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The Role of Carbohydrate in the Blood Group N-Related Epitopes Recognised by Three New Monoclonal Antibodies

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The specificity of three new monoclonal anti-glycophorin antibodies, reacting preferentially with blood group N antigen, was characterized by means of untreated, enzymatically and chemically modified M and N glycoproteins. All antibodies recognized the NH_2-terminal Leu residue and its amino group, but differed in some other features, including the role of carbohydrate in the epitopes. One of the antibodies (631/3B4, IgM) showed an unusual two-directional dependence of activity on the degree of antigen desialylation. The progressive desialylation of N glycoprotein first caused a strongly increased binding to the epitope, followed by a complete loss of activity. The epitopes for the two remaining antibodies (648/4B5 and 650/4B5, both IgG_1) showed reactivity independent of sialylation, but dependent on the presence of Gal-GalNAc-units. Release of the disaccharide by *O*-glycanase treatment of N glycoprotein abolished its reactivity with both antibodies.

Antibodies related to the MN blood group system recognize various epitopes in the genetically differentiated NH_2-terminal portion of glycophorin A. The basic condition for specific or preferential reactivity of an antibody with blood group M or N determinant in glycophorin is the recognition of the 1st or 5th amino-acid residue, which are the only residues that differ in blood group M and N (see [1]). The anti-M or anti-N antibodies show different subspecificities, since their epitopes differ in contribution of individual amino-acid residues, polar groups, and carbohydrate components (see [1, 2]). The blood group M or N-related NH_2-terminal fragment of glycophorin A is glycosylated at amino-acid residues 2, 3, and 4 with oligosaccharides of the structure NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc [3, 4]. Studies on the significance of carbohydrate for M and N epitopes have been limited so far to the effect of desialylation. The release of sialic acid from M and N antigens results in a loss or significant decrease of their reactivity with most antibodies (particularly anti-M), but a number of other antibodies show unchanged or even enhanced reaction with the

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desialylated antigen (see [1, 2]). It is not known, however, how many and which of the six sialic acid residues present at the NH_2-terminus of glycophorin A are essential for various sialic acid-dependent M and N epitopes. The significance of Gal-GalNAc-disaccharide units in the sialic acid-independent epitopes is also unknown. These problems are interesting, since M and N antigens may serve as models for elucidating the possible modes of immunological recognition of glycosylated peptide antigens. On the other hand, the knowledge of carbohydrate contribution to the epitopes recognized by the anti-glycopeptide monoclonal antibodies may make some of the antibodies convenient as reagents for defining the degree of glycosylation, e.g. during biosynthesis or cell differentiation.

We attempted to obtain answers to some of the above questions during characterization of three new monoclonal anti-glycophorin antibodies reacting preferentially with blood group N antigen. One of the antibodies (631/3B4) showed an unusual property of maximal reactivity with a partially desialylated antigen. The two other antibodies (648/4B5 and 650/4B5) did not require sialic acid in the antigen and were used for testing the role of Gal-GalNAc-chains in the epitopes.

Materials and Methods

Monoclonal Antibodies

The hybridoma clone 631/3B4 was obtained after immunization of BALB/cABom mice with purified glycophorin B, and the clones 648/4B5 and 650/4B5 derived from mouse immunized with cord red cells. The details of immunization, fusion and cloning procedures were described earlier [5-7]. The selected clones were grown as expanded cell cultures under standard conditions. Double immunodiffusion in agarose with the use of rabbit antisera against isotypes of mouse Ig (Miles, Elkhart, IN, USA) showed that 631/3B4 clone produced IgM, while the antibodies 648/4B5 and 650/4B5 were of IgG_1 subclass.

Other monoclonal antibodies used were N/61 with anti-N specificity [8], 22.19 against Thomsen-Friedenreich antigen [9], and GPA105 recognizing a non-glycosylated peptide fragment of glycophorin A around the 40th amino-acid residue (Wasniowska *et al.*, unpublished results).

Antigens

Glycophorin A of blood group M and N type (M and N glycoproteins) and glycophorin B were products of BioCarb Chemicals AB (Lund, Sweden). Alternatively, glycophorin A was prepared from outdated human M and N erythrocytes by the phenol extraction of membranes [7]. The NH_2-terminal M and N glycopeptides were purified from tryptic digests of crude M and N glycoproteins, respectively [10].

Modifications of Glycoproteins

Desialylation, *N*-acetylation, and Edman degradation of M and N glycoproteins were done as reported previously [6, 7]. Decomposition of amino groups with nitrous acid was

performed by treatment of 1 ml 1% glycoprotein solution in 15% acetic acid with 0.3 ml saturated sodium nitrite, added dropwise with constant stirring on an ice-bath.

Erythrocytes

Blood group M and N erythrocytes used in tests were obtained from healthy blood donors. Treatments of red cells with neuraminidase from *Vibrio cholerae* (Serva, Heidelberg, W. Germany), TPCK-trypsin or chymotrypsin (Worthington, Freehold, NJ, USA) were done under conditions described earlier [6].

