on thin layer chromatograms although at a weaker intensity than afucoB structures (Figure 5, panel A, lane 2). IgY did not detect glycolipids of blood group O and A individuals (data not shown). Blood group B glycolipids were no longer labelled by IgY which had been pre-absorbed by blood

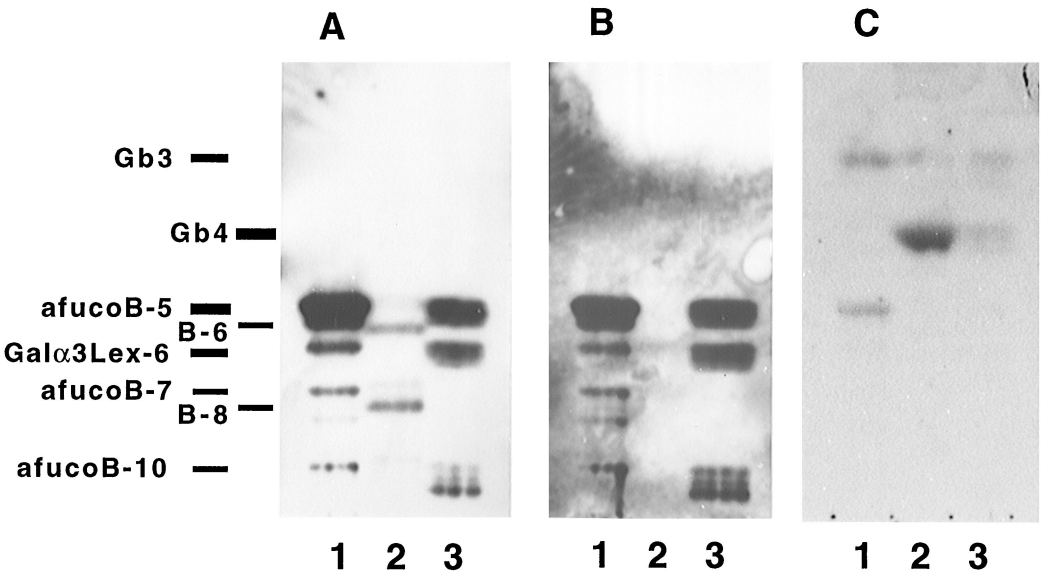

Figure 5. High performance thin layer chromatography of glycosphingolipids and immunostaining with affinity-purified IgY. Glycolipids of rabbit erythrocyte membranes (lane 1), blood group P_1 and B human erythrocyte membranes (lane 2), and pig kidney cortex (lane 3). Panel A, immunostaining with affinity-purified IgY (1 μg/ml). Panel B, immuno staining with affinity-purified IgY which have been absorbed by human blood group B erythrocytes. Panel C, chemical detection of the glycolipids in chromatogram A. On the left margin, identity of glycolipids, the structures of which are listed in Table 1. Glycolipids were analysed in the solvent chloroform/methanol/water (60:35:8).

group B human erythrocytes (Figure 5, panel B, lane 2). Labelling with IgY antibody was abolished after hydrolysis of glycolipids with green coffee bean α-galactosidase, demonstrating that antibodies recognized exclusively Galα-terminated determinants (data not shown).

Chemical detection after immunostaining (Figure 5, panel C) detected globotriaosylceramide (Table 1, P^k antigen) and afucoB-5 in rabbit erythrocyte, and globotetraosylceramide (P antigen) in human erythrocyte glycolipids. P^k and P antigens, which are also the major glycolipids of pig kidney, were not detected by IgY antibodies in any of the samples (Figure 5, panel A and B). Isoglobotriaosyceramide, Galα1-3Galβ1-4Glcβ1-Cer, generated from the rat stomach isogloboside [20] by β-hexosaminidase hydrolysis, was labelled (data not shown). Blood group P_1 antigen, an isomer of afucoB-5 (Table 1), which migrates above B-6 [21], was not labelled by IgY. Therefore, on thin layer chromatograms, affinity-purified IgY reacted primarily with Galα1-3Gal- and Galα3Le^x-terminated glycolipids, and, to a lesser extent, with blood group B-active glycolipids, but not with Galα1-4Gal-terminated glycolipids.

