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Anti-Pr cold agglutinins recognize immunodominant α2,3- or α2,6-sialyl groups on glycophorins

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Anti-Pr cold agglutinins (CAs) with the subspecificities anti-Pr_{1h}, -Pr_{1d}, -Pr_2, -Pr_{3h}, -Pr_{3d}, -Pr^M and anti-Sa CAs recognize immunodominant *N*-acetylneuraminic acid (Neu*N*Ac) groups of tetra and/or trisaccharides (O-glycans) of glycophorin. These O-glycans are sialylated in α2,3- and/or α2,6-linkages. Sa and most Pr antigens have been inactivated by α2,3-specific sialidases. Antigenicity was reconstituted on desialylated glycophorin by α2,3-specific Galβ1,3Gal*N*Ac-sialyltransferase indicating that α2,3-linked Neu*N*Ac groups are the immunodominant components of Sa and most Pr antigens. Some Pr antigens were resistant to α2,3-specific sialidase and were not reconstituted by α2,3-specific Galβ1,3Gal*N*Ac-sialyltransferase, which indicates that α2,6-linked Neu*N*Ac group represents an immunodominant component of some Pr antigens.

Keywords: Anti-Pr cold agglutinins, sialidase, sialyltransferase.

Introduction

Autoimmune haemolytic anaemia (AIHA) is caused by autoantibodies to red blood cells (RBCs). The main groups of these human autoantibodies are warm-type, cold-type and Donath-Landsteiner antibodies [1]. Cold-type autoantibodies or cold agglutinins (CAs) account for 20% of AIHA. The pathological conditions leading to the production of CAs are well defined. CAs are occurring transiently in connection with certain infections or persistently during chronic B lymphocyte proliferation ranging from benign to highly malignant gammopathies. Nearly all CAs belong to the IgM class and are capable of complement activation which is responsible for the CA-induced RBC destruction. Regarding their restricted heterogeneity CAs are unique among human RBC autoantibodies. Postinfection CAs are oligoclonal antibodies, whereas CAs in chronic lymphoproliferation are invariably monoclonal antibodies (MoAbs). Since individual MoAbs recognize identical epitopes, detailed structural analyses of the epitopes recognized by CAs were possible. Based on serological and biochemical studies, three main groups of CA antigens have been identified (for review, see [2]).

The first group consists of the Ii antigens which are protease- and sialidase-resistant developmentally regulated structures. The i antigen is fully expressed on new-born RBCs and consists of linear poly-*N*-acetyllactosamine or type 2 chains (Table 1). The I antigen is fully expressed on adult RBCs originating from the linear i antigen after branching (Table 1) during the first year of life. The antigen j (linear and branched type 2 chains) has been described recently [3].

The second group consists of the Sia-I1, -b1, Ib1, (formerly termed Vo, Fl, Gd) antigens which result from sialylation of linear and/or branched type 2 chains (cf. Table 1).

The third main group are the Pr and Sa antigens (Table 1). These are protease- and sialidase-labile antigens on human RBCs, except for the sialidase-resistant Pr_a antigen. They are tetra/trisaccharides which are O-glycosidically bound to serine or threonine of glycophorin (O-glycans) [2] (see No. 1 in Table 2 and Fig. 1). It has been reported that anti-Pr and anti-Sa also detect sialylated glycolipids (gangliosides) [4, 5]. Because Pr antigens are destroyed by treatment of human RBCs with proteases, it can be assumed that Pr epitopes are carried by glycophorin on the human red cell surface. Contrasting anti-Pr, anti-Sa could be inhibited by 2,3-sialyllactose (see No. 5 in Table 2). Since Pr and Sa antigens are inactivated by sialidase-treatment of RBCs, it is apparent that Neu*N*Ac serves as the immunodominant component of these epitopes. Therefore, glycophorin has been

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Table 1. Serological and biochemical characterization of antigens recognized by human CAs. For subclassification of Pr antigens into Pr_1, Pr_2, Pr_3 see Fig. 2.

