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Presence of natural anti-Galα1-4GalNAcβ1-3Gal (anti-NOR) antibodies in animal sera

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Abstract Rare polyagglutinable NOR erythrocytes contain unusual globoside extention products terminating with a Galα1-4GalNAcβ1-3Gal- unit. This trisaccharide epitope is recognized by recently characterized antibodies naturally occurring in most human sera (*Duk et al., Glycobiology, 15, 109, 2005*). These antibodies represent two major types of fine specificity. All these antibodies are most strongly inhibited by Galα1-4GalNAcβ1-3Gal (NOR-tri), and weakly by Galα1-4Gal. However, the type 1 antibodies are strongly inhibited by Galα1-4Galβ1-3Gal-R and weakly by Galα1-4GalNAc, while the type 2 antibodies show the opposite reactivities with these two oligosaccharides. Similar antibodies have now been found in horse, rabbit and pig sera. The antibodies were purified from animal sera by affinity chromatography on Galα1-4GalNAcβ1-3Gal-human serum albumin(HSA)-Sepharose 4B conjugate. The specificity of the antibodies was determined by binding to ELISA plates coated with several α-galactosylated oligosaccharide-polyacrylamide (PAA) or -HSA conjugates and by inhibition with synthetic oligosaccharides. The purified antibodies bound specifically to conjugates containing NOR-tri. The inhibition of binding showed that the animal sera also contain two types of anti-NOR antibodies: type 2 was found in the horse serum, and a mixture of both types was present in rabbit and pig serum. These results indicate that anti-NOR, a new and distinct kind of anti-αGal antibody, are present in animal sera and show similar specificties and diversity as their counterparts found in human sera.

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Introduction

Oligosaccharide chains terminating with an α-galactose residue are limited in humans to blood group B antigens and blood group P system-related glycosphingolipids. By consequence, human sera contain various natural antibodies directed against α-galactosylated oligosaccharide chains. Besides the blood group anti-B isoantibodies commonly present in B-negative persons and the anti-P1 antibodies occurring in the sera of some P2 individuals, there are several antibodies commonly present which are directed against structures absent in humans. The best known example are anti-Galα1-3Gal antibodies recognizing epitopes present in the glycoproteins and glycolipids of mammals but absent in humans. Therefore, the anti-Galα1-3Gal antibodies are present only in human sera and do not occur in animal sera. These antibodies have been widely studied because they are a major cause of acute xenotransplant rejection [1]. They were shown to be a heterogeneous group of antibodies with recpect to their fine specificity, and other anti-αGal antibodies have been identified in human sera [2,3].

Recently, we found that rare erythrocytes with an inherited NOR characteristic [4] contain two unusual glycosphingolipids which are recognized by antibodies present in most human sera [5]. These glycolipids are globoside extension products terminating with the unique sequence Galα1-4GalNAcβ1-3Gal [6,7]. Two types of human anti-NOR antibodies were identified that are specific either to Galα1-4GalNAc/Galβ1-3Gal or to Galα1-4GalNAc [8,9]. The Galα1-4GalNAc sequence has not been found so far in mammals, but anti-NOR antibodies seem to be widespread

in animal sera. In this report, the identification of anti-NOR antibodies in horse, rabbit, and pig sera is described.

Materials and methods

Animal sera

Horse and pig sera were obtained from the Veterinary Department of the School of Agriculture in Wroclaw. Rabbit blood was withdrawn from experimental gray rabbits at our Institute. Before use, the sera were incubated for 30 min at 56°C and filtered through a Syringer polyvinylidene difluoride (0.45 μm) filter (Millipore, Bedford, MA).

Oligosaccharides and oligosaccharide conjugates

The NOR-related oligosaccharides, Galα1-4GalNAc (NOR-di), Galα1-4GalNAcβ1-3Gal (NOR-tri), and Galα1-4Galβ1-3Galβ1-4Glc-sp [$(Gal)_3Glc$], and conjugates of NOR-tri with polyacrylamide (NOR-tri-PAA) and human serum albumin (NOR-tri-HSA) were obtained by chemical syntheses [9,10]. The disaccharides Galα1-4Gal and Galα1-3Gal were purchased from Glycorex (Lund, Sweden). The conjugates of the other oligosaccharides with PAA (of M_r around 30 kDa, containing ~20% sugars) were kindly supplied by Dr. N.V. Bovin (Moscow).