Assays for Antigen-Antibody Reaction

Reaction of antibodies with untreated and modified M and N antigens was tested by hemagglutination and hemagglutination-inhibition, microtitre plate enzyme-linked immunosorbent assay (ELISA), and immunoblotting. Hemagglutination tests and incubation of antigen with antibody in ELISA [6, 7] were performed in 0.15 M NaCl containing 0.01 M phosphate at the pH indicated in the Results section. Binding of antibodies to the ELISA plates coated with M or N glycoprotein was measured using a conjugate of rabbit anti-mouse Ig or anti-mouse IgM antibodies with horseradish peroxidase (Dakopatts, Copenhagen, Denmark) and *o*-phenyldiamine (Sigma, St. Louis, MO, USA) as a substrate; the plates were scanned at 490 nm. Immunoblotting of red cell membranes fractionated by SDS-polyacrylamide gel electrophoresis [11] was based on the method of Hawkes *et al.* [12]: the nitrocellulose (BA85, Schleicher & Schuell, Dassel, W. Germany) blots were overlayed with Whatman 3MM paper soaked with the monoclonal antibody and were incubated overnight at 4°C. The antibody bound was detected with rabbit anti-mouse Ig, or anti-mouse IgM, antibodies conjugated with peroxidase, using 4-chloro-1-naphthol (Sigma) as a substrate.

Glycosidases from Diplococcus pneumoniae

The glycosidases were isolated from the culture supernatant of *D. pneumoniae* type 1 by precipitation with ammonium sulfate and gel filtration of the sediment on the Sephadex G-200 column [13]. The fractions containing glycosidases, including endo-α-*N*-acetylgalactosaminidase (E.C. 3.2.1.97, *O*-glycanase) were pooled and concentrated by ultrafiltration. This preparation does not show any proteolytic activity and degrades the *N*- and *O*-linked oligosaccharide chains of glycophorin; the products of hydrolysis of *O*-linked chains are N-acetylneuraminic acid and Galβ1-3GalNAc [13]. The enzyme sample used in the present experiments showed activity of neuraminidase (with glycophorin as a substrate) of 0.04 U/ml, and of *N*-acetyl-β-glucosaminidase (tested with *o*-nitrophenyl-β-GlcNAc) - 0.18 U/ml.

Purified *O*-glycanase from *D. pneumoniae* was purchased from Genzyme (Boston, MA, USA).

Treatment of Glycoprotein-coated ELISA Plates with Glycosidases

The following enzymes were used: neuraminidase from *Vibrio cholerae* (Serva , Heidelberg, W. Germany), *Arthrobacter ureafaciens* (Calbiochem, La Jolla, CA, USA), or *Clostridium perfringens* (Sigma; Type V), which were serially diluted with 0.1 M sodium acetate buffer, pH 5.5; the β-galactosidase from *Aspergillus niger* (Sigma), diluted with 0.05 M sodium

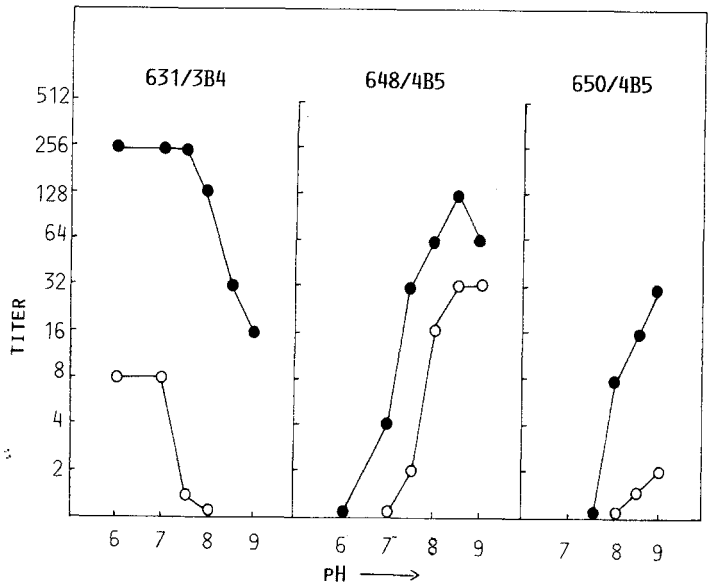

Figure 1. The pH-dependence of agglutination of M (○) and N (●) erythrocytes by 631/3B4, 648/4B5 and 650/4B5 antibodies.

