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Inhibitory effects of carrier-immobilized synthetic histo-blood group A-, B-, H-, and SiaLe-oligosaccharides on H_2O_2 generation by human polymorphonuclear leukocytes

A.V. Timoshenko[a,*], S.Yu. Vakhrushev[a], N.V. Bovin[b], H.-J. Gabius[c]

[a]*Institute of Photobiology, National Academy of Sciences of Belarus, ul. Akademicheskaya 27, 220072 Minsk, Belarus, Russian Federation*
[b]*Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, 117871 Moscow, Russian Federation*
[c]*Institute of Physiological Chemistry, Ludwig-Maximilians-University, D-80539 Munchen, Germany*

Abstract

Carbohydrate-bearing polymers of biologically inert design are versatile tools to delineate functional aspects of oligosaccharides. Binding of synthetic N-substituted polyacrylamide (PAA) conjugates of histo-blood group (A_{di}, A_{tri}, B_{di}, B_{tri}, H_{di}, SiaLe^a, and SiaLe^x) to human polymorphonuclear leukocytes (PMNs), and effects on H_2O_2 generation elicited by different agonists such as digitonin, N-formyl-Met-Leu-Phe (FMLP) and the galactoside-specific lectin from *Viscum album* L. (VAA) were assessed. PMNs expressed binding sites for blood group-related neoglycoconjugates in the range of $N \sim 10^6$–10^7/cell with K_D-values in the μM range. Treatment of PMNs (2×10^6 cells/ml) with PAA-probes (50 μg/ml) for 5 min did not activate the "respiratory burst". However, it led to suppression (range 20–70%) of H_2O_2 generation of cells in the presence of elicitors. In detail, the FMLP-induced response was significantly decreased by A_{di}, A_{tri}, B_{tri}, H_{di}, SiaLe^a, and SiaLe^x conjugates, whereas for digitonin one only by A_{di}, A_{tri}, B_{tri}. All the seven tested PAA-probes were found to inhibit significantly VAA-mediated release of H_2O_2 from PMNs. In this case, interference can take place already, at the stage of initial binding, especially for B- and H-epitopes, but less prominently for A- and SiaLe-epitopes. These results support the notion that PAA-immobilized histo-blood group oligosaccharides can serve as effector molecules with the ability to reduce the H_2O_2-generation of PMNs, warranting further studies on the involved reaction pathway.

Keywords: Blood group oligosaccharides; Digitonin; Hydrogen peroxide; Lectin; Leukocytes; Neoglycoconjugate

1. Introduction

Biological information encoded in glycan determinants can readily be deciphered by endogenous lectins and translated into cellular responses by various lectin-dependent signaling cascades (Gabius, 1997; Villalobo & Gabius, 1998). Due to the functional significance of this information-transfer route, carrier-immobilized glycans (neoglycoconjugates) have found widespread application in biology and medicine (Gabius, 1988; Gabius, Unverzagt & Kayser, 1998; Lee & Lee, 1997). Relative to protein vehicles such as albumin polyacrylate-type scaffolds such as substituted poly(2-hydroxyethyl acrylamide) harbor the advantage of defined assembly of a non-biodegradable backbone without any inherent receptor properties, immunogenicity and pronounced changes of the overall isoelectric point (Bovin, 1998; Bovin & Gabius, 1995). Synthetic hybrids of an inert matrix and a putative bioeffector can thus be valuable to help delineate the spectrum of functions of carbohydrate epitopes in the absence of the protein part of glycoproteins (Reuter & Gabius, 1999; Varki, 1993). Several examples, document the potential of this approach. Neoglycoconjugates expose glycans of α_1-acid glycoprotein enhanced monocyte synthesis of interleukins 1,2,4, and 6 and tumor necrosis factor α (Shiyan & Bovin, 1997). With lacto-N-fucopentaose III (Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc) as key determinant on a neoglycoprotein, the production of interleukin-10 by murine spleen cells was markedly stimulated (Vellupillai & Harn, 1994). Moreover, sugar-selective inhibition of zymosan-induced chemiluminescence of rat peritoneal macrophages was elicited by carrier-immobilized mannose and fucose (Mikhalchik, Korkina, Shiyan & Bovin, 1994). In addition, galactoside-exposing neoglycoconjugates as well as galectins were found to modulate the proliferation of selected sarcoma cell lines (Remmelink et al., 1999). Further cell feature migration of rabbit alveolar macrophages was responsive to fucosylated and mannosylated

* Corresponding author.
E-mail address: timoshenko@phys.bsu.unibel.by (A.V. Timoshenko).