Mouse monoclonal anti-NOR antibody

The antibody nor118 (IgG1) was obtained by immunization of BALB/c mice with NOR-tri-HSA. The details of immunization, cell-fusion and clone selection have been described [9].

Purification of animal anti-NOR antibodies

The antibodies from horse, rabbit and pig sera were isolated by affinity chromatography on a NOR-tri-HSA-Sepharose 4B column using a procedure applied previously to human anti-NOR antibodies [9]. Briefly, the diluted serum sample was applied to a 1-ml column which was washed with PBS (10 mM phosphate buffer/0.15 M NaCl, pH 7.4) to elute unbound proteins, then with 0.02 M galactose in PBS to elute weakly bound, less specific antibodies, and finally with 5 M guanidine hydrochloride in PBS which eluted specific anti-NOR antibodies.

Microtiter plate ELISA

The antibody binding to oligosaccharide conjugate-coated plates and inhibition of the binding with oligosaccharides were determined by previously described procedures [8,9]. Generally, after incubation of the tested antibody or antibody/inhibitor samples on the coated plate, two versions of binding detection were applied: (1) overlaying the plates with alkaline phosphatase-conjugated secondary antibody (goat anti-rabbit Ig, Dako; rabbit anti-pig Ig, Sigma) or (2) with biotinylated rabbit antibody against horse Ig and then with ExtrAvidin-alkaline phosphatase conjugate (both from Sigma). The p-nitrophenyl phosphate (Sigma 104 Phosphatase Substrate tablets) was used as an enzyme substrate.

Binding of antibodies to human erythrocyte glycolipids

The isolation of erythrocyte neutral glycolipds, high-performance thin-layer chromatography (HPTLC) on Kieselgel 60 (Merck) plates and the detection of glycolipids reacting with antibodies on the plate have been desribed [5,6]. The detection of antibody binding was performed with the same reagents as those applied for the microtiter plate ELISA, except that nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Sigma) was used as a substrate.

Results

Purification of animal anti-NOR antibodies

Immunoglobulins from horse, rabbit, and pig sera, besides the binding to NOR-tri-HSA, showed differentiated binding to other α-galactosylated synthetic glycoconjugates. Horse immunoglobulins showed the highest binding to Galα1-4GlcNAc-PAA and Galα1-6Glc-PAA, rabbit immunoglobulins bound most strongly to Galα1-3GalNAc-PAA and Galα1-4Galβ1-4GlcNAc-PAA (P1-tri-PAA), and those from pig serum to Galα1-6Glc-PAA and P1-tri-PAA (Figures 1–3). Passing the sera through the NOR-tri-HSA-Sepharose 4B column gave retention of all or most anti-NOR antibodies, while other anti-αGal antibodies were eluted with the buffer, indicating that they represent distinct antibody species, different from anti-NOR. Similarly as in the purification of human anti-NOR antibodies, the affinity column was then washed with 20 mM galactose to elute loosely bound, less specific antibodies, followed by elution of strongly bound anti-NOR antibodies with 5 M guanidine hydrochloride. In the case of horse serum, the galactose- and guanidine-eluates contained apparently specific anti-NOR antibodies (Figure 1). No rabbit antibodies were eluted with galactose, and anti-NORs were eluted with 5 M guanidine (Figure 2). Pig anti-NORs were also eluted with 5 M guanidine, but they seemed to be less specific than horse and rabbit anti-NOR because they showed a weak binding to other conjugates (Figure 3).