Antigen	*Expression on RBCs*		*Effect of enzymes*		*Structure*	*Designation*
	Adult	*Newborn*	*prot.*	*VC sialid.*		
I	+	↓	↑	↑	(1) O-□ branched	Branched type 2 chains
i	↓	+	↑	↑	(2) O-□-O-□-O-	Linear type 2 chains
j	+	+	↑	↑	(1) and (2)	Linear and branched type 2 chains
Sia-b1	+	↓	+	–	(3) Siaα2,3 O-□ branched, Fucα1,2	Sialylated branched type 2 chains
Sia-l1	↓	+	+	–	(4) Siaα2,3 O-□-O-□-O-	Sialylated linear type 2 chains
Sia-lb1	+	+	+	–	(3) and (4)	Sialylated linear and branched type 2 chains
$Pr_{1,2,3}$;Pr^{M*}	+	+	–	–	Tetra/trisaccharides of glycophorins, gangliosides	O-glycans
Pr_a	+	+	–	+	Glycophorins	O-glycans?
Sa	+	+	↓	–	Trisaccharides of glycophorins, gangliosides	O-glycans

*, preferential reaction with M + RBCs at higher temperatures; O, Galβ1–4; □, GlcNAcβ1–3; Sia, *N*-acetylneuraminic acid; prot., proteases; VC sialid., *Vibrio cholerae* sialidase; +, present; –, inactivated; ↑, increased; ↓, decreased.

Table 2. Glycan structures of the compounds employed for CA inhibition studies.

No.	*Structure*	*Designation*
1	Neu*N*Acα2-3Galβ1-3[α2,6 Neu*N*Ac]Gal*N*Ac-Ser/Thr	O-glycan bound via Ser/Thr to glycophorin
2	Galβ1-3Gal*N*Ac-Ser/Thr	O-glycan on asialo-glycophorin
3	Neu*N*Acα2-3Galβ1-3Gal*N*Ac-Ser/Thr	α-2,3-resialylated-glycophorin
4	Galβ1-3[α2,6 Neu*N*Ac]Gal*N*Ac-Ser/Thr	α-2,3-desialylated-glycophorin
5	Neu*N*Acα2-3Galβ1-4Glc	3′-*N*-acetylneuraminyl-lactose
6	Neu*N*Acα2-6Galβ1-4Glc	6′-*N*-acetylneuraminyl-lactose
7	Neu*N*Acα2-3Galβ1-3Gal*N*Ac-Ser-Leu	O-linked type glycopeptide

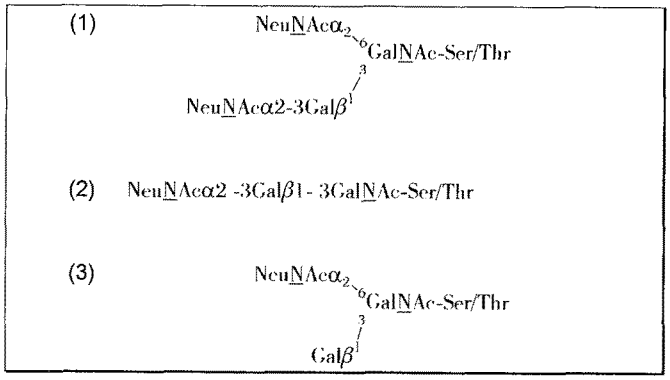

Figure 1. Tetra/trisaccharides of glycophorins. The O-glycans with the structures 1, 2 and 3 are found in a ratio of 8:3:1 on glycophorin A [24].

chemically modified regarding its Neu*N*Ac groups by periodate oxidation and carbodiimide-treatment. The effect of this treatment on binding of several anti-Pr samples was investigated [6, 7]. The effects of these treatments are shown in Fig. 2. Most anti-Pr did not react with modified glycophorin, and were termed anti-Pr_1. Some reacted 100–200-fold stronger with oxidized compared to untreated glycophorin, termed anti-Pr_2, and some reacted 100–200-fold stronger with carbodiimide-treated glycophorin, termed anti-Pr_3. Some anti-Pr_1 CAs reacted also with carbodiimide-treated glycophorin, but their reaction is not increased compared to untreated glycophorin [8]. A further subclassification of Pr antigens is based on agglutination studies with animal (dog) RBCs, extending the subclassification into Pr_{1h}, Pr_{1d}, Pr_2, Pr_{3h}, Pr_{3d} [2]. A further Pr antigen, Pr^M, is characterized by full expression only in the presence of the M antigen of the MN blood groups [9]. Anti-Pr^M recognizes O-glycans with immunodominant Neu*N*Ac groups located preferentially at the M specific peptide backbone, whereas anti-Pr CAs of all other subspecificities detect immunodominant Neu*N*Ac groups of glycophorin irrespective of the amino acid sequence of the peptide backbone [10]. Glycophorins are a family of RBC