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Table 1
Structures of blood group antigen-related oligosaccharides attached to the polyacrylamide matrix

Oligosaccharides	Chemical structure
A_{di}	GalNAcα1-3Galβ1-R
A_{tri}	GalNAcα1-3[Fucα1-2]Galβ1-R
B_{di}	Galα1-3αGalβ1-R
B_{tri}	Galα1-3[Fucα1-2]Galβ1-R
H_{di}	Fucα1-2Galβ1-R
SiaLe[a]	Neu5Acα2-3Galβ1-3[Fucα1-4]GlcNAcβ1-R
SiaLe[x]	Neu5Acα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-R

neoglycoproteins (Gordon, Chida, Takata & Myrvik, 1987; Takata, Chida, Gordon, Myrvik, Ricardo & Kusera, 1987).

Access to carbohydrate ligands with increased sequence complexity has made it possible, especially, to pursue research on growth regulation. Blood group A- and H-trisaccharide binding was positively correlated with prognosis in lung cancer (Kayser & Gabius, 1999; Kayser, Bovin, Korchagina, Zeilinger, Zeng & Gabius, 1994a; Kayser, Bovin, Zemlyanukhina, Donaldo-Jacinta, Koopmann & Gabius, 1994b), and an A/Le[x]-related tetrasaccharide, designated as TS4, inhibited proliferation of astrocytes, gliomas and neuroblastomas at micromolar concentrations (Santos-Benito, Fernandez-Mayoralas, Martin-Lomas & Nieto-Sampedro, 1992). Having mastered this synthetic problem, it is feasible to extend our knowledge of the effects of histo-blood group epitopes in immune cells, which express a variety of lectins (Gabius, 1987, 1997; Shiyan, Khaidukov, Pukhalsky, Toptygina & Bovin, 1996). As listed above, several aspects of their activity appear to be responsive to exposure to neoglycoconjugates. In this report, we focus on polymorphonuclear leukocytes, addressing two issues. Firstly, we quantitatively assessed expression of binding sites for neoglycoconjugates exposing histo-blood group epitopes. Secondly, we investigated whether and to what extent the presence of these probes could modulate the H_2O_2 generation of these cells.

2. Experimental

PMNs were isolated from citrate-stabilized donor blood O(1), purchased at the Republican Station of Blood Transfusion (Minsk, Belarus), by centrifugation of leukocyte-rich plasma through Histopaque-1077 as described by Timoshenko, Kayser and Gabius (1997). The obtained cells were suspended in the phosphate-buffered saline (PBS), pH 7.3, containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4/KH_2PO_4, and were kept at 4–6°C.

To assess binding of labeled neoglycocojugates to cell surface of PMNs the avidin-biotin technique was used, as described by André et al. (1997); Gabius, André, Danguy, Kayser and Gabius (1994). In detail, aliquots of cell-containing solutions (100 μl, 10^5 cell in PBS with 0.1% BSA) were incubated in polystyrene Eppendorf tubes on melting ice for 30 min, with nine different concentrations in the range of 0–160 μg/ml of biotinylated neoglycoconjugates. It was prepared according to the description of Galanina, Kaltner, Khraltsova, Bovin and Gabius (1997); Weitz-Schmidt, Stokmaier, Scheel, Nifant'ev, Tuzikov and Bovin (1996). The unbound neoglycoconjugates were separated from the cells by three-fold centrifugation for 4 min at 1500 rpm on a centrifuge OPN-3, and there was a thorough washing of cell pellets with 500 μl of 0.1% BSA in PBS. Thereafter, the washed cells were incubated in streptavidin-peroxidase-containing solution (0.5 μg/ml) for 90 min on melting ice. These cells were washed again three times by centrifugation, as indicated above, and the color reaction was developed at 37°C by the addition of 150 μl of a solution containing 1 mg/ml 1,2-phenylenediamine dihydrochloride, 0.3% H_2O_2, and 50 mM Na_2HPO_4 adjusted to pH 5.0 by citric acid and stopped after 10 min by the addition of 50 μl of 0.25 M H_2SO_4. The obtained colored solutions were centrifuged for 5 min at 3000 rpm to remove any turbidity and were diluted 1:29 with 0.25 M H_2SO_4. The optical density of diluted aliquots was measured at 490 nm in a spectrophotometer PV1251C from Solar (Minsk, Belarus) using a semi-microcuvette. To translate the optical density into amount of bound neoglycoconjugates, calibration curves were determined for each biotinylated probe. The number of available binding sites (N) and the dissociation constant (K_d) were estimated according to Scatchard (1949). The mean molecular weight of PAA-neoglycoconjugates was considered to be 35 *k*Da.