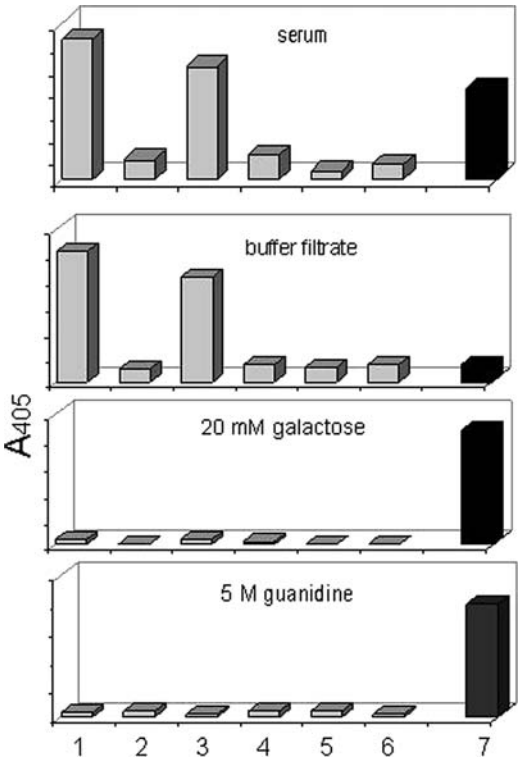

Fig. 1 Purification of horse anti-NOR antibodies on the NOR-tri-HSA-Sepharose 4B column. Horse serum (10 ml) was diluted 3 times with PBS and applied to the 1-ml affinity column, which was then washed with PBS, 20 mM galactose in PBS, and finally with 5 M guanidine hydrochloride. The serum and the column eluate samples were tested for binding to ELISA plates coated with the following conjugates: 1, Galα1-4GlcNAc-PAA; 2, Galα1-3GalNAc-PAA; 3, Galα1-6Glc-PAA; 4, Galα1-2Gal-PAA; 5, Galα1-3Gal-PAA; 6, Galα1-4Galβ1-4GlcNAc-PAA (gray bars); 7, Galα1-4GalNAcβ1-3Gal-HSA (NOR-tri-HSA, black bars). For the binding assay the serum was diluted 100 times, the buffer eluate was adjusted to the same dilution as the serum; the galactose and guanidine eluates were tested at an approx. 10-times lower dilution. The A_{405} units shown on ordinate axes coorespond to 0.1.

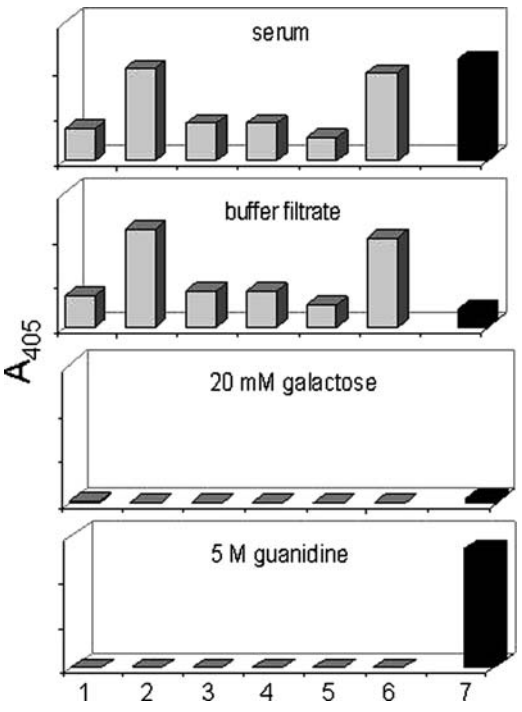

Fig. 2 Purification of rabbit anti-NOR antibodies on the NOR-tri-HSA-Sepharose 4B column. Other details as in Figure 1.

Inhibition of animal anti-NOR antibodies by oligosaccharides

The fine specificity of horse, rabbit, and pig anti-NOR antibodies was determined by inhibition of their binding to NOR-tri-PAA-coated ELISA plates with synthetic oligosaccharides and galactose (Figure 4).

Two fractions of horse anti-NOR were tested. The antibodies eluted from the affinity column with 20 mM galactose were inhibited (in a decreasing order) with Galα1-4GlNAcβ1-3Gal (NOR-tri), Galα1-4GalNAc (NOR-di), Galα1-4Gal, Galα1-4Galβ1-3Galβ1-4Glc [$(Gal)_3Glc$] and galactose. For 50% binding inhibition, an about 100-times higher concentration of galactose than of NOR-tri was required. On the other hand, the guanidine-eluted horse anti-NOR antibodies showed a high specificity to NOR-tri and NOR-di (50% inhibition at concentrations of 0.05 mM and 0.1 mM, respectively), were weakly inhibited by Galα1-4Gal