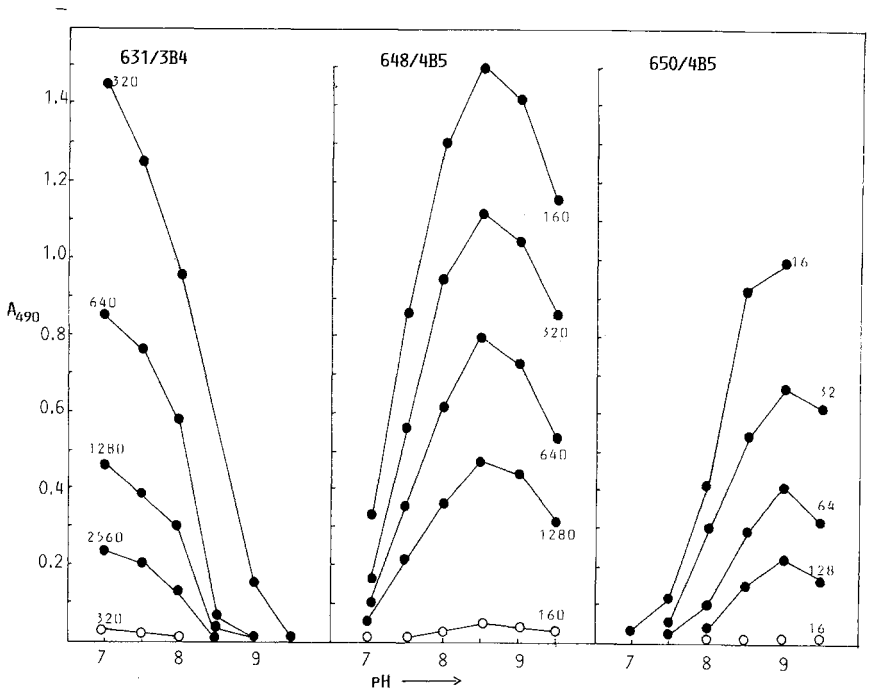

Figure 2. The pH-dependence of binding of 631/3B4, 648/4B5 and 650/4B5 antibodies to M (○) and N (●) glycoprotein-coated ELISA plates. The numbers by the curves denote dilutions of the antibody.

Table 1. The effect of treatment of M and N erythrocytes with trypsin (tr) or chymotrypsin (ch) on their agglutination by the monoclonal antibodies.

Erythrocytes	Antigen present on glycophorin		Titre of antibodies[a]		
	A(M or N)	B(N)	631/3B4	648/4B5	650/4B5
M	+	+	64	16	Ø[b]
M-tr	-	+	256	64	8
M-ch	±	-	Ø	16	Ø
N	+	+	1000	128	16
N-tr	-	+	128	128	8
N-ch	±	-	32	128	4

[a] The antibodies 631/3B4, 648/4B5 and 650/4B5 were tested at pH 7.0, 7.5 and 8.0, respectively.
[b] Ø denotes lack of agglutination.

acetate buffer of pH 4.0; the mixture of glycosidases from *D. pneumoniae* described above, diluted 20-fold with 0.1 M sodium phosphate/citrate buffer of pH 6.0 and then serially diluted with the same buffer.

The plates coated with N glycoprotein (2 μg/well) were blocked with 5% bovine serum albumin for 1 h at 20°C and washed. The wells were filled with 100 μl portions of the serially diluted enzyme solution and were incubated for 2 h at 37°C with neuraminidase, or for five days at 37°C with *D. pneumoniae* glycosidases or β-galactosidase, with the exchange of the enzyme solution for the fresh one on the 3rd day. The plates were washed and the binding of a constant amount of monoclonal antibody was tested as described above.

Other Analytical Methods

Free sialic acid was determined colorimetrically by the method of Warren [14], total and bound sialic acid were determined by the method of Jourdian *et al.* [15]. In order to evaluate the proportion of periodate-resistant (i.e. substituted with sialic acid) galactose residues, the untreated and neuraminidase-treated N glycoprotein samples in 0.05 M sodium periodate (0.5 mg/ml) were incubated overnight at 4°C, dialyzed, and dried. The samples were hydrolyzed in 1 M sulfuric acid for 7 h at 100°C, monosaccharides were transformed into alditol acetates, and galactose was determined by gas liquid chromatography, using xylose as an internal standard.

Results

Blood Group Specificity and pH-Dependence of the Antibodies

The three monoclonal antibodies obtained agglutinated blood group N more strongly than M erythrocytes and showed different patterns of pH-dependence (Fig. 1). The antibody 631/3B4 was most active at pH 6-7/7.5 and its agglutination titre decreased at higher pH values,