Discussion

IgY antibodies are typical low molecular weight serum antibodies of oviparous vertebrates such as birds, reptiles and amphibians. IgY (molecular mass of 180 kDa) has two heavy and two light chains, and consequently, two antigen-binding sites. It is considered an evolutionary ancestor both of mammalian IgG and IgE [22]. IgY is transported at high concentration into the egg yolk, providing passive immunity for the embryo in a manner similar to the transfer of IgG from mother to foetus through the placenta in mammals.

Immunization of hens and recovery of IgY from egg yolk has been recognized as a way to produce large quantities of these antibodies [23, 24]. In the field of carbohydrate antigens, Fujii *et al.* [25] immunized hens with purified gangliosides and recovered sera with activity against gangliosides containing N-glycolylneuraminic acid, a sialic acid absent in man and birds. Yasawa *et al.* [26] obtained anti-blood group H antibody from eggs of hens immunized with human blood group O erythrocytes.

In the present work, hens were immunized with rabbit erythrocyte membranes in order to obtain anti-Galα1-3Gal antibodies. Although egg yolk antibodies were affinity-purified on Synsorb carrying the disaccharide Galα1-3Gal, it was clear that their major target was the trisaccharide Galα1-3Galβ1-4GlcNAc (Table 2). IgY antibodies also recognized the afucoB epitope fucosylated on C3 of the N-acetylglucosamine residue. This property led to the detection and characterization of glycolipids carrying the Galα-3Le^x determinant [19]. IgY contained an anti-B activity that was absorbed by blood group B erythrocytes (Figure 5). This finding was in agreement with previous data showing that chicken can produce anti-B antibodies [27]. As the proportion of anti-Galα1-3Gal activity absorbed by

Table 2. Binding to different carbohydrate structures of IgY affinity-purified on Galα1-3Galβ-Synsorb

Structure	*Synsorb*	*Free saccharide*	*Red blood cells*	*HPTLC of glycolipids*
Galα1-3Gal	+++	+++		
Galα1-3Galβ1-4Gal	+++			
Galα1-3Galβ1-4Glc				+++
Galα1-3Galβ1-4GlcNAc (afucoB)	+++	+++	+++	+++
Galα1-3Galβ1-4(Fucα1-3)GlcNAc (Galα3Le^x)				+++
Galα1-3(Fucα1-2)Galβ1-4GlcNAc (B)			(+)	+
Galβ1-4GlcNAc	–			
Galα1-4Gal	++			
Galα1-4Galβ1-4Glc (P^k)	++		–	–
Galα1-4Galβ1-4GlcNAc (P$_1$)	++		–	–
Galα1-6Glc		+		
Galα/β		(+)		

B erythrocytes was very small (Figure 2), it was concluded that the antibodies responsible for the anti-B activity were quantitatively minor but displayed a high affinity for their target.

On the contrary, the large part of the activity that was lost upon absorption on P_1 and P^k Synsorb columns was of low affinity as neither anti-P^k nor anti-P_1 activity could be detected upon immunostaining of glycolipids on thin layer chromatograms, a technique that involves overlay and multiple washing steps. It was also found that free galactose, a mixture of α and β anomers at equilibrium, and melibiose (Galα1-6Glc) were weak inhibitors. Therefore, IgY affinity-purified on Galα1-3Gal Synsorb contained antibodies that have affinities for other Galα-terminated structures. The cross-reactive antibodies could be removed by absorption whenever a narrower specificity was needed.

The ubiquitous distribution of the afucoB epitope in mammals makes it difficult to produce a monoclonal [14] or polyclonal antibody in species other than primates. Immunization of hens, which lack Galα1-3Galβ1-4GlcNAc- [7], with rabbit erythrocyte membranes, which are rich in this determinant [10], allowed the recovery from egg yolks of large quantities of IgY. Over a 1 month period, a hen laying a minimum of 20 eggs produced 1 g of IgY and 20 mg of anti-Galα1-3Gal antibodies. This is a cheap and abundant source of antibodies which, as an analytical reagent, could complement the limited murine monoclonal antibodies [14] or lectins [28] available.

Acknowledgements

The authors thank Dr Rafael Oriol for supplying some of the Synsorbs. This work was supported by the Région des Pays de la Loire, and the Fondation Transvie (Fellowships to C.R.).

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Received 29 September 1997