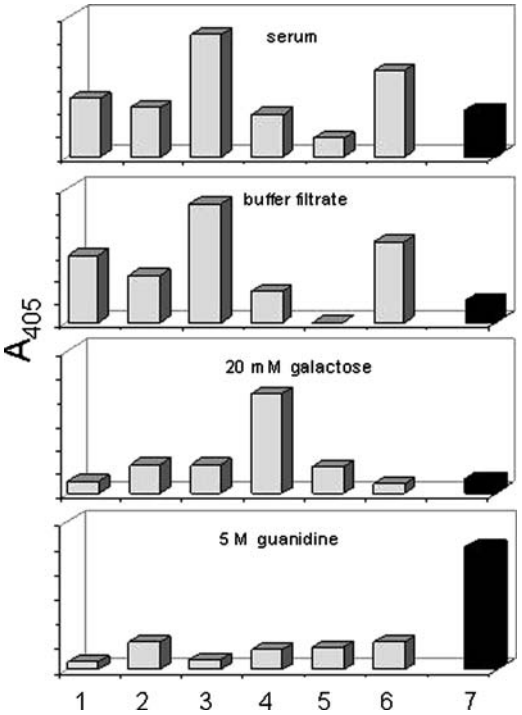

Fig. 3 Purification of pig anti-NOR antibodies on the NOR-tri-Sepharose 4B column. Other details as in Figure 1.

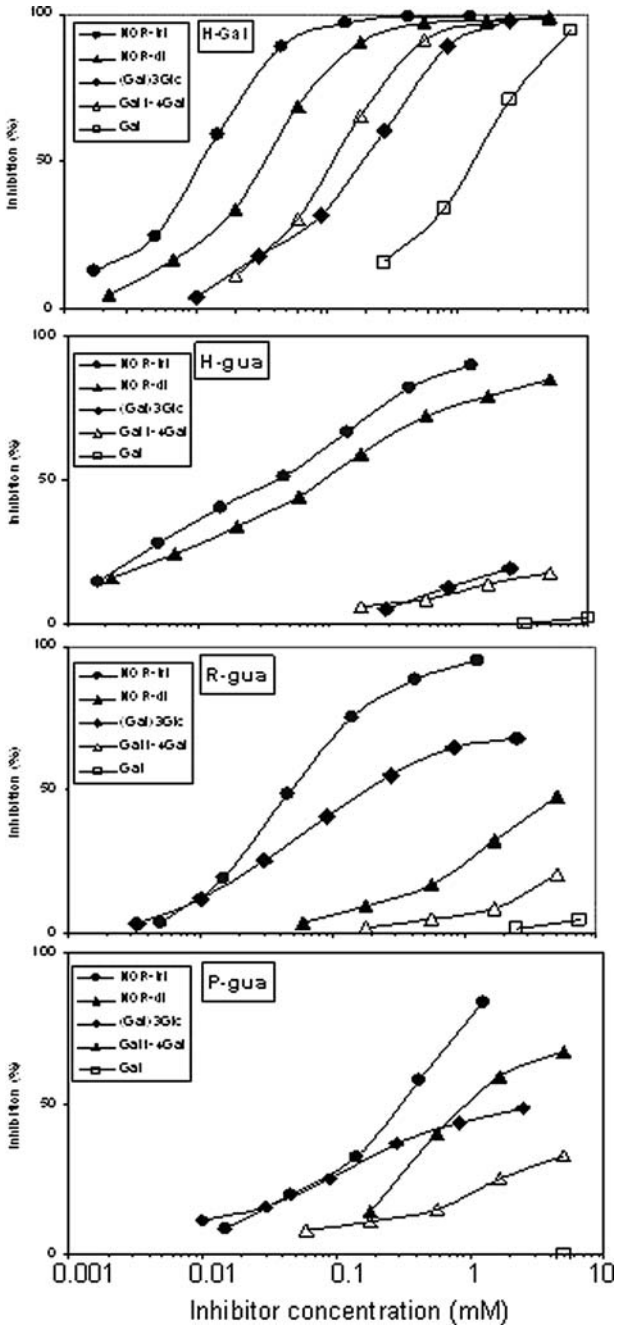

Fig. 4 Inhibition of binding of purified animal anti-NOR antibodies to NOR-tri-PAA-coated ELISA plates. H-Gal, horse antibodies eluted with galactose from the affinity column; H-gua, R-gua, and P-gua, horse, rabbit, and pig antibodies, respectively, eluted with guanidine from the affinity column. The following serially diluted inhibitors were used: Galα1-4GalNAcβ1-3Gal (NOR-tri), Galα1-4GalNAc (NOR-di), Galα1-4Galβ1-3Galβ1-4Glc [$(Gal)_3Glc$], Galα1-4Gal and galactose (Gal).