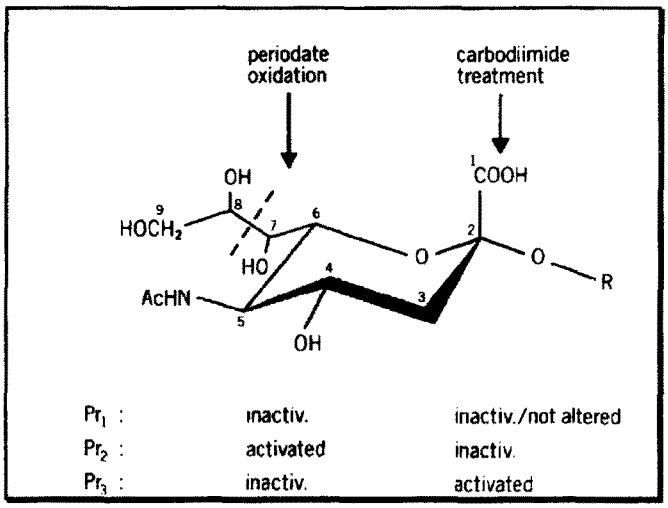

Figure 2. Chemical modifications of Neu*N*Ac and their influences on Pr_1, Pr_2, and Pr_3 antigenicity.

membrane sialoglycoproteins consisting of the major glycophorin A (GPA) and the minor glycophorins GPB, GPC, and GPD [11]. Carbohydrate moieties of glycophorin are N-glycans attached to Asn and O-glycans attached to Ser or Thr. Both, N- and O-glycans are carrying Neu*N*Ac residues. One N-glycan per molecule is found on GPA and GPC but is missing on GPB and GPD [11]. Single Anti-$Pr_{1,2,3}$-Pr^M samples reacted with all external fragments of GPA including those without N-glycan [9, 10]. Furthermore, they reacted with GPB missing N-glycans. Anti-Sa has been shown to recognize a fragment of GPA which is close to the plasma membrane [10] and does not carry the N-glycan. Thus it is obvious that the O-glycans are responsible for expression of these $Pr_{1,2,3}$, Pr^M and Sa antigens. Each glycophorin carries several O-glycans which are consisting of the disialotetrasaccharide and the monosialotrisaccharides shown in Fig. 1. These O-glycans are sialylated in α2,3 and/or α2,6 position.

Despite the extensive studies on Pr antigens, the main question concerning the structure of Pr epitopes has not been answered. It is not known whether anti-Pr CAs recognize the Neu*N*Acα2,3Gal or the Neu*N*Acα2,6Gal-*N*Ac sequence or both of these structures.

To address this question we used α2,3-linkage specific sialidase and α2,3-specific Galβ1,3Gal*N*Ac-sialyltransferase for de- and resialylation of O-glycans. The data presented demonstrate that individual monoclonal anti-Pr CAs recognize different sialylated sequences of O-glycans.

Materials

Cold agglutinins

Sera from patients with CAs of the specificities anti-Pr and anti-Sa were taken from our collection [8]. Several anti-Pr samples used in this study have not been published previously. Their specificities and subspecificities were determined in this laboratory according to methods introduced also by this laboratory [6, 7]. Titres of the individual sera with untreated RBCs at 0 °C ranged from 8 to >2000. CAs were purified by cold binding to and warm elution from untreated RBCs [12].

Blood samples

RBCs were taken without anticoagulants by peripheral veinpuncture from healthy probands, and were washed immediately three times with phosphate buffered saline solution (PBS) to prevent coagulation.

All chemicals used were of analytical grade and purchased from Boehringer (Mannheim), Serva (Heidelberg) and Merck (Darmstadt).

Enzymes

α2,3-specific Newcastle disease virus sialidase (NDV sialidase, complete virus suspension, EC 3.2.1.18) was obtained from Boehringer (Mannheim); α2,3-specific recombinant sialidase expressed in *E. coli* (EC 3.2.1.18) was purchased from Glyko Inc. (USA); α2,3-specific Galβ1,3Gal*N*Ac-sialyltransferase from porcine liver (ST, EC 2.4.99.4) and sialidase from Athrobacter ureafaciens (AU sialidase, EC 3.2.1.18) were purchased from Sigma (Deisenhofen) and sialidase from *Vibrio cholerae* (VC sialidase, EC 3.2.1.18) from Behringwerke (Marburg); papain (EC 3.4.22.2) was obtained from Merck (Darmstadt).

