Naturally occurring hemagglutinins in the hemolymph of the scorpion *Paruroctonus mesaensis* Stahnke[1]

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Summary. Lectins which bind sialic acid-containing receptors are present in the hemolymph of the scorpion *Paruroctonus mesaensis* Stahnke. Glycoproteins like bovine submaxillary mucin, fetuin and human orosomucoid behave as strong inhibitors for *Paruroctonus* lectins; desialylation of glycoproteins results in a drastic reduction of their inhibitory capabilities confirming that sialic acids are the terminal monosaccharides of *Paruroctonus* lectin receptors.

The presence of naturally occurring humoral agglutinins has previously been reported in the scorpions *Androctonus australis*[3] and *Centruroides sculpturatus*[4]. Specificity for sialic acids containing receptors appears to be a common feature between these and the lectins from the merostomes *Limulus polyphemus*[5,6], *Tachypleus tridentatus*[7], and *Carcinoscorpius rotundicauda*[8]. In the present report the partial determination of the specificity of hemagglutinins detected in the hemolymph of the scorpion *Paruroctonus mesaensis* Stahnke is described.

Material and methods. Paruroctonus and *Limulus* sera: scorpions were collected near Mesa, Arizona, and bled from pedipalps. Scorpion hemolymph was allowed to clot at room temperature (25±2 °C), the serum was cleared by centrifugation at 5000× g for 15 min, and stored at −25 °C until used. *Limulus* serum was received by courtesy of Dr J. Granberry, Limulus Laboratories, Florida.

Erythrocytes (RBC). Human blood samples were collected in ACD. All other blood samples from diverse vertebrate species were obtained by venous or cardiac puncture and collected in Alsevers. RBC were washed twice with saline 0.85% and twice with tris-buffered saline (TBS): 100 mM tris-HCl, 50 mM NaCl, 10 mM $CaCl_2$, pH 7.6) and suspended at concentrations of 5×10^6 RBC/ml in TBS.

Reagents. Neuraminidase from *Vibrio cholerae* (VCN) 500 U/ml and fetuin were purchased from GIBCO, Grand Island, New York. N-Acetylneuraminic acid β-methyl glycoside (NANAβMeGly) was kindly supplied by Dr W. Korytnyk, RPMI, Buffalo, New York. Bovine lung galactan was a gift from Hoffman-La Roche, Nutley, NJ. All other reagents were purchased from Sigma Chemical Co, St. Louis, Missouri, at the highest purity available.

Enzyme treatment of RBC. RBC were treated with pronase P (Pr)(protease type VI from *Streptomyces griseus*, 3–4 units/mg) and VCN by the procedure of Uhlenbruck et al.[9]. Pr-treated RH_o(D) human RBC showed satisfactory agglutination titers with an incomplete anti-D serum (Ortho).

Agglutination test. 5 µl of 2-fold serial dilutions of *Paruroctonus* or *Limulus* serum in TBS were placed in Terasaki 96 well trays (Robbins Scientific) and equal volumes of RBC suspension were added. The trays were vortex-mixed for 10 sec at speed 1 and incubated at room temperature for 45 min. Agglutination was read under the microscope and graded from 0 (negative) to 4. Controls for all titrations were the substitution of sera by TBS.

Desialylation of glycoproteins. Glycoproteins were disolved in 0.1 N H_2SO_4, 0.85% NaCl at concentrations of 10 mg/ml. Hydrolysis was carried out for 1 h at 80 °C. Released sugars were analyzed by paper chromatography in ethyl acetate:pyridine:acetic acid:H_2O (5:5:1:3) for neutral sugars[10] using 1 µg of D-galactose, D-glucose, D-mannose (D-Man) and L-fucose (L-Fuc) as standards developing with the $AgNO_3$ and alcoholic KOH reagents[11], and in n-butanol:n-propanol:0.1 N HCl (1:2:1) for sialic acids[12], using 1 µg of N-acetylneuraminic acid (NANA) and N-glycolylneuraminic acid (NGNA) as standards developing with the resorcinol reagent. Sialic acids were the only sugars detected in the chromatograms. No neutral hexoses were released as measured by the anthrone reagent[13]. Released and total sialic acids were determined by the thiobarbituric acid[14] and resorcinol[15] methods respectively. Percentages of released sialic acids are as follows: bovine submaxillary mucin (BSM): 72.1%; fetuin: 78.1% and human orosomucoid: 98.3%. Hydrolyzed glycoproteins were exhaustively dialyzed against 0.85% NaCl and TBS, aliquoted and stored at −25 °C.