and $(Gal)_3Glc$ (around 20% inhibition at concentrations of 2.5 mM and 1.25 mM, respectively), and were not inhibited by galactose at a 10 mM concentration. These results indicated that the examined horse serum contained anti-NOR type 2 antibodies (specific to Galα1-4GalNAc and cross-reacting weakly with Galα1-4Gal-containing oligosaccharides), and less specific antibodies showing preferential reaction with NOR-tri and NOR-di, but also reacting distinctly with Galα1-4Gal and galactose.

Inhibition of rabbit and pig anti-NOR antibodies eluted with guanidine from the affinity column showed that these antibodies represent a mixture of type 1 and 2 specificities (Figure 4). The antibodies were most strongly inhibited by NOR-tri, and only a part of them was inhibited by $(Gal)_3Glc$. Moreover, the lower inhibition of pig than rabbit anti-NOR by $(Gal)_3Glc$, a strong inhibitor of type 1 anti-NOR, was accompanied by relatively stronger inhibition by NOR-di, a strong inhibitor of type 2 antibodies. Rabbit and pig anti-NOR antibodies were most weakly inhibited by Galα1-4Gal, but the inhibition of pig antibodies was relatively higher, which was in accord with the less restricted specificity of pig anti-NOR, compared with rabbit and horse antibodies. Rabbit and pig antibodies were not inhibited by galactose.

None of the studied animal anti-NOR antibodies were inhibited by Galα1-3Gal at a 5 mM concentration (not shown).

Reactivity of animal anti-NOR antibodies with NOR glycolipids

Total neutral glycosphingolipids from NOR (blood group A) and control blood group A erythrocytes were fractionated by HPTLC. Overlaying the plate with mouse monoclonal anti-NOR antibody (nor118) showed binding to two major unique glycolipids representing the globoside elongated by Galα1-4 (NOR1) or Galα1-4GalNAcβ1-3Galα1-4 (NOR2) (Figure 5). Staining the plate with orcinol showed two distinct additional bands in glycolipids from NOR erythrocytes, representing NOR1 and a glycolipid terminating with GalNAc (precursor to NOR2), which does not react with anti-NOR

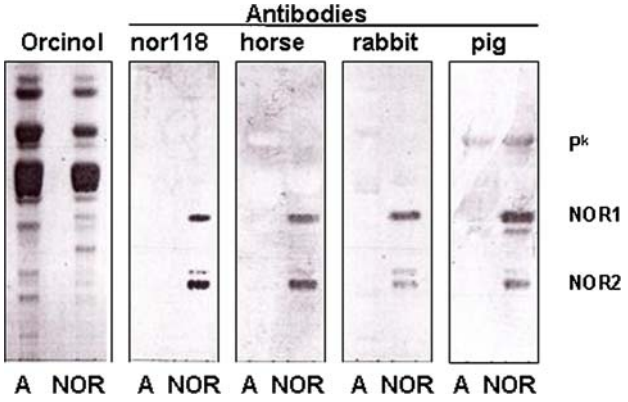

Fig. 5 Binding of horse, rabbit and pig anti-NOR antibodies (eluted with guanidine from the affinity column) to human erythrocyte neutral glycolipids fractionated by HPTLC. Lanes A and NOR, glycolipids from control blood group A and NOR erythrocytes, respectively. For comparison, orcinol-stained plate and binding of murine monoclonal anti-NOR antibody (nor118) are shown.

antibodies [7]. The NOR2 band is hardly seen which indicates its much smaller amount. Horse and rabbit anti-NOR antibodies specifically recognized NOR1 and NOR2 glycolipids. Pig antibodies, besides the reaction with NOR1 and NOR2, also showed reactivity with P^k glycolipid (Galα1-4Galβ1-4Glc-Cer), present in control and NOR erythrocytes, and with a glycolipid migrating closely below NOR1, which is most likely the P1 glycolipid (Galα1-4Galβ1-4GlcNAcβ1-3Galβ1-4Glc-Cer) (Figure 5). This confirmed the inhibition results indicating that pig anti-NOR antibodies showed higher degree of cross-reactivity with Galα1-4Gal than horse and rabbit antibodies.