Peanut-lectin for T antigen detection

Peanut-lectin (PNA) (1 mg ml^{-1} solution in Tris, 50 mM NaCl, pH 7) was purchased from Boehringer (Mannheim).

Materials for fixation of RBCs

Glutaraldehyde 25% (v/v) solution in water was purchased from Serva (Heidelberg) and bovine serum albumin (BSA) from Boehringer (Mannheim).

Materials for inhibition studies

The glycophorin mixture was obtained by phenol/saline extraction of human RBC ghosts [13]; 3′-*N*-acetylneuraminyl-lactose (No. 5 in Table 2) and 6′-*N*-acetylneuraminyl-lactose were purchased from Sigma (purity 95%) (No. 6 in Table 2); O-linked type glycopeptide (No. 7 in Table 2) purified from human urine was obtained from Oxford Glyco Systems Ltd.

Methods

Haemagglutination tests

Haemagglutination and haemagglutination inhibition

studies were performed using the standard tube technique [14].

Determination of cold agglutinin specificities

Titres of individual anti-Pr and anti-Sa sera were determined using untreated, sialidase- and papain-treated human adult and newborn RBCs. Determination of specificities was based on the reaction patterns shown in Table 1. Anti $Pr_{1,2,3}$ and $-Pr^M$ subspecificities were defined according to Ebert *et al.* [7], Roelcke *et al.* [6] and Roelcke *et al.* [9]. Further subclassification into anti-Pr_{1h}/-Pr_{1d} and anti-Pr_{3h}/-Pr_{3d} was performed as described earlier [2]. Anti-Sa specificity was defined according to Roelcke *et al.* [15].

Desialylation of RBCs

3% (v/v) RBC suspension (1400 μl) PBS was incubated in the presence of 14 mU ml^{-1} of NDV sialidase for 3 h at 37 °C or in presence of 5 mU ml^{-1} recombinant α2,3-specific sialidase for 24 h.

For complete desialylation, a 3% (v/v) RBC suspension in PBS (2 ml) was incubated at 37 °C for 60 min with 25 mU ml^{-1} VC sialidase. After sialidase treatment, RBCs were washed four times with PBS.

The enzymatic treatment of RBCs was performed sequentially. Native RBCs were first treated with α2,3-specific NDV sialidase, washed thoroughly and subsequently treated with VC sialidase as above.

Determination of T antigen

Efficiency of enzymatic Neu*N*Ac release by NDV- or VC sialidase from intact RBCs was controlled by peanut agglutinin binding which specifically detects Galβ1,3Gal*N*Ac sites. 30 μl of a 3% (v/v in PBS) suspension of RBCs in PBS were incubated for 30 min with 30 μl of serial twofold dilutions of peanut agglutinin (stock solution 1 mg ml^{-1}) dissolved in PBS. Titres were read macroscopically after brief centrifugation.

Fixation of RBCs

10 ml glutaraldehyde (0.045% (v/v) dilution in PBS) were added to 200 μl RBCs (4% v/v solution in PBS). The cells were suspended immediately and incubated for 4 min at room temperature. Subsequently fixation was stopped by addition of 2.6 ml 5% (g per 100 ml) BSA and the cells were collected (3000 rpm per 3 min). Fixed RBCs were resuspensed in a mixture of 2 ml 5% (g per 100 ml) BSA and 11 ml PBS, collected as above and finally washed three times with PBS.

Resialylation of glycophorin

Asialoglycophorin mixture was enzymatically resialylated to carry only α2,3-linked Neu*N*Ac and was subsequently used for inhibition of haemagglutination.

The glycophorin mixture extracted from human RBCs was first desialylated by acid hydrolysis (0.1 N HCl, 45 min at 80 °C) and extensively dialysed in twice distilled water to remove Neu*N*Ac liberated. After lyophilization the asialo-glycophorin preparation was resuspended in twice distilled water (40 mg lyophilisate per ml), and insoluble compounds were separated (5 min, 10000 g).

