Glycoconjugate Journal (1994) **11**:371–374

Vicia villosa B4 lectin is the second anti-Tn lectin shown to react better with blood group N than M antigen

MARIA DUK[1,2] ALBERT M. WU[2] and ELWIRA LISOWSKA[1]*

[1] *Department of Immunochemistry, Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Czerska 12, 53-114 Wroclaw, Poland*

[2] *Glycoimmunochemistry Research Laboratory, Institute of Molecular and Cellular Biology, Chang-Gung College of Medicine and Technology, Kwei-san, Tao-yuan, Taiwan R.O.C. 33332*

Received 2 May 1994, revised 23 June 1994

Earlier studies showed that *Moluccella laevis* lectin, which has anti-Tn specificity, reacts more strongly with native or desialylated blood group N glycophorin A than with the respective glycophorins of blood group M. We now present results indicating that *Vicia villosa* B4 anti-Tn lectin, which does not show detectable reaction with untreated glycophorins or erythrocytes, reacts better with desialylated blood group N antigen than with asialo M antigen. This was demonstrated by three assays: (1) agglutination of asialoerythrocytes; (2) binding of biotinylated lectin to asialoerythrocytes immobilized on ELISA plates; and (3) inhibition of lectin binding to asialo-agalactoglycophorin with asialoglycophorins M and N. These results supply further support for the conclusion that glycophorin of blood group N has more GalNAc residues unsubstituted with Gal (Tn receptors) than glycophorin of blood group M.

Keywords: blood groups MN; glycophorin; lectins; Tn antigen; *Vicia villosa* lectin

Abbreviations: GPA, glycophorin A; GPA-M and GPA-N, GPA from OM and ON erythrocytes, respectively; MLL, *Moluccella laevis* lectin; PBS, 0.02 M phosphate buffer/0.15 M NaCl, pH 7.4; PNA, peanut agglutinin; RBC, erythrocytes; TBS, 0.05 M Tris buffer/0.15 M NaCl, pH 7.4; TBS-T, TBS containing 0.02% Tween 20; VVL, *Vicia villosa* B4 lectin.

Introduction

The experiments described in this report were prompted by the results obtained with *Moluccella laevis* lectin (MLL). This lectin showed surprising serological specificity in agglutinating blood group A-positive and N-positive erythrocytes [1, 2]. Such specificity was difficult to explain because blood group A and N antigens are structurally unrelated. Blood group A determinants are located in the oligosaccharide chains terminated with the structure GalNAcα1-3[Fucα1-2]Gal- [3]. Blood group M and N antigens are represented by two forms of glycophorin A (GPA) differing in amino acid residues at positions 1 and 5 [reviewed in 4]:

M: **Ser**-Ser*-Thr*-Thr*-**Gly**-Val-Ala-Met-His-

N: **Leu**-Ser*-Thr*-Thr*-**Glu**-Val-Ala-Met-His-

The amino acid residues 2–4 (marked with asterisks) carry *O*-linked oligosaccharide chains of the following structure: NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAcα-.

Agglutination of blood group A- and N-positive erythrocytes by the purified MLL was similarly inhibited by several monosugars tested, among which GalNAc was the strongest inhibitor [2]. These results indicated that the same binding site of the lectin is involved in the reactions with A and N antigens and were in agreement with the blood group A specificity of MLL. However, the blood group N specificity of MLL was still mysterious. Immunochemical studies with glycophorins and other glycoproteins revealed the anti-Tn specificity of MLL, with a weak cross-reactivity with blood group A antigen [5, 6]. The lectin reacted most strongly with Tn antigens (i.e. glycoproteins carrying multiple GalNAc-Ser/Thr residues), including asialo-agalactoGPA, and showed much weaker reactivity with asialoGPA and the weakest one with untreated GPA. In spite of this, stronger binding of MLL was observed with GPA-N and asialoGPA-N than with the respective blood group M antigens. The resistance of MLL-receptors present in asialoGPA to *O*-glycanase suggested that these receptors

* To whom correspondence should be addressed.

are *O*-linked GalNAc residues unsubstituted with Gal which are present in GPA in minor amount. Moreover, the results indicated that GPA-N has a higher content of these minor components than GPA-M.