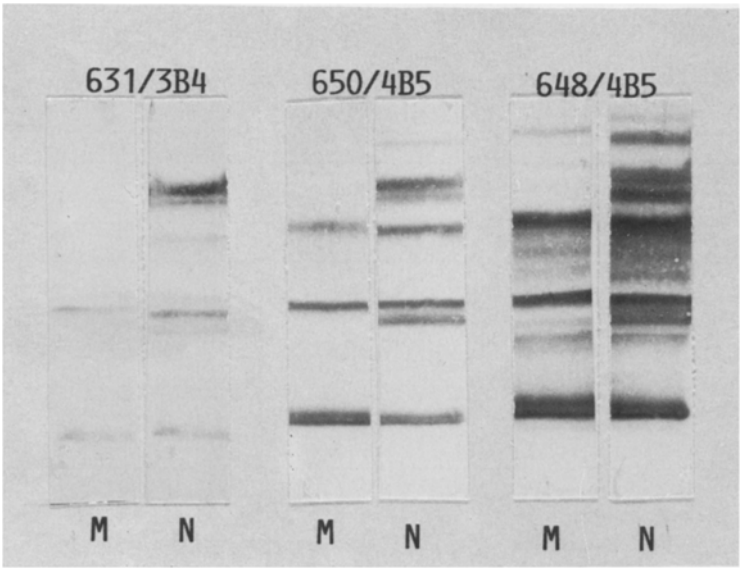

Figure 3. Immunoblotting of M and N erythrocyte membranes with the monoclonal antibodies. Binding of the antibodies to the blots was performed at pH 7.5 with 631/3B4 and 648/4B5, and at pH 8.0 with 650/4B5. The binding was detected immunoenzymatically, for details see the Materials and Methods section.

while 648/4B5 and 650/4B5 were inactive or weakly active at pH 6-6.5 and their reactivity strongly increased up to pH 8.5 and 9, respectively. Analogous pH-dependence and specificity for N antigen were shown in binding of the antibodies to M and N glycoprotein-coated ELISA plates (Fig. 2).

In order to evaluate the contribution of glycophorin B (carrying the blood group N determinant) to agglutination of M red cells by the antibodies, trypsin- and chymotrypsin-treated erythrocytes were tested. Trypsin digests glycophorin A at the cell surface, and chymotrypsin digests glycophorin B and a substantial part of glycoprotein A [1]. The agglutination of protease-treated M and N red cells by 631/3B4 and 650/4B5 antibodies was typical for anti-N specificity and indicated that agglutination of M erythrocytes occurred mostly due to the interaction with glycophorin B (Table 1). The antibody 648/4B5 did not show the decrease of agglutination titre with protease-treated cells (Table 1). The lack of response to the decreased number of epitopes may have resulted from the higher affinity of this antibody, and agglutination of chymotrypsin-treated M erythrocytes indicated its cross-reactivity with blood group M antigen. Similar conclusions on the blood group specificity of the three antibodies can be drawn from the results of the immunoblotting assay (Fig. 3).

Characterization of the Epitopes

The effect on activity of N antigen-modifications was tested by inhibition of agglutination and inhibition of binding of the antibodies to N glycoprotein-coated ELISA plates (Table 2). The N glycoprotein lost the activity after *N*-acetylation, degradation of amino groups with

Table 2. Inhibition of the antibodies by untreated and modified M and N glycoprotein, tested by hemagglutination assay (a), or microplate ELISA (b).

Inhibitor	Inhibitor concentration (μg/ml[a]) Antibodies 631/3B4 a	631/3B4 b	648/4B5 a	648/4B5 b	650/4B5 a	650/4B5 b
M glycoprotein						
untreated	>5000		>5000	500	>5000	2500
tryptic glycopeptide 1-39	>5000	>5000	5000	300	>5000	700
N glycoprotein						
untreated	300	50	160	15	1250	130
desialylated	>5000	>5000	160	6	300	35
N-acetylated	>5000	>5000	5000	300	>5000	>5000
treated with HNO_2	>5000		5000		>5000	
Edman degraded	>5000		>5000		>5000	
control to Edman degradation[b]	80		80		300	
tryptic glycopeptide 1-39	5000	2500	300	25	2500	80

[a] Minimal concentration of an inhibitor giving a complete inhibition of agglutination (a), or concentration giving 50% inhibition of the antibody binding (b).
[b] Sample submitted to acidic conditions of Edman degradation (control for a partial desialylation).

nitrous acid, or removal of the NH_2-terminal Leu residue by Edman degradation. The results indicated that the three antibodies tested reacted with epitopes in which terminal Leu residue, including its amino group, was an essential component. The requirement, however, for the free amino group was less rigourous in the 648/4B5 epitope than in the remaining ones (Table 2). Furthermore, the epitopes differed in the contribution of sialic acid residues. Desialylation of the antigen abolished its reactivity with the 631/3B4 antibody, while the reaction with 648/4B5 and 650/4B5 antibodies was unchanged, or even enhanced.

Changes in Epitope Activity as a Result of Different Degrees of Desialylation

The antibodies tested showed increased reactivity with the preparation used as a control of the partial desialylation during Edman degradation (Table 2) that was surprising in the case of 631/3B4 antibody. This control preparation was N glycoprotein submitted to acidic conditions of cyclization/degradation step performed in anhydrous trifluoroacetic acid. During such treatment,10-15% of sialic acid is released and the activity of sialic acid-dependent epitopes may be decreased [7].