Specific resialylation of asialo-glycophorin O-glycans in α2,3-linkage was achieved by employing purified Galβ1,3Gal*N*Ac α2,3-specific ST (200 mU ml^{-1}) as follows: The reaction mixture (1.6 ml) contained 60 mM sodium cacodylate pH 6.8, 6.8 mg asialoglycophorin, 1.35 mM CMP-Neu*N*Ac, 100 μM 2,3-dehydro-Neu*N*Ac, and 10 mU α2,3-specific ST. After incubation at 37 °C for 24 h, further 6 mU enzyme and 1.2 μmol CMP-Neu*N*Ac were added and incubation was extended for another 24 h. Finally, the reaction mixture was dialysed intensively using twice distilled water (8 °C, 48 h) and lyophilized. The resialylated glycophorin preparation was resuspended in twice distilled water (40 mg lyophilisate per ml) and insoluble material was removed by centrifugation as above.

Sialylation efficiency was determined after enzymatic release of transferred Neu*N*Ac from the resialylated glycophorin. For this purpose sialidases without linkage specificity from AU and VC as well as NDV sialidase specific for α2,3-linked Neu*N*Ac were employed. Sialidase treatment of glycophorin preparation (about 60 μg) was performed in 0.1 M sodium acetate pH 5.5 (25 μl total assay) employing 200 mU ml^{-1} VC- or AU sialidase or 40 mU ml^{-1} NDV sialidase, respectively. Reaction mixture was incubated at room temperature for up to 24 h. The Neu*N*Ac liberated was determined by analytical HPLC with an aminopropyl-phase column at 200 nm as outlined previously [16–18].

Results

α2,3-specific desialylation of RBCs

α2,3-Linked Neu*N*Ac of human RBCs was specifically released by NDV sialidase. CAs from 29 patients were assayed for their binding to α2,3-desialylated RBCs applying standard haemagglutination assay. No agglutination was achieved with 25 CAs, only 4 CAs yielded almost identical titres with untreated and α2,3-desialylated RBCs (Table 4). These four CAs lost agglutination capacity only after treatment of RBCs with VC sialidase which splits α2,3- and α2,6-linkages of Neu*N*Ac.

Resialylation of glycophorin O-glycans in α2,3-linkage

Sialylation extent of the starting glycophorin mixture, of the chemically desialylated and of the enzymatically resialylated glycophorin preparation was compared by determining Neu*N*Ac released after sialidase treatment. After acidic hydrolysis of starting glycophorin prepara-

tion less than 5% of bound Neu*N*Ac remained. Specific resialylation of O-linked glycans (for structure see No. 2 in Table 2) of desialylated glycophorin in α2,3-linkage was achieved employing purified Galβ1,3Gal*N*Ac α2,3-specific ST. Linkage specificity of enzymatic sialyl transfer was proved comparing treatment of the resialylated glycophorin by sialidases without linkage specificity (VC/AU sialidase) as well as by NDV sialidase with specificity for α2,3-linked Neu*N*Ac. Both types of sialidases released nearly equal amounts of bound Neu*N*Ac from the resialylated glycophorin mixture which confirmed the linkage specificity of the ST employed (Table 3). The resialylation degree reached at least 65% related to the potential galactose acceptor sites exposed in O-linked glycans of the starting glycophorin preparation. (Galactose sites were calculated according to the puplished glycan structure) [2].

A second enzymatic sialylation procedure performed with glycophorin once resialylated yielded only 3% further incorporation of Neu*N*Ac, indicating a saturating extent for the resialylation.

Haemagglutination inhibition studies with glycophorin resialylated in α2,3-linkage

These studies were performed using glutaraldehyde-fixed RBCs, as the resialylated glycophorin contained Triton X-100 which would cause haemolysis of untreated RBCs. The glutaraldehyde fixation did not result in a loss of RBC agglutinability by CAs. Anti-Pr CAs of seven patients were selected for haemagglutination inhibition studies with native, desialylated and resialylated glycophorin. Native glycophorin inhibited binding of 6 CAs to a low and one to a high degree (Table 5). As expected, no inhibition of haemagglutination was observed with the asialo-glycophorin preparation.

Pr antigen activity of desialylated glycophorin could be reconstituted by α2,3-specific ST in four cases (labelled ◆ in Table 5). In these cases, treatment of RBCs with α2,3-specific sialidases caused inactivation of the Pr-antigens (Table 4). In contrast, Pr antigenicity could not be reconstituted by α2,3-specific ST in three cases (labelled * in Table 5). Accordingly, α2,3-specific sialidase did not abolish Pr antigenicity in the case of these three CAs (Table 4).