If the latter conclusion is true, other anti-Tn lectins showing a detectable reaction with GPA, or at least with asialoGPA, should react more strongly with GPA of blood group N than of blood group M. In this report we present data indicating that *Vicia villosa* B4 lectin (VVL), which is one of the most specific anti-Tn reagents [7–9], reacts weakly with asialoGPAs and shows a distinct preference in its reaction with asialoGPA-N over that with asialoGPA-M.

Materials and methods

Erythrocytes (RBC)

Blood group OM and ON erythrocytes were obtained from healthy donors. Erythrocytes were desialylated by incubation of 0.4 ml 50% RBC suspension in PBS with 0.04 U *Vibrio cholerae* sialidase (Serva, Germany) for 1 h at 37 °C.

Glycophorin A (GPA)

GPA was prepared from the membranes of outdated blood group OM and ON erythrocytes [10]. GPA was desialylated by mild acid hydrolysis in 0.025 M sulfuric acid for 4 h at 60 °C; over 90% of sialic acid was removed under these conditions. Asialo-agalactoGPA was obtained from asialoGPA by Smith degradation [11].

Lectins

Vicia villosa B4 lectin (VVL) and peanut agglutinin (PNA) were products of Sigma (USA). *Salvia sclarea* lectin was purified from the seed extract by affinity chromatography on immobilized asialo-agalactoGPA (the details of purification will be published elsewhere). VVL was biotinylated with biotinamidocaproate-*N*-hydroxysuccinimide ester (Sigma, USA) by incubating the lectin sample (200 µg per 250 µl PBS) with 400 µl 0.025% ester solution in PBS for 30 min at room temperature. The sample was dialysed against water and then against TBS (Tris-buffered saline, pH 7.4), and was supplemented with sodium azide to a final concentration of 0.1%.

Haemagglutination

The test was performed in U-well microtitre plates. Serially diluted lectin (20 µl per well) was mixed with an equal volume of 2% asialoRBC suspension and agglutination was read after 60 min at 20 °C.

Microtitre plate biotin/avidin-mediated lectin assays

Inhibition of VVL by asialoGPA-M and -N

The test was performed as recently described [12]. The ELISA plates (Nunc MaxiSorp, Austria) were coated with asialo-agalactoGPA (0.1 µg in 50 µl 0.05 M carbonate buffer of pH 9.6 per well) overnight at 4 °C. All further steps were performed at 20 °C and the volume of each reagent applied to the plate was 50 µl per well. All reagents, except the substrate, were diluted with TBS containing 0.05% Tween 20 (TBS-T) and the same buffer was used for washing the plates between incubations.

Serially diluted inhibitor samples (and TBS-T as a control) were mixed with an equal volume of biotinylated VVL (1 µg ml^{-1}). After 1 h these samples were tested for binding of VVL to the coated plates, by consecutive incubation of the plates with: (1) inhibitor-VVL sample (30 min); ExtrAvidin/alkaline phosphatase conjugate (Sigma, USA, diluted 1:10000, 1 h); and (3) Sigma 104® phosphatase substrate in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ (5 mg tablets, 1 tablet per 5 ml, 30 min). The plates were read at 405 nm in a microtitre plate reader.

Binding of VVL to immobilized erythrocytes

The wells of ELISA plates were coated with asialoRBC according to a slightly modified procedure of Bigbee *et al.* [13]. Briefly, the wells were consecutively filled with 50 µl PBS containing: (1) poly-L-lysine, 50 µg ml^{-1}, 1 h at 37 °C, and washed with PBS; (2) asialoRBC, about 10^6 per 50 µl, 1 h at 20 °C, excess unbound RBC were rejected; (3) 0.025% glutaraldehyde, 30 min at 20 °C, and washed. The coated wells were filled with 100 µl samples of 1% BSA in TBS-T and left for 1 h at 20 °C, or overnight at 4 °C. After washing with TBS-T, binding of serially diluted biotinylated VVL was measured as described above. The lectin was tested in the presence and absence of 0.2 M GalNAc, and the difference between both values for each lectin dilution was assumed as specific binding. Since the binding of VVL to asialoRBC was low, the plate was read at 405 nm after 2.5–3 h incubation with the substrate.

Both tests were performed in duplicate and the results are mean values. The standard deviation in most cases was less than 5% of the mean value.