In order to study the effect of gradual desialylation, the N glycoprotein-coated ELISA plates were treated with increasing concentrations of neuraminidase from *V. cholerae* or *A. ureafaciens* and the binding of the antibodies was tested (Fig. 4). The binding of 631/3B4 antibody increased 10-20-fold with progressing desialylation, and then dropped to zero.

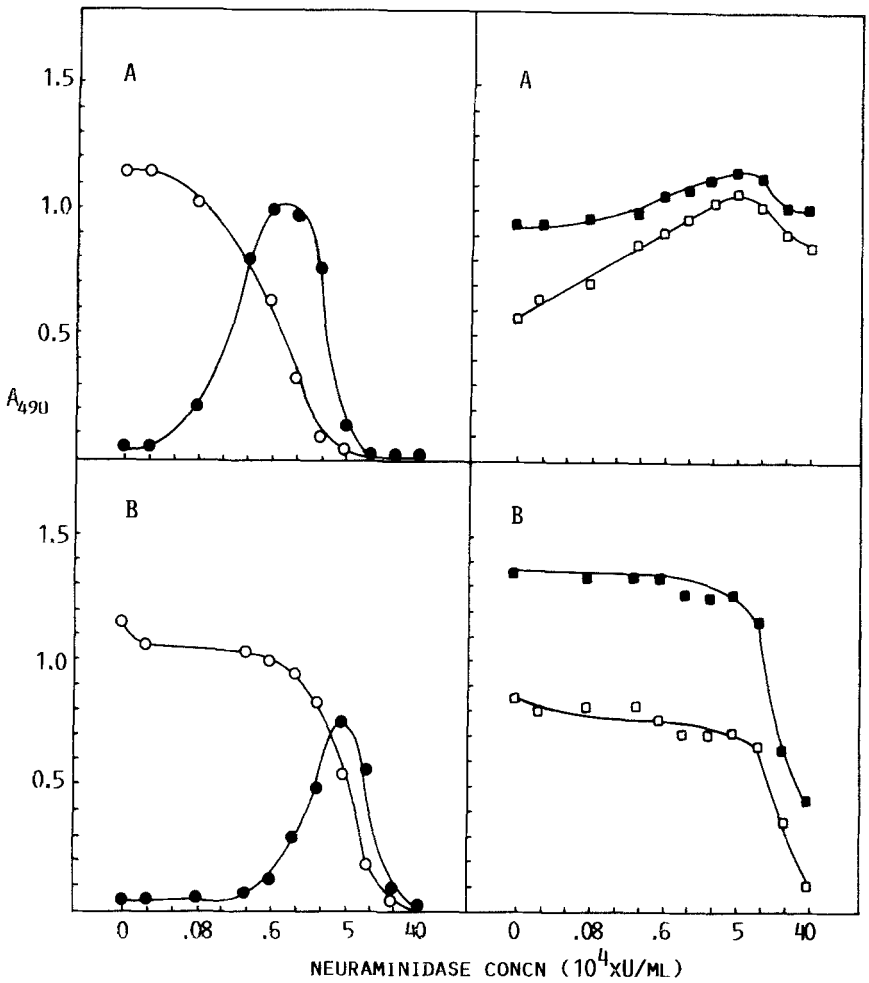

Figure 4. Binding of monoclonal antibodies 631/3B4 (●), 648/4B5 (■), 650/4B5 (□) and N/61 (○) to N glycoprotein-coated ELISA plates pretreated with increasing concentrations of neuraminidase from *V. cholerae* (A) or *A. ureafaciens* (B). The binding of the antibodies was carried out at pH 7.5, except 450/4B5 which was tested at pH 8.0. Other details are described in the Materials and Methods section.

Such behaviour was definitely not a typical one, another monoclonal anti-N (N/61, reacting with sialic acid-dependent epitopes [8]) showed gradually decreasing binding with increasing degree of desialylation (Fig. 4). The antibodies 648/4B5 and 650/4B5 showed a relatively low transient increase of binding to N glycoprotein progressively desialylated by *V. cholerae* neuraminidase, and the binding to the exhaustively desialylated antigen was similar or slightly higher than to the untreated one (Fig. 4). Desialylation of N glycoprotein

