An agglutinin with unique specificity for N-glycolyl sialic acid residues of thyroglobulin in the hemolymph of a marine crab *Scylla serrata* (Forskal)

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Abstract. A novel agglutinin with specificity for sialic acid sequence of sugars in thyroglobulin is identified in the hemolymph of *Scylla serrata*. The physico-chemical characteristics of its binding affinity, such as pH and temperature optima, and cationic requirements are defined. N-glycolyl neuraminic acid (NeuGc) (at 0.6 mM), in contrast to N-acetyl neuraminic acid (NeuAc) (at > 5.0 mM), is the potent inhibitor of hemagglutination. Bovine and porcine thyroglobulins containing NeuGc, inhibited the agglutination. NeuGc-acid glycoprotein fraction (bovine) but not NeuAc-acid glycoprotein fraction (human) inhibited the hemagglutination. The inability of other NeuGc-glycoproteins (bovine submaxillary mucin) to inhibit the agglutination suggests that the agglutinin may also recognize glycosidic linkage associated with NeuGc. The potential of the agglutinin in identifying NeuGc containing human tumor associated antigens is discussed.
Key words. Hemolymph; agglutinin; sialic acids; N-acetyl neuraminic acid (NeuAc); N-glycolyl neuraminic acid (NeuGc); thyroglobulin; glycosidic linkages.

Invertebrate agglutinins are sugar-binding proteins with multiple binding sites (multivalent), diverse biological roles[1-7], and biomedical applications[8]. An agglutinin may recognize a part of a sugar[9], a whole sugar[10], their glycosidic linkage[11], or a sequence of sugars[1,5]. Among invertebrates, hemolymph of arthropods and mollusks contain agglutinins that specifically recognize a unique family of sugars called sialic acids. Although sialic acids are not synthesized by these invertebrates, they are found on the cell surface of many pathogenic bacteria and vertebrate tissues. The type of sialic acid and the glycosidic linkages with the adjacent sugar in an oligosaccharide differ among pathogenic bacteria[8] and human cancer tissues[12,13]. Therefore, agglutinins with specificity for different kinds of sialic acids and their glycosidic linkages serve as potential diagnostic tools.

While developing strategies for affinity purification of an hemolymph agglutinin of a marine crab *Scylla serrata*, we have described an agglutinin with unique specificity for sialyl residues of thyroglobulin. We have studied the physico-chemical properties of the agglutinin with a view to increase its binding affinity. The potent glycoprotein inhibitor of the agglutinin can be used as an affinity matrix for purification.

Materials and methods

Hemagglutination assays (HA) were performed in Tris buffer-saline (TBS) with or without metal ions. The pH (5.5–9.0) and temperature (25 °C–45 °C) dependence of agglutinating activity were measured by incubating the serum at specific pH or temperature for an hour before adding erythrocyte suspension. Erythrocyte suspension and asialo erythrocytes for adsorption assays were prepared as described earlier[14,15].

Results

Hemolymph of *S. serrata* selectively agglutinated human B, rabbit and mouse erythrocytes (table 1). Adsorption studies were carried out to assess whether the agglutination activity is due to the presence of one or more agglutinins. When the hemolymph was adsorbed to human B or mouse or dog erythrocytes, it failed to agglutinate erythrocytes from the other two species. However it continued to agglutinate those of rabbit and guinea pig even after adsorption with human B and rabbit erythrocytes. However, when the hemolymph was adsorbed twice with human B or dog or mouse erythrocytes, its ability to agglutinate rabbit or guinea pig erythrocytes was lost (table 2) suggesting the presence of one hemagglutinin in the hemolymph.

Sialic acid affinity of the agglutinin is indicated when hemolymph poorly agglutinated sialidase treated human B (HA: 32) in contrast to untreated control human B cells (HA: 128). Therefore, agglutination inhibitory potency of different sialic acids (N-acetyl neuraminic acid

Table 1. Hemagglutination titers of hemolymph of *S. serrata*. 20 µl of serum suspended in TBS (pH 7.2) were serially diluted in a microtiter plate and mixed with 25 µl of 1.5% suspension of erythrocytes. The HA titer was determined as the reciprocal of the highest dilution of serum giving complete agglutination after 60 min at 30 °C.

Erythrocytes	HA titers of the hemolymph
Human A	0
Human B	64–128
Human O	8
Sheep	2
Goat	4
Ox	0
Water buffalo	16
Horse	64–128
Rabbit	64–128
Guinea pig	4
Pig	32
Dog	32
Cat	32
Rat	32
Mouse	128
Chicken	2