The generation of H_2O_2 by PMNs ($2x10^6$ cells/ml) was assayed by a fluorescent method, using scopoletin as a substrate of horseradish peroxidase, as described elsewhere (Timoshenko et al., 1997; Timoshenko, Bovin, Shiyan, Vakhrushev, André & Gabius, 1998). Digitonin (2.3 μg/ml), FMLP (26 nM), and VAA (2.5 μg/ml), isolated as described by Gabius (1990), were used as agonists of the plasma membrane NADPH-oxidase system. Kinetics of H_2O_2-mediated oxidation of 1 μM scopoletin resulting in a decrease of fluorescence at 450 nm (excitation setting to 360 nm), were recorded continuously for 10–15 min on a spectrofluorimeter LSF 1211A from Solar (Minsk, Belarus).

PAA-immobilized oligosaccharides (about 20% of carbohydrate per molecule) were from Syntesome GmbH (Munich, Germany); FMLP, digitonin, scopoletin, horseradish peroxidase, streptavidin-peroxidase were from Sigma (Deisenhofen, Germany); 1,2-phenylenediamine dihydrochloride was from Fluka (Buchs, Switzerland); 30% H_2O_2 was from Merck (Germany).

Statistical management of obtained results was carried out by using the NCSS 5.03 statistics package through multiple regression analyses and Student's t-test considering P value of less than 0.05 as indicative of significant differences between compared values.

Table 2
Determination of the apparent number of binding sites at saturating ligand concentration (N), and the dissociation constant (K_d) for carrier-immobilized blood group A-, B-, H-, and SiaLe-oligosaccharides. The data are presented as mean ± SD of two measurements, with different donors running in duplicate

Oligosaccharides	$N \times 10^6$	$K_D \times 10^{-6}$ M
A_{di}	3.93 ± 1.29	1.28 ± 0.27
A_{tri}	7.52 ± 0.41	2.24 ± 1.58
B_{di}	1.56 ± 0.68	1.72 ± 0.04
B_{tri}	15.4 ± 6.2	2.09 ± 0.19
H_{di}	3.66 ± 0.60	1.07 ± 0.06
SiaLe[a]	2.00 ± 1.01	2.55 ± 1.14
SiaLe[x]	1.72 ± 0.50	2.15 ± 0.64

3. Results and discussion

The chemical structures of the studied blood group oligosaccharides are presented in Table 1. Binding studies with increasing concentrations of labeled neoglycoconjugates were instrumental to determine the number of accessible binding sites at saturation (N) and the dissociation constant (K_d) for each of the seven biotinylated glycoprobes. Evidently, PMNs expressed specific binding sites for blood group related neoglycoconjugates ($N \sim 10^6$–10^7/cell) with K_d-values in the μM-range (Table 2). The extension form the di- to the trisaccharide led to an increase in the number of bound probe molecules at saturation. Having answered the first question on presence and quantitative assessment of specific binding sites positively, we can now proceed to examine the functional implications of this binding. Based on the capacity of α_1-acid glycoprotein to modulate stimulus-dependent H_2O_2 production (Timoshenko et al., 1998) and the relevance of this product for host defense, we focused on this property.

To test any activity of the probes in this respect, neoglycoconjugates were dissolved in 0.9% NaCl solution at a concentration of 1 mg/ml and added up to the final concentration of 50 μg/ml, to a PMNs suspension (2×10^6 cells/ml) maintained at 37°C. In initial assays, it was found that the treatment of PMNs with aliquots of this panel of neoglycoconjugates for up to 10–15 min failed to activate cells examined for H_2O_2 release, using the scopoletin method.