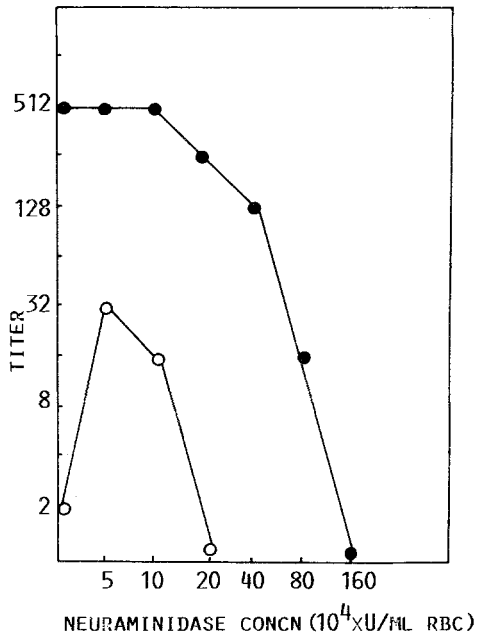

Figure 5. Agglutination of progressively desialylated M (○) and N (●) erythrocytes by the 631/3B4 antibody. The red cells were treated with *V. cholerae* neuraminidase (at indicated concentrations) for 30 min at 37°C, washed, and treated with the antibody dilutions at pH 7.5.

with *A. ureafaciens* neuraminidase did not affect the binding of 648/4B5 and 650/4B5 antibodies up to a certain enzyme concentration, but at higher enzyme doses the binding significantly decreased (Fig. 4); similar results were obtained with *C. perfringens* neuraminidase (not shown). In conclusion, treatment of the N antigen-coated plates with *A. ureafaciens* or *C. perfringens* neuraminidases reduced the binding of all anti-N antibodies tested, but the antibodies which recognize sialic acid-dependent epitopes showed a decrease of binding in the range of several-fold lower enzyme concentrations (Fig. 4B, N/61 versus 648/4B5 and 650/4B5). The most likely explanation of this finding is a contamination of the neuraminidase preparations with proteases. The most suitable reagent for the studies described above appeared to be the neuraminidase from *V. cholerae*, which is free of proteolytic activity.

The effect of progressive desialylation of erythrocytes with increasing doses of *V. cholerae* neuraminidase on the agglutinability by the 631/3B4 antibody is shown in Fig. 5. The higher affinity of the antibody to the partially desialylated antigen was demonstrated in agglutination of M erythrocytes only. Most probably, agglutination titre of N erythrocytes does not respond to an increasing antibody affinity due to a higher number of epitopes at the N red cell surface.

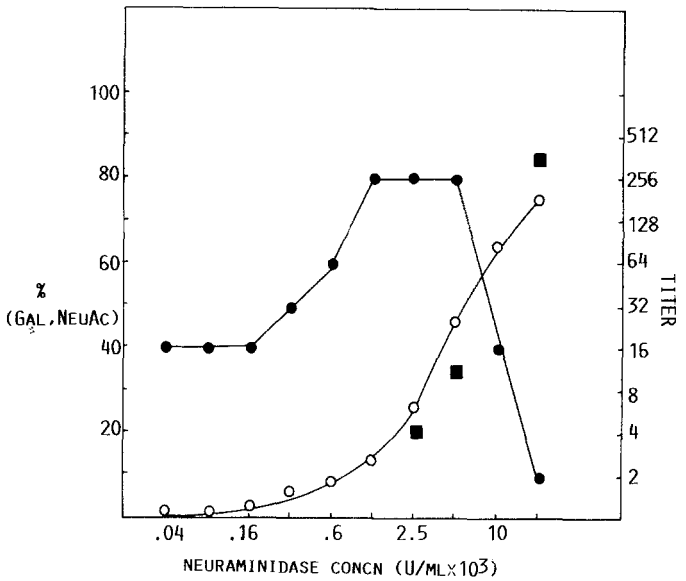

Figure 6. The effect of progressive enzymatic desialylation of N glycoprotein in solution on its reactivity with 631/3B4 antibody. The samples of 0.5% solution of N glycoprotein containing *V. cholerae* neuraminidase at concentrations indicated in the Figure were incubated for 100 min at 37°C, and then for 5 min in a boiling water bath, and were used for the following determinations: % sialic acid released (○), % galactose ozidized with periodate (■), inhibitory titre towards 631/3B4 antibody (●). The activity was measured by hemagglutination-inhibition at pH 7.5, the titre denotes dilution of 1% glycoprotein solution at which the inhibition of an equal volume of the antibody (diluted to the titre 4) was obtained. For other details see the Materials and Methods section.

The enhanced reactivity of 631/4B5 antibody with partially desialylated N glycoprotein was also shown for the antigen in solution. The glycoprotein samples were treated with increasing amounts of *V. cholerae* neuraminidase and their activity was tested by hemagglutination inhibition (Fig. 6). The highest (16-fold increased) activity was found in the samples which had 15-45% of their sialic acid released, and after losing about 75% of sialic acid the glycoprotein was almost inactive. Determination of periodate-resistant galactose in some of the samples showed that the percentage of periodate-oxidized galactose was roughly proportional to the percentage of sialic acid released (Fig. 6). Taking into account that about half of the sialic acid is linked to the C3 of galactose, the result obtained did not suggest a preferential enzymatic release of sialic acid linked either to galactose or to *N*-acetylgalactosamine. On the other hand, the high enhancement of reactivity with 631/3B4 antibody was not reproduced if N glycoprotein was gradually desialylated in sulfuric acid at 60°C, when either time of hydrolysis, or concentration of sulfuric acid was increased. The activity was not changed, or only 2-4 times increased, when up to approximately a third of the sialic acid released, and then progressively decreased.