As shown in Table 5, the haemagglutination inhibition capacity of native glycophorin is in the range of 0.3–10.0 mg ml^{-1}. This is in agreement with our previous findings [8]. An exception is the anti-Pr$_{1d}$No. which is at least 100-fold more sensitive to inhibition by native glycophorin than all other anti-Pr tested [8]. This CA was, therefore, included in this study. It recognizes α2,3-specific sialidase-resistant Pr epitopes which are not as common as the α2,3-specific sialidase-labile Pr-epitopes. However, further data on its strong reactivity with native glycophorin are not available.

As is also shown in Table 5, the inhibitory potency of α2,3-resialylated glycophorin is at least 10-fold higher than that of native glycophorin regarding the CAs Fa., Pe., Wa. with the anti-Pr subspecificities anti-Pr$_3$ and anti-Pr^M, respectively. The limited number of anti-Pr CAs with these rare subspecificities does not allow to investigate whether the strong inhibitory potency of α2,3-resialylated glycophorin could be correlated with distinct anti-Pr subspecificities.

3′-*N*-Acetylneuraminyl-lactose and 6′-*N*-acetylneuraminyl-lactose (for structures see No. 5/6 in Table 2) did not inhibit any anti-Pr CA tested (Table 5).

The O-linked glycopeptide (structure 7 in Table 2) was employed as inhibitor for two representative CAs (Table 5). No inhibition of anti-Pr$_{1h}$ Rh. up to 11.5 mM glycopeptide could be achieved, anti-Pr$_{1d}$ No. was inhibited by 11.5 mM glycopeptide. The latter CA is highly sensitive towards inhibition by native glycophorin and recognized Pr-antigens resistant towards α2,3-specific sialidase. The inhibition by the glycopeptide containing only α2,3-linked Neu*N*Ac could be due to small amounts of a contaminating molecule with α2,6-linked Neu*N*Ac.

Discussion

VC sialidase, which has been used in our laboratory for several years to characterize Pr and Sa antigens, cleaves all types of Neu*N*Ac linkage. Using α2,3-specific sialidases as well as a sialyltransferase specific for α2,3-linkage and O-glycan acceptors we were able to demonstrate in this paper that most anti-Pr CAs (22/26) and three anti-Sa CAs recognize immunodominant α2,3-Neu*N*Ac groups attached to galactose. It can be concluded that the other anti-Pr CAs (4/26) recognize immunodominant α2,6-Neu*N*Ac groups attached to the internal Gal*N*Ac as binding was abolished by VCN. Another possibility

Table 3. Release of Neu*N*Ac from glycophorin preparations [nmol Neu*N*Ac/mg lyophilised material] by non linkage specific VC- and AU sialidase, or by α2,3-specific NDV sialidase (values of 3 independent assays).

	VC sialidase or AU sialidase	*NDV sialidase*
Native glycophorin mixture (starting preparation)	450–490	300
Desialylated glycophorin mixture (asialo-glycophorin)	15–25	<5
Glycophorin mixture resialylated in α2,3-linkage	230–250	237

Table 4. Titres of several anti-Pr and anti-Sa cold agglutinins achieved with untreated, NDV sialidase and VC sialidase treated human unfixed RBCs.

Patient	*Specificity of cold agglutinins*	*Untreated RBCs*	*NDV sialidase treated α2,3-desialo RBCs*	*VC sialidase treated asialo RBCs*
Rh◆	-Pr_{1h}	128	0	0
St	-Pr_{1h}	128	0	0
Cl	-Pr_{1h}	1000	0	0
Po	-Pr_{1h}	1000	0	0
Vk	-Pr_{1h}	>2000	0	0
Do	-Pr_{1d}	32	0	0
Ma*	-Pr_{1d}	32	32	0
St	-Pr_{1d}	32	0	0
Bi	-Pr_{1d}	64	0	0
Tö	-Pr_{1d}	64	0	0
Jo	-Pr_{1d}	128	0	0
We	-Pr_{1d}	128	0	0
Br*	-Pr_{1d}	256	128	0
No*	-Pr_{1d}	256	256	0
Pi	-Pr_{1d}	512	256	0
Jü	-Pr_{1d}	>2000	0	0
A5	-Pr_2	8	0	0
Re	-Pr_2	128	0	0
A3	-Pr_2	>2000	0	0
Li	-Pr_2	>2000	0	0
Pe◆	-Pr_{3h}	>2000	0	0
Ra	-Pr_{3d}	64	0	0
Hü	-Pr_{3d}	128	0	0
Fa◆	-Pr_{3d}	512	0	0
Wa◆	-Pr^M	256	0	0
Th	-Pr^M	512	0	0
Sa	-Sa	>512	0	0
Co	-Sa	>2000	0	0
Mo	-Sa	>2000	0	0