Results

VVL did not agglutinate untreated erythrocytes, but agglutinated quite strongly asialoRBC. Comparison of agglutination of five random OM and five ON desialylated RBC samples (Fig. 1) showed that the minimal VVL concentration required for agglutination of OM RBC was in the range of 1.2–3 µg ml^{-1}, while ON RBC were agglutinated at concentration 0.15–0.3 µg ml^{-1}. PNA, used as a control lectin, agglutinated almost identically all erythrocytes tested. The receptors for PNA are Gal-GalNAc-chains of asialoGPA [8] and the results showed that M and N RBC did not differ in GPA content or degree of desialylation.

The biotinylated VVL showed distinctly stronger binding

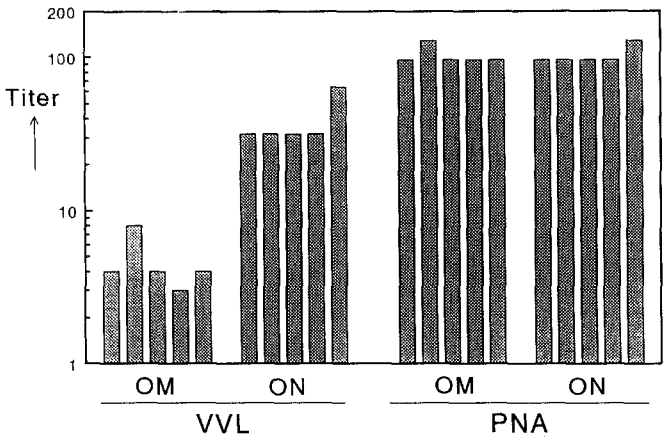

Figure 1. Agglutination of neuraminidase-treated OM and ON erythrocytes by VVL and PNA. The erythrocyte samples derived from 10 unrelated donors. The initial concentration of lectins was 10 μg ml^{-1}.

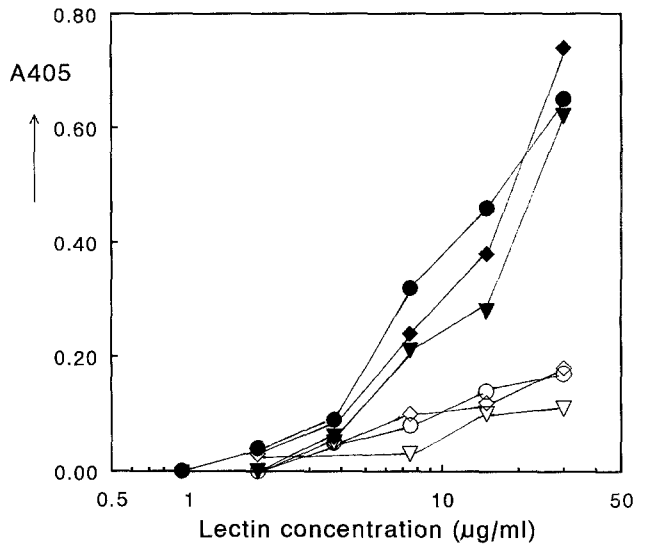

Figure 2. Binding of biotinylated VVL to OM and ON asialo-erythrocytes immobilized on ELISA plate. The erythrocyte samples were obtained from three OM donors (open symbols) and three ON donors (closed symbols).

to ON asialoRBC immobilized on ELISA plates than to OM asialoRBC (Fig. 2), while the binding of PNA to both types of erythrocytes was comparable (not shown).

VVL did not give any detectable reaction with isolated untreated GPA, and reacted weakly with asialoGPA. For comparison of the reactivity of VVL with asialoGPA-M and asialoGPA-N, inhibition of lectin binding to asialo-agalactoGPA-coated ELISA plates was chosen as the most sensitive assay. Here again, asialoGPA-N inhibited VVL more strongly than asialoGPA-M, showing about a six- to 15-fold difference in concentration of both antigens required for 50% inhibition of binding (Fig. 3).