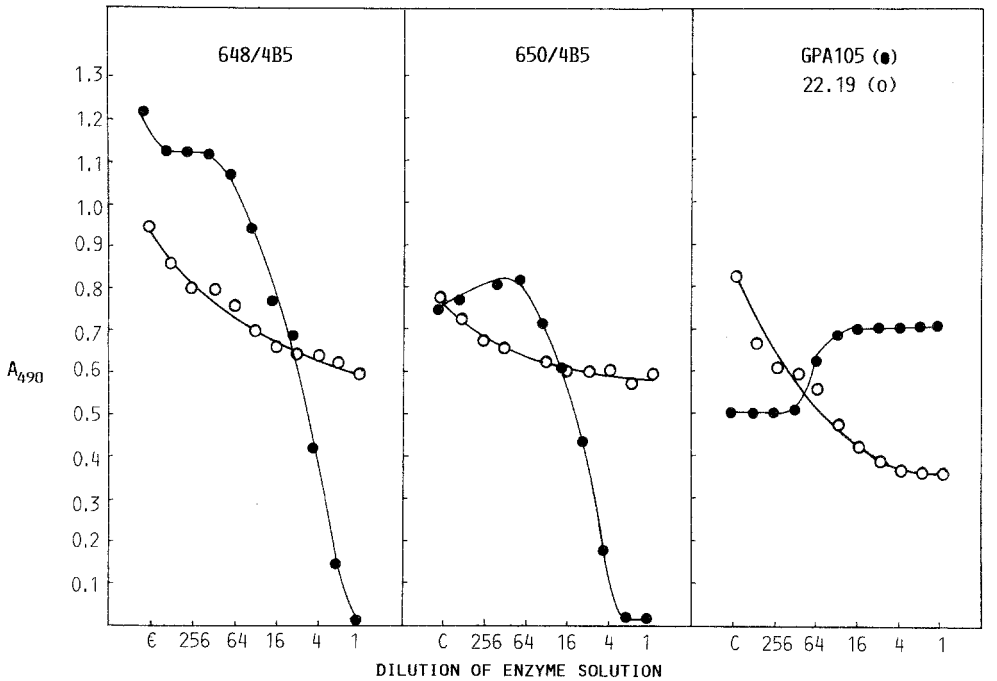

Figure 7. The effect of treatment of N glycoprotein-coated ELISA plates with the mixture of *D. pneumoniae* glycosidases (●), and N asialoglycoprotein-coated plates with β-galactosidase (○), on the binding of 648/4B5 and 650/4B5 antibodies. The antibodies GPA105 and 22.19 were used as controls. The starting concentration of β-galactosidase was 1 U/ml (0.1 U/well), the solution of *D. pneumoniae* glycosidases and other details of the assay are described in the Materials and Methods section. C denotes control wells not treated with glycosidases.

The Significance of Gal-GalNAc-Chains in Sialic Acid-independent 648/4B5 and 650/4B5 Epitopes

To elucidate this problem, asialoglycoprotein N was treated with *O*-glycanase or β-galactosidase. The antibodies 648/4B5 and 650/4B5, which reacted with desialylated antigens, showed a distinctly decreased binding to glycophorin bands on the nitrocellulose blots treated with *V. cholerae* neuraminidase and *D. pneumoniae* *O*-glycanase (not shown). Since the reaction was not completely abolished, it raised the question whether the result indicated an incomplete deglycosylation (due to insufficient amount of *O*-glycanase),

or a decreased, but persisting reaction with deglycosylated peptide. In further experiments the microtitre ELISA plate was used, and purified enzymes were replaced with a mixture of *D. pneumoniae* glycosidases, containing neuraminidase and *O*-glycanase. Treatment of N glycoprotein-coated plates with increasing amounts of the mixture of glycosidases progressively reduced the subsequent binding of 648/4B5 and 650/4B5 antibodies, down to the lack of binding at higher enzyme concentrations (Fig. 7). Despite the lack of detectable proteolytic activity in the enzyme preparation [13], the binding of the monoclonal antibody GPA105 was tested as an additional control. This antibody, directed against a trypsin-sensitive peptidic epitope of glycophorin A, showed a slightly increased binding to N glycoprotein treated with the glycosidases (Fig. 7), that confirmed the lack of proteolytic degradation of the antigen. The results obtained showed that 648/4B5 and 650/4B5 antibodies do not react with the deglycosylated N antigen, and that a crude mixture of *D. pneumoniae* glycosidases can be successfully used for deglycosylation of glycophorin, instead of expensive purified *O*-glycanase.