Discussion

Earlier studies on natural human anti-αGal antibodies showed their diversity and differentiated level in individual sera [2,3,9]. The α-galactosylated oligosaccharide-conjugates used in the present study were previously tested with human sera, and each serum showed a different pattern and level of antibody binding [9]. Generally, many individuals possess high, medium, or low levels of all or most of the examined anti-αGal antibodies, which seems to reflect individual differences in response to α-galactosylated immunogens. However, some sera showed a selectively high level of antibody binding to one or few of the glycoconjugates tested [9,11]. The recently characterized human anti-NOR antibodies represent a new group of anti-αGal antibodies. All of them react strongly with a unique Galα1-4GalNAcβ1-3Gal glycotope (NOR-tri) and show two major types of subspecificity. There are antibodies that require the third Gal residue in the epitope, but the second GalNAc residue can be replaced by Gal. The antibodies that do not see a difference between Gal and GalNAc residues in the epitope were already described, *e.g.* there are anti-AB antibodies reacting specifically with blood group A and B glycotopes [12]. The second type of anti-NOR antibodies is specific for Galα1-4GalNAc, the GalNAc residue cannot be replaced by Gal, but the third Gal residue of NOR-tri has a negligible contribution to the activity [9]. These two types of anti-NOR antibodies can be identified by that type 1 antibodies react weakly with Galα1-4GalNAc and strongly with Galα1-4Galβ1-3Galβ1-4Glc, while type 2 antibodies show reverse reactions. Some of the tested human sera showed the presence of type 1 or type 2 antibodies only, and most sera contained a mixture of both types in various proportions [9].

The experiments described in this report showed that closely similar anti-NOR antibodies are present in animal sera. Similarly as in human sera [9], less specific anti-NOR antibodies inhibitable by galactose were identified in horse serum. These antibodies were less strongly bound to the NOR-tri affinity column and were eluted with 20 mM galactose. Moreover, animal sera contained more specific antibodies that bound strongly to the affinity column and were eluted with 5 M guanidine. The latter antibodies showed two major types of fine specificity, similar to those of human anti-NORs. Anti-NOR of type 2 antibodies were found in horse serum, while rabbit and pig sera contained a mixture of both types. The anti-NOR specificity of these antibodies was confirmed by their selective binding to unique glycolipids present in NOR erythrocytes. These results showed that animal, as well as human anti-NORs represent a heterogeneous group of antibodies differing with respect to affinity and fine specificity. The presently found differences between horse, rabbit and pig anti-NOR antibodies were not greater than those found earlier between anti-NOR antibodies in individual human sera. This suggests that these differences have rather inter-individual, and not inter-species character.

The widespread presence of anti-NOR antibodies in human and animal sera suggests that the respective immunogen(s) must be more frequent in nature than currently known. To our best knowledge, the Galα1-4GalNAc- sequence was found only in one of the 25 oligosaccharide chains of *Rana ridibunda* mucin [13], and as an internal sequence in lipopolysaccharide O-chains of two bacterial strains, *Proteus vulgaris* 019 [14] and *Proteus mirabilis* 014 [15]. Recently, we found that lipids reacting on a thin-layer plate with mouse monoclonal anti-NOR antibody (highly specific for Galα1-4GalNAc) are present in several edible plants [16], but the origin and character of these compounds are not yet known. Therefore, the reason for the widespread existence of 'natural' anti-NOR antibodies and their eventual biological significance remain to be elucidated.