[NeuAc] and N-glycolyl neuraminic acid [NeuGc]), sialyl oligosaccharide (sialyl lactoses with NeuAc 2–3 and NeuAc 2–6 linkages) and sialoglycoproteins (table 3) were tested. While NeuGc inhibited the hemagglutination at a molar concentration of 0.6 mM, NeuAc and sialyl lactoses did not show any inhibition at 5 mM. Other sugars such as glucose, glucose-6-phosphate, fructose, galactose, mannose, methyl mannoside, mannosamine, ManNAc, GlcNAc, GalNAc and sucrose and lactose failed to inhibit the agglutinating activity of the hemolymph even at concentrations as high as 100 mM. Of the various glycoproteins tested (table 3) bovine and porcine thyroglobulin showed the highest inhibitory potency. Among acid glycoproteins, bovine but not human acid glycoprotein fraction inhibited the hemagglutination. The hemagglutination inhibitory potency (HAI) of other glycoproteins can be graded as follows: bovine and porcine thyroglobulin > fetuin > bovine acid glycoprotein > bovine submaxillary mucin > porcine stomach mucin (contains 1% of sialic acids). Human acid glycoprotein, MM and NN glycophorins and colominic acid (polysialic acid with NeuAc2–8NeuAc linkages) failed to inhibit HA activity of the hemolymph.

Table 2. Hemagglutination (HA) of hemolymph of *S. serrata* after adsorption with different kinds of erythrocytes (E).

E absorbed	E tested for HA				
	Hu-B	Mouse	Guinea pig	Dog	Rabbit
None	64	128	64	32	64
Hu-B	0	0	16(0)	0	64(0)
Mouse	0	0	16(0)	0	64(0)
Guinea pig	0	0	0	0	64(0)
Dog	0	0	16(0)	0	64(0)
Rabbit	0	0	2(0)	0	0

Values in parentheses refer to HA titers after second adsorption.

Table 3. Hemagglutination inhibition (HAI) of hemolymph agglutinin by various sialo-glycoproteins. The glycoproteins (5 mg/ml), reconstituted in TBS were serially diluted in microtiter plates and mixed with serum previously adjusted to 2 HA units. After 60 min of incubation at 30 °C, the erythrocyte suspension (1.5%) was added. The HAI was determined after 1 h of incubation and expressed as the highest reciprocal of the lowest dilution of inhibitors giving complete inhibition of the agglutination after 1 h.

Glycoprotein	HAI titer of erythrocytes tested		
	Human-B	Rabbit	Mouse
Thyroglobulin			
bovine	4096	128	8
porcine	4096	128	16
Fetuin	2048	32	0
Acid glycoprotein			
bovine	1024	16	0
human	0	0	0
BSM	8	16	2
BSM-de-O-acetylated	32	128	0
Human glycophorin			
MM blood group	0	0	0
NN blood group	0	0	0
Porcine stom. mucin	4	0	0

BSM: bovine submaxillary mucin.

Thermal stability of the hemagglutinin (against human B and rabbit erythrocytes) was tested at 25, 30, 35, 40, 45, and 50 °C. Peak activity was observed at 30 °C and the activity was minimal or lost at or above 45 °C. Similarly maximum agglutination was observed between pH 7.0 and 7.5. Like other invertebrate agglutinins, the activity of *S. serrata* agglutinin was enhanced by the addition of Ca^{2+} (10 mM) but not Mg^{2+} (1 mM–100 mM), indicating a preferential cation requirement for the binding of the agglutinin to the sugar residues. HA was inhibited by mercuric chloride (1 mM).

Discussion

The ability of the hemolymph of *S. serrata* to agglutinate specifically human B, mouse and rabbit erythrocytes argue for the presence of a hemagglutinin. Repeated adsorption with these erythrocytes entirely removes agglutinability of the hemolymph, suggesting the presence of a single hemagglutinin. The ability of the hemolymph to agglutinate sialylated erythrocytes but not its asialo counterpart argues for the affinity for sialic acids. N-glycolyl neuraminic acid (NeuGc) in contrast to NeuAc inhibited the agglutination significantly. Similarly, NeuGc containing thyroglobulins[16] in contrast to NeuAc containing glycoproteins showed remarkable inhibition of hemagglutination. Similarly NeuGc-acid glycoprotein (bovine) but not NeuAc-acid glycoprotein (human)[16] inhibited the agglutination. Inability of other NeuGc containing bovine submaxillary mucin[16], to inhibit the agglutination of the hemolymph suggest that specific linkage of sialic acids found in terminal oligosaccharide of thyroglobulin could be responsible for the inhibition. If the agglutinin is purified, it would serve as a valuable diagnostic tool to identify NeuGc containing tumor associated antigens found in the cell surface of human neoplasms[13]. It should be noted that the sialic acid of human tissues contain only NeuAc, whereas neoplastic transformation results in production of NeuGc as in melanoma and other human cancers[13].

Conclusion

Although the sugar specificity of the agglutinin can be better characterized only after purification, this study established physico-chemical requirements for affinity purification of the agglutinin (lectin) from the hemolymph. We plan to use bovine thyroglobulin with agarose as affinity matrix. The presence of sialic acid binding agglutinins in arthropods[17] such as *S. serrata*, that are not capable of synthesizing sialic acids suggests that these agglutinins may be involved in humoral defense mechanisms in these animals. Purification of these agglutinins would be of interest because of their biomedical potential for the identification of NeuGc residues in pathogenic bacteria and human tumor associated antigens.