Treatment of N asialoglycoprotein-coated ELISA plates with β-galactosidase from *A.niger* gave a 35% and 23% reduction of binding of 648/4B5 and 650/4B5 antibodies, respectively, and a 57% reduction of binding of 22.19 antibody [9], which is directed against Thomsen-Friedenreich antigen and does not react with asialo-agalacto-glycophorin (Fig. 7). These results suggested a partial release of galactose residues from the antigen under the conditions used, and therefore, they are less conclusive.

Discussion

The routine serological and immunochemical characterization of the three new monoclonal anti-N antibodies revealed the properties which have been already reported for other anti-N monoclonal antibodies (see [1, 2]). As most anti-N, the antibodies recognized epitopes in which NH_2-terminal Leu residue with its amino group was an essential component. The significant difference was found between the antibody 631/3B4 and the antibodies 648/4B5 and 650/4B5, which was reflected in different requirements for sialylation of the antigen and opposite patterns of pH dependence. On the other hand, the antibodies 648/4B5 and 650/4B5, albeit similar, also showed some differences, such as slightly different pH optima, different response of agglutination to treatment of erythrocytes with trypsin and chymotrypsin, or different degree of requirement for the free amino group in the antigen. Various combinations of the properties mentioned above give rise to a great diversity of MN-related monoclonal antibodies.

Concerning the role of glycosylation in MN-related epitopes, it has been known that desialylation of the antigens affected their reactivity with various antibodies in different ways, from an enhancement to the loss of activity, the latter effect being most frequent (see [1, 2]). We have described here the first example of a strong "two-direction" effect of desialylation, namely the strong increase of affinity of the 631/3B4 antibody to the partially desialylated antigen, followed by the reduction and then abolishment of the reaction on further desialylation. This effect may have been related to a specific sequence of enzymatic desialylation, since it was less pronounced if the gradual desialylation was carried out by mild acid hydrolysis. Gahmberg and Andersson [16] reported that treatment of glycophorin

with *V. cholerae* neuraminidase gives a preferential release of sialic acid residues attached to galactose in *O*-linked chains. Therefore, it was tempting to speculate that two sialic acid residues present in the tetrasaccharide chain may play an opposite role in the 631/3B4 epitope: the residue linked to galactose decreases the reaction, while that linked to *N*-acetylgalactosamine is necessary. Although our results on determination of periodate-resistant galactose residues in progressively desialylated glycophorin did not support this concept, this possibility cannot be ruled out. On the other hand, it is also likely that the effect observed is not connected with any ordered sequence of enzymatic desialylation. For example, a decreasing net negative charge may result in such spatial reorganization of glycophorin molecule that leads initially to a better exposure or better fitting shape of the site reacting with 631/3B4 antibody, and then the effect is reversed. In such a case a weaker and less reproducible activation of glycophorin during partial desialylation by mild acid hydrolysis could be explained by side-effects of acidic conditions on the spatial arrangement of glycophorin. It is also possible that several reasons contribute to the effect which is quite unusual, since it has not been observed with many other anti-M and anti-N antibodies tested by us.

The antibodies 648/4B5 and 650/4B5, which recognized epitopes independent of sialylation, were tested for binding to N glycoprotein immobilized on ELISA plates and treated with neuraminidase and *O*-glycanase or β-galactosidase. The enzymatic treatment of immobilized antigen was the method of choice, since it was possible to avoid the problems of an increased aggregation and decreased solubility of deglycosylated glycophorin [17]. The antibodies did not bind to the antigen deglycosylated with *O*-glycanase, and showed only a partial reduction of binding to the (asialo)antigen treated with β-galactosidase.

These results indicated that the epitopes for 648/4B5 and 650/4B5 antibodies require the presence of *O*-linked *N*-acetylgalactosamine residues which either participate directly in the reaction, or contribute to the proper conformation of the peptidic epitope. The role of galactose residues is less certain, since inability of the complete inactivation of the antigen by β-galactosidase may indicate either a minor significance of the galactose residues, or their resistance to enzymatic hydrolysis. Some of anti-M and anti-N antibodies tested in the 1st Workshop on Monoclonal Antibodies against Red Cell and Related Antigens (Paris, 1987) were reported to agglutinate Tn red cells which lack NeuAc-Gal-residues in *O*-linked chains of glycophorins. It suggested that these antibodies did not require the presence of galactose residues in blood group MN-related epitopes. Comparison of the effect of β-galactosidase and the ability of various anti-M and anti-N antibodies to react with Tn red cells could help to elucidate this problem. The role of galactose residues in blood group M- and N-related epitopes may be diverse, similar to the role of sialic acid residues. Testing more antibodies with neuraminidase- and *O*-glycanase-treated antigens should show whether such diversity also concerns the *O*-linked *N*-acetylgalactosamine residues of glycophorin.

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