Hemagglutination-inhibition tests. *Paruroctonus* and *Limulus* sera were diluted to 4–8 agglutination units. All substances to be tested were dissolved in TBS (at concentrations up to 200 mM for mono- and oligosaccharides and 1% (w/v) for polysaccharides and glycoproteins and brought to pH 7.6 with concentrated NaOH. Equal volumes of diluted sera and inhibitor solution were mixed and incubated for 45 min at room temperature. The mixtures were titrated as described before, with untreated or enzyme treated RBC. Minimal concentrations required for the inhibition of 2 agglutination units were recorded. Controls were the substitution of the inhibitor solution by TBS, and as described before, substitution of the sera by TBS.

Results and discussion. Paruroctonus and *Limulus* agglutination profiles are very similar (table 1): except for horse and rat RBC, titers are increased by Pr-treatment of the RBC and agglutination is abolished after VCN-treatment.

Table 1. Hemagglutination patterns of *Paruroctonus* and *Limulus* hemolymph

Erythrocytes	*P. mesaensis*			*L. polyphemus*		
	Unt	Pr	VCN	Unt	Pr	VCN
Human A_1	+++	++++	0	+++	++++	0
B	+++	++++	0	+++	++++	0
O	+++	++++	0	+++	++++	0
Stump tail monkey	+	++++	0	+++	++++	0
Baboon	+	++++	0	+++	++++	0
Rhesus monkey	+	++++	0	+++	++++	0
Bovine	++	+++	0	+	++	0
Cat	+++	++++	0	+++	+++	++
Horse	++++	++++	++++	+++	+++	+++
Rat	++++	++++	++++	+++	++++	+++

Figures are titer ranges: +: 1–8; ++: 16–64; +++: 128–512; ++++: 1024–4096. Unt, untreated; Pr, pronase; VCN, neuraminidase.