Preferential reaction with asialoGPA-N was not observed with such Tn-reactive lectins as *Helix pomatia* agglutinin or

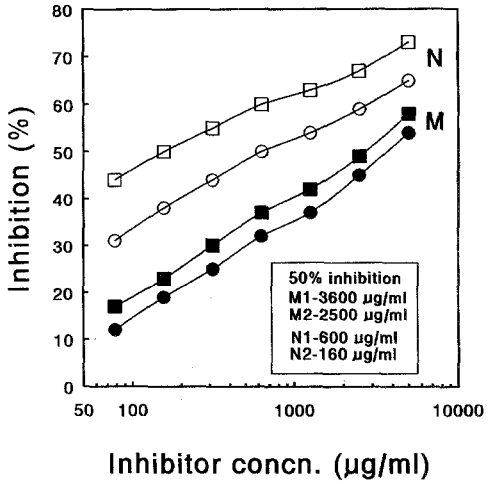

Figure 3. Inhibition of VVL binding to asialo-agalactoGPA-coated ELISA plates by serially diluted asialoGPA-M (M, closed symbols) and asialoGPA-N (N, open symbols). M1 and N1 (circles), crude GPA preparations, M2 and N2 (squares), purified GPA preparations. Insert: concentrations of GPA-M and GPA-N giving 50% inhibition of the control lectin binding.

jacalin (results not shown). However, these lectins are not Tn-specific, because they react similarly with GalNAc- (present in asialo-agalactoGPA) and Galβ1-3GalNAc- (present in asialoGPA) [12, 14, 15 and our unpublished data]. The stronger reactivity of GPA-N than that of GPA-M seems to be limited to lectins with restricted anti-Tn specificity which do not react with GalNAc substituted at C3 and Gal. To strengthen this conclusion we tried to include *Salvia sclarea* anti-Tn lectin [14, 16] to these studies. However, *S. sclarea* lectin did not give detectable reaction with asialoglycophorins and could be checked only in agglutination assays with asialoRBC at relatively high lectin concentration. The lectin tested at concentrations of 160, 80, and 40 μg ml^{-1} gave the agglutination patterns 2+, + or −, −, respectively, with four samples of blood group M asialoRBC, and 3+, 3+, + or −, with four samples of N asialoRBC. These results suggested that *S. sclarea* lectin also reacts more strongly with GPA-N than with GPA-M, but due to its generally weaker reactivity with asialoRBC the difference in agglutination was less spectacular than that obtained with *V. villosa* lectin.

Discussion

The results obtained showed that VVL, like MLL, reacts more strongly with asialoGPA of blood group N than M. Since both lectins have well documented anti-Tn specificity, the results support the conclusion that GPA-N has more Tn receptors than GPA-M. These receptors (GalNAc-Ser/Thr) may be (at least in part) sialylated in untreated GPA to give the NeuAcα2-6GalNAc-Ser/Thr structure. This may be the reason for the lower reactivity of GPAs

relative to asialoGPAs with anti-Tn lectins, because these lectins were shown to react less strongly with sialyl-Tn than with Tn receptors [5, 12].

The major *O*-glycosidic chain of GPA is the tetrasaccharide described in the Introduction but the presence of minor amounts of 'incomplete' oligosaccharide chains is quite possible in view of the well known microheterogeneity of protein glycosylation. The structural studies on the oligosaccharides of human glycophorins did not give unequivocal evidence for the presence of non-galactosylated *O*-linked chains, since due to their low content they could escape detection [17, 18], or if detected as minor components, they were considered to be the degradation products of larger chains [19, 20]. This problem has been recently re-investigated with a focus on the identification of free GalNAc-ol in the products of the β-elimination of GPA-M, GPA-N and N-terminal tryptic glycopeptides of these glycoproteins [21]. A small amount of GalNAc-ol (corresponding to the presence of one residue per several GPA molecules) was identified by glc-ms in native GPA, its content was higher in asialoGPA and, interestingly, was about double in GPA-N or GPA-N-derived preparations in comparison with the respective GPA-M preparations. It is tempting to speculate that a minor number of non-galactosylated GalNAc molecules is present randomly in GPA-M and -N, and in addition there is some site(s) of defective galactosylation present in GPA-N only. This hypothetical site, if dependent on MN status, should be located in the N-terminal portion of GPA in proximity to one of the blood group-typical amino acid residues at positions 1 and 5 of the polypeptide chain. Such a location in the most exposed portion of GPA would be in agreement with the preferential contribution of this 'N-related' GalNAc residue to reaction with anti-Tn lectins. The Tn receptors are not detectable in GPA or asialoGPA by anti-Tn monoclonal antibodies [11]. However, most anti-Tn antibodies may require clusters of GalNAc residues [22], whereas anti-Tn lectins are able to recognize single residues.