0, no agglutination; RBC, red blood cell; NDV, Newcastle disease virus; VC, *Vibrio cholerae*.

Table 5. Haemagglutination inhibition of anti-Pr agglutinins by native or enzymatically modified glycophorins and by sialo-oligosaccharides.

Patient	*Specificity of cold agglutinins*	*native glycophorin (mg ml^{-1})*	*asialo glycophorin (mg ml^{-1})*	*α2,3-resialo glycophorin (mg ml^{-1})*	*3'N-acetyl-neuraminyl-lactose (mM)*	*6'N-acetyl-neuraminyl-lactose (mM)*	*O-linked type glycopeptide (mM)*
Rh◆	-Pr_{1h}	0.6	–	0.3	○	○	●
Ma*	-Pr_{1d}	5.0	–	–	○	○	ND
Br*	-Pr_{1d}	10.0	–	–	○	○	ND
No*	-Pr_{1d}	0.0025	–	–	○	○	11.5
Fa◆	-Pr_3	0.6	–	0.01	○	○	ND
Pe◆	-Pr_3	0.3	–	0.02	ND	ND	ND
Wa◆	-Pr^M	0.3	–	0.04	○	○	ND

–, no inhibition by 5 mg ml^{-1}; ○, no inhibition by 15 mM; ●, no inhibition by 11.5 mM; ND = not determined.
Haemagglutination inhibition studies were performed using glutaraldehyde-fixed RBCs.

could be that the Pr-antigens, which are resistant to α2,3-specific sialidase, are represented by N-glycans carrying α2,6-Neu*N*Ac groups [19]. Studies with GPA fragments or GPB missing N-glycans are nesessary to prove or falsify this possibility. Recognition of N-glycans would not correlate with any anti-Pr-subspecificity defined, because examples of all subspecificities reacted with fragments of glycophorin A missing N-glycans [10]. Recognition of α2,3-Neu*N*Ac groups by anti-Sa and anti-Pr_2 is in agreement with earlier findings. Anti-Sa has been inhibited by α2,3-sialyllactose but not by the α2,6-sialyllactose isomer [20]. The antibody also recognizes the Neu*N*Acα2-3Galβ1-4Glc*N*Ac/Glc sequence as found in G_{M3} and in gangliosides of the neolacto series [4]. Furthermore, it reacts with those O-glycans of GPA which are located close to the red cell membrane [10]. These O-glycans are incompletely sialylated and consist predominantly of the monosialotrisaccharide with the structure Neu*N*Acα2-3Galβ1-3Gal*N*Ac-Ser/Thr [11]. Because the Sa antigen is only partially destroyed by protease treatment of red cells, gangliosides and/or glycophorin portions not accessible for proteases may contribute to Sa antigenicity on RBCs. It has also been shown previously that anti-Pr_2 reacts with an array of Neu*N*Ac groups on gangliosides including the Neu*N*Acα2-3Galβ1-3Gal*N*Ac sequence also found on the O-glycans of glycophorin [4].

The identification of the type of immunodominant Neu*N*Ac group responsible for $Pr_{1,2,3,}Pr^M$ and Sa antigenicity may be of general importance, because selectively the Neu*N*Ac group mediates binding of these autoantibodies (CAs) to its target on RBCs. Moreover, it would be an intriguing step to define the conditions which are involved in induction of postinfection CAs. Based on earlier findings which demonstrate a definite association between *Mycoplasma pneumoniae* and anti-I induction as well as Epstein Barr virus and anti-i induction [see review 2], we have focused on infectious agents capable of anti-Pr induction. Rubella virus [21] and varicella virus [22, 23] could be identified. It is now possible to examine whether these viruses would select distinct Neu*N*Ac groups for anti-Pr induction.

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