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References

1. Galili, U.: Immune response, accommodation, and tolerance to transplantation carbohydrate antigens. Transplantation **78**, 1093–8 (2004)
2. Parker, W., Lateef, J., Everett, M.L., Platt, J.L.: Specificity of xenoreactive antiGalα1-3Gal IgM for α-galactosyl ligands. Glycobiology **6**, 499–506 (1996)
3. Parker, W., Lin, S.S., Yu, P.B., Sood, A., Nakamura, Y.C., Song, A., Everett, M.L., Platt, J.L.: Naturally occurring anti-α-galactosyl antibodies: relationship to xenoreactive anti-α-galactosyl antibodies. Glycobiology **9**, 865–73 (1999)
4. Harris, P.A., Roman, G.K., Moulds, J.J., Bird, G.W.G., Shah, N.G.: An inherited characteristic, NOR, resulting in erythrocyte polyagglutination. Vox Sang **42**, 134–40 (1982)

5. Kusnierz-Alejska, G., Duk, M., Storry, J.R., Reid, M.E., Wiecek, B., Seyfried, H., Lisowska, E.: NOR polyagglutination and St^a glycophorin in one family. Relation of NOR polyagglutination to terminal α-galactose residues and abnormal glycolipids. Transfusion **39**, 32–8 (1999)
6. Duk, M., Reinhold, B.B., Reinhold, V.N., Kusnierz-Alejska, G., Lisowska, E.: Structure of a neutral glycosphingolipid recognized by human antibodies in polyagglutinable erythrocytes of the rare NOR phenotype. J. Biol. Chem. **276**, 40574–582 (2001)
7. Duk, M., Reinhold, B.B., Reinhold, V.N., Lisowska, E.: Unusual glycosphingolipids in polyagglutinable NOR erythrocytes. Glycoconjugate J. **19**, 118 (2004)
8. Duk, M., Westerlind, U., Norberg, T., Pazynina, G., Bovin, N.N., Lisowska, E.: Specificity of human anti-NOR antibodies, a distinct species of 'natural' anti-α-galactosyl antibodies. Glycobiology **13**, 279–84 (2003)
9. Duk, M., Kuśnierz-Alejska, G., Korchagina, E.Yu, Bovin, N.V., Bochenek, S., Lisowska, E.: Anti-α-galactosyl antibodies recognizing epitopes terminating with α1-4-linked galactose: human natural and mouse monoclonal anti-NOR and anti-P1 antibodies. Glycobiology **15**, 109–18 (2005)
10. Westerlind, U., Hagback, P., Duk, M., Norberg, T.: Synthesis and inhibitory activity of a di- and a trisaccharide corresponding to an erythrocyte glycolipid responsible for the NOR polyagglutination. Carbohydr. Res. **337**, 1517–522 (2002)
11. Lisowska, E., Duk, M.: Polyagglutination NOR: new glycosphingolipid antigens recognized by a new type of common human anti-α-galactosyl antibodies. Arch. Biochem. Biophys. **426**, 142–7 (2004)
12. Korchagina, E.Y., Pochechueva, T.V., Obukhova, P.S., Formanovsky, A.A., Imberty, A., Rieben, R., Bovin, N.V.: Design of the blood group AB glycotope. Glycoconjugate. J. **22**, 127–33 (2005)
13. Mourad, R., Morelle, W., Neveu, A., Strecker, G.: Diversity of O-linked glycosylation pattern between species. Characterization of 25 carbohydrate chains from oviductal mucins of Rana ridibunda. Eur. J. Biochem. **268**, 1990–2003 (2001)
14. Vinogradov, E.V., Kaca, W., Knirel, Y.A., Rozalski, A., Kochetkov, N.V.: Structural studies on the fucosamine-containing O-specific polysaccharide of Proteus vulgaris 019. Eur. J. Biochem. **190**, 95–9 (1989)
15. Perepelov, A.V., Ujazda, E., Senchenkova, S.N., Shashkov, A.S., Kaca, W., Knirel, Y.A.: Structural and serological studies on the O-antigen of Proteus mirabilis 014, a new polysaccharide containing 2-[(R)-1-carboxyethylamino]ethyl phosphate. Eur. J. Biochem. **261**, 347–353 (1999)
16. Duk, M., Lisowska, E.: Mouse monoclonal anti-NOR antibodies and their application in detecting reactive glycolipids in plants. Glycoconjugate J. **22**, 346 (2005)