This fact would suggest sialic acid containing receptors for *Paruroctonus* lectins. *Paruroctonus* does not differentiate between ABO groups in untreated or enzyme-treated RBC. Horse and rat RBC are agglutinated at high titers even if VCN-treated. This has already been observed for other sialic acid binding lectins[4] and VCN-resistant sialic acid is probably responsible for this pattern, since sialic acids or sialocompounds are still the best inhibitors when testing with VCN-treated horse RBC (table 2). Horse RBC are known to contain VCN-resistant lipid bound sialic acid[16]. Rat RBC, although very different from horse RBC in their lack of N-glycolylneuraminic acid (NGNA) still exhibit O-acetylated sialic acid which is VCN-resistant[17]. This indicates that the type of acyl group, acetyl or glycolyl, on the N does not affect the binding of the lectins. This is confirmed in the hemagglutination-inhibition experiments since N-acetylneuraminic acid (NANA) and NGNA exhibit similar inhibitory power. However the N-acyl group appears to be an absolute requirement for the binding since methoxyneuraminic acid does not inhibit while the NANAβMeGly inhibits at low concentration. Better inhibitory capibilities of the latter compared to NANA would indicate that a substitution on C_2 hydroxyl is also important for *Paruroctonus* specificity. 2-keto-3-deoxyoctonate (KDO) inhibits only when testing with human RBC, suggesting heterogeneity of these agglutinins. KDO is present in gram positive bacteria, and according to Rostam-Abadi and Pistole[18], the receptor for agglutinins present in *Limulus* hemolymph, and thus another possible common feature between these and *Paruroctonus* agglutinins. Inhibition by colominic acid, a homopolymer of NANA linked 2-8, and sialyllactose (NANA-α-2-3(6)-Gal-β1-4-Glc] indicates that the type of binding (2-3, 2-6 or 2-8) of sialic acid to a 2nd carbohydrate molecule, is not the main factor which defines *Paruroctonus* specificity. N-Acetyl-D-galactosamine (GalNAc) and N-acetyl-D-glucosamine (GlcNAc) are as good inhibitors as sialic acids. However they are not likely to be the main receptors of *Paruroctonus* since porcine stomach mucin and desialylated BSM (both rich in terminal GalNAc) do not inhibit at all. *Paruroctonus* and *Limulus* differ in their inhibition by hexuronic acids: the former is not inhibited by glucuronic or galacturonic acids at concentrations up to 100 mM while the latter is inhibited by glucuronic acid but not galacturonic acid[19]. Expressed in terms of sialic acid content, BSM, fetuin and human orosomucoid exhibit much higher inhibitory power than the free sialic acids indicating that probably not only the terminal sugar is important in the binding, but also subterminal sugars and conformational aspects of the oligosaccharide chains, and in this aspect *Paruroctonus* behaves as *Limulus*. Desialylation of glycoproteins results in a drastic reduction of inhibitory capabilities, which confirms that sialic acid is the terminal sugar of *Paruroctonus* receptors. Sialic acid poor glycoproteins like ovomucoid do not inhibit at all. Binding of *Paruroctonus* lectins to glycoproteins opens the possibility of their isolation and purification by affinity chromatography, in order to achieve a complete characterization of their specificity.

Table 2. Hemagglutination-inhibition of *Paruroctonus* hemolymph agglutinins with carbohydrates and glycoconjugates

Inhibitors (mM)	Human (O Rh_o) Pr	Horse Pr	VCN	Specificity factor (for Hu O Rh_o)
NANA	100	100	100	1
NGNA	100	100	100	1
GalNAc	100	100	100	1
GlcNAc	100	100	100	1
Sialyllactose	50	50	50	2
NANAβMeGly	25	NI	25	4
KDO	50	NI	NI	2
Colominic acid*	4.0	4.0	4.0	25
Orosomucoid (human)*	0.4	0.4	0.4	250
Fetuin*	0.3	0.3	0.3	333
BSM*	0.14	0.3	0.14	714

NANA, N-acetylneuraminic acid; NGNA, N-glycolylneuraminic acid; NANAβMeGly, N-acetylneuraminic acid β-methylglycoside; GalNAc, N-acetyl-D-galactosamine; GlcNAc, N-acetyl-D-glucosamine; KDO, 2-keto-3-deoxyoctonate; BSM, bovine submaxillary mucin. *Expressed in terms of NANA content. Figures are minimal concentrations (mM) that inhibit 2 agglutination units. NI, no inhibition at tested concentrations. D-galactose, D-glucose, L-fucose, methoxyneuraminic, glucuronic and galacturonic acids did not inhibit at concentrations up to 200 mM. Bovine lung galactan, orosomucoid and porcine stomach mucin did not inhibit at concentrations up to 10 mg/ml.

Table 3. Hemagglutination-inhibition of *Paruroctonus* hemolymph agglutinins with untreated and desialylated glycoproteins

	Human* Pr	Horse* Pr	VCN	Sialic acid content (%)
BSM	0.06	0.125	0.06	7.7
Asialo-BSM	0.50	1.00	1.00	2.2
Fetuin	0.125	0.125	0.125	8.5
Asialo-fetuin	1.00	NI	NI	2.46
Orosomucoid (human)	0.125	0.125	0.125	11.4
Asialo-orosomucoid	1.00	NI	NI	0.2

*Figures are minimal concentrations (% w/v) required for the inhibition of 2 agglutination units of *Paruroctonus;* BSM, bovine submaxillary mucin. NI, no inhibition at tested concentrations.

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