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1 Kobiler, D., and Mirelman, D., Infect. Immun. *29* (1980) 221.
2 Ravdin, J. I., John, J. E., Johnston, L. I., Innes, D. J., and Guerrant, L., Infect. Immun. *48* (1985) 292.
3 Lov, B., Ward, H., Gerald, T., Keusch, T., and Pereira, M. E. A., Science *232* (1986) 71.
4 Muller, W. E. G., Muller, I., and Zahn, R. K., Experientia *30* (1974) 899.
5 Mauchamp, B., Biochimie *64* (1982) 1001.
6 Prokop, V. O., Uhlenbruck, G., and Kohler, W., Vox Sang. *14* (1968) 321.
7 Arimoto, R., and Tripp, M. R., J. Invertebr. Path. *30* (1977) 406.
8 Ravindranath, M. H., and Cooper, E. L., Prog. clin. Biol. Res. *157* (1984) 83.
9 Shimizu, S., Ito, M. I., and Niwa, M., Biochim. biophys. Acta *500* (1977) 71.
10 Bretting, H., and Kabat, E. A., Biochemistry *15* (1976) 3228.
11 Koch, O. M., Lee, C. K., and Uhlenbruck, G., Immunobiology *163* (1982) 53.
12 Ravindranath, M. H., Paulson, J. C., and Irie, R. F., J. biol. Chem. *263* (1988) 2079.
13 Ravindranath, M. H., and Irie, R. F., in: Malignant Melanoma, p. 17. Ed. L. Nathanson. Kluwer Acad. Publishers, Boston 1988.
14 Ravindranath, M. H., Higa, H. H., Cooper, E. L., and Paulson, J. C., J. biol. Chem. *260* (1985) 8850.
15 Ravindranath, M. H., and Paulson, J. C., Meth. Enzymol. *138* (1987) 520.
16 Gottschalk, A., Glycoproteins, p. 516. Elsevier Publishing Co., Amsterdam 1966.

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Resistance of in vivo-selected spontaneously transformed cells and Rous sarcoma virus-transformed cells to macrophage-mediated cytotoxicity

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Abstract. The cytotoxic activity (CTA) of activated peritoneal macrophages (MP) on variant lines of Syrian hamster embryo (HE) cells of differing malignant characteristics was studied. The target cells were a line of low-malignant cells resulting from spontaneous transformation of HE cells in vitro (STHE strain), and malignant variants selected from them in vivo (STHE-LM-4, STHE-LM-8, and STHE-75/18 strains). In addition, we used cells of the HET-SR-1 strain; these are HE cells transformed in vitro by a tumorigenic Rous sarcoma virus (Schmidt-Ruppin strain, RSV-SR), or the TU-SR strain induced by RSV-SR in vivo. Thioglycollate-elicited peritoneal MP from Syrian hamsters were activated in vitro with bacterial levan, LPS or MDP and used as effector cells. MP-mediated cytolysis was determined by means of a 42-h radioactivity release assay with ^{3}H-thymidine-labeled target cells. We found that only the parental STHE cells were susceptible towards fully-activated MP-mediated CTA. All three of the in vivo-selected malignant variants of the STHE cell sublines, as well as the tumorigenic RSV-SR transformants, were resistant to cytolysis by activated MP. Non-activated thioglycollate-elicited MP did not lyse any of the tumor cells studied.
Key words. Natural resistance; tumors; spontaneously- and Rous sarcoma virus-transformed cells; Syrian hamsters; peritoneal exudate cells; macrophages; activation; cytotoxicity.

One of the factors involved in tumor progression is in vivo selection of tumor cells. Effectors of the host's natural resistance (NR) (macrophages, monocytes, natural killer (NK) cells, and neutrophils) may play an essential role in this process [1–3]. It has been shown recently that hamster embryo (HE) cells in vitro, spontaneously transformed during in vivo selection, acquired characteristics of a malignant phenotype. These include tumorigenic activity (TGA) and metastasizing activity (MA), both experimental (EMA) and spontaneous (SMA). Moreover, such in vivo-selected cells variants were resistant to hydrogen peroxide (H_2O_2) damage, and they were also able to release prostaglandin E_2 (PGE_2) when in contact with NK cells [4, 5]. It appears that a number of characteristics of tumor cells (i.e., resistance to H_2O_2 and PGE_2 release, frequently united in clusters), at least partly determine their resistance to NR effector cells. A good correlation of these characteristics with TGA and EMA has been demonstrated [2, 4, 5]. HE cells transformed by Rous sarcoma virus (Schmidt–Ruppin strain) (RSV-SR) in vitro, without any in vivo selection, have also acquired the malignant characteristics, and both the resistance to H_2O_2 damage and the PGE_2-releasing activity [2, 6].

Certain activated cytotoxic macrophages (MP) are well-known effector cells of the host's natural resistance to tumor progression. However, data on their cytotoxic potential for transformed and malignant tumor cells are contradictory [7–9]. Therefore, we examined the susceptibility of two different types of transformed cells, and their in vivo-selected malignant variants, to MP-mediated cytotoxic activity (CTA). Previously, we showed that the low-malignant spontaneously transformed in vitro cells of the STHE strain are a highly susceptible target for MP-mediated CTA [10]. In this study, we compared the