These considerations need direct experimental evidence but the problem is interesting with respect to the regulation of glycosylation by amino acid residues adjacent to the glycosylated site. The dependence of the extent of processing on the accessibility of the oligosaccharides for glycosyltransferases has been already suggested by differences in the patterns of *N*-glycans identified in individual glycosylation sites of Thy-1, a neural surface glycoprotein [23]. Quite recently, Granovsky *et al.* [24] reported that 3-β-D-galactosyltransferase activity synthesizing *O*-glycan core 1 is controlled by the amino acid sequences of glycopeptide substrates, using as substrates synthetic peptides with GalNAc attached to Ser or Thr residues. The reactivity of anti-Tn lectins with GPA draws attention to the possibility that the extent of processing of *O*-glycans *in vivo* is also affected by the microenvironment of the glycosylated site.

Acknowledgements

This work was supported by Grants from the National Science Council (NSC 82-0412-B-182-088 and 82-0418-B-182-009), Taipei, Taiwan, and by the Grant No. 4-0622-91-01 of the Committee of Scientific Studies (KBN), Warsaw, Poland.

References

1. Bird GWG, Wingham J (1970) *Vox Sang* **18**:235–9.
2. Lis H, Latter H, Adar R, Sharon N (1988) *FEBS Lett* **233**:191–5.
3. Watkins WM, Greenwell P, Yates AD (1981) *Immunol Commun* **10**:83–100.
4. Lisowska E (1988) In *Molecular Immunology of Complex Carbohydrates* (Wu AM, ed.) pp. 265–315. New York and London: Plenum Press.
5. Duk M, Mitra D, Lisowska E, Kabat EA, Sharon N, Lis H (1992) *Carbohydr Res* **236**:245–58.
6. Lis H, Sharon N (1993) *Trends Glycosci Glycotechnol* **6**:65–74.
7. Tollefsen SE, Kornfeld R (1983) *J Biol Chem* **258**:5172–6.
8. Wu AM, Sugii S, Herp A (1988) In *Molecular Immunology of Complex Carbohydrates* (Wu AM, ed.) pp. 819–53. New York and London: Plenum Press.
9. Puri KD, Gopalakrishnan B, Surolia A (1992) *FEBS Lett* **312**:208–12.
10. Lisowska E, Messeter L, Duk M, Czerwiński M, Lundblad A (1987) *Molec Immunol* **24**:605–13.
11. Duk M, Steuden D, Duś D, Radzikowski C, Lisowska E (1992) *Glycoconjugate J* **9**:148–53.
12. Duk M, Lisowska E, Wu JH, Wu AM (1994) *Anal Biochem*, in press.
13. Bigbee WL, Vanderlaan M, Fong SSN, Jensen RH (1983) *Molec Immunol* **20**:1353–62.
14. Piller V, Piller F, Cartron JP (1990) *Eur J Biochem* **191**:461–6.
15. Ahmed H, Chatterjee BP (1989) *J Biol Chem* **264**:9365–72.
16. Piller V, Piller F, Cartron JP (1986) *J Biol Chem* **261**:14069–75.
17. Krotkiewski H, Lisowska E, Nilsson G, Grönberg G, Nilsson B (1993) *Carbohydr Res* **239**:35–50.
18. Lisowska E, Duk M, Dahr W (1980) *Carbohydr Res* **79**:103–13.
19. Thomas DB, Winzler RJ (1969) *J Biol Chem* **244**:5943–6.
20. Fukuda M, Lauffenburger M, Sasaki H, Rogers ME, Dell A (1987) *J Biol Chem* **262**:11952–7.
21. Krotkiewski H, Nilsson B, Lisowska E (1993) *Glycoconjugate J* **10**:285 (abstr).
22. Nakada H, Numata Y, Inoue M, Tanaka N, Kitagawa H, Fukanoshi I, Fukui S, Yamashina I (1991) *J Biol Chem* **266**:12402–5.
23. Williams AF, Parekh RB, Wing DR, Willis AC, Barclay AN, Dalchau R, Fabre JW, Dwek RA, Rademacher TW (1993) *Glycobiology* **3**:339–48.
24. Granovsky M, Bielfeldt T, Peters S, Paulsen H, Meldal M, Brockhausen J, Brockhausen I (1994) *Eur J Biochem* **221**:1039–46.