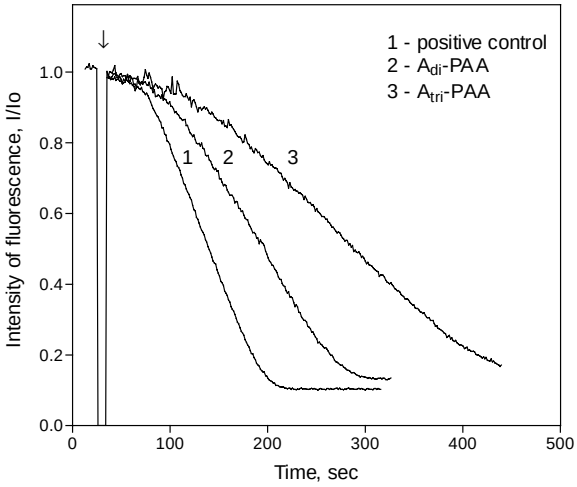

Fig. 1. Effect of A_{di}-PAA and A_{tri}-PAA on digitonin-induced oxidation of scopoletin by PMNs. The cells (2×10^6 cells/ml) were incubated at 37°C with neoglycoconjugates (50 μg/ml) for 5 min prior to addition on digitonin (2.3 μg/ml). Digitonin was added at the moment indicated by the arrow. For details of measurements see Experimental.

However, certain immobilized blood group-specific oligosaccharides were effective to decrease the rate of scopoletin oxidation by human PMNs in response to VAA, FMLP, and digitonin. Typical traces of the reaction triggered by digitonin as example of an elicitor in controls and in the presence of 50 μg/ml A_{di}-PAA and A_{tri}-PAA are shown in Fig. 1. Table 3 summarizes the obtained data concerning the rates of H_2O_2 generation by human PMNs, in the presence of the studied neoglycoconjugates. Obviously, they were in the range of 30–80% of the control values. Only three probes, namely A_{di}-PAA, A_{tri}-PAA, and B_{tri}-PAA, were found to inhibit significantly the H_2O_2 generation, independent of the nature of agonist of plasma membrane NADPH-oxidase. Terminal positions of GalNac or Gal(α1-2)Fuc may thus be essential to affect the functional activity of PMNs, in this case. For VAA-dependent effects, the data reflected the inhibitory potency of lectin binding, rendering it difficult to distinguish lectin effects from post-binding interference. For example, B_{di}-PAA was a potent inhibitor of VAA-induced H_2O_2 generation by PMNs (38.4 ± 19.5%, $n = 4$), and a potent inhibitor of binding VAA to lactose

Table 3
Effects of carrier-immobilized blood group A-, B-, H-, and SiaLe-oligosaccharides (tested at a final concentration of 50 μg/ml), on H_2O_2 generation by PMNs (2×10^6 cells/ml), stimulated by digitonin (2.3 μg/ml), FMLP (26 nM), or VAA (2.5 μg/ml). The presented values are expressed as percentage of respective control responses, without any addition of neoglycoconjugates (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Oligosaccharides	Digitonin	FMLP	VAA
A_{di}	73.3 ± 1.2(3)***	58.8 ± 20.7(5)*	70.1 ± 10.4(5)**
A_{tri}	58.5 ± 6.6(3)**	44.6 ± 11.8(3)*	60.5 ± 17.5(4)*
B_{di}	65.0 ± 20.1(3)	78.8 ± 23.0(4)	32.1 ± 10.1(4)***
B_{tri}	61.9 ± 4.3(3)**	35.6 ± 16.9(3)*	38.4 ± 19.5(4)**
H_{di}	84.5 ± 16.9(4)	51.7 ± 18.7(5)**	40.6 ± 19.3(3)*
SiaLe[a]	74.2 ± 18.6(3)	68.5 ± 9.7(3)*	77.7 ± 6.8(3)*
SiaLe[x]	80.6 ± 27.2(4)	81.0 ± 8.4(4)*	71.4 ± 4.8(3)**

($I_{50} = 6.9 \times 10^{-7}$ M for a neoglycoconjugate with 30% incorporation yield) (Galanina et al., 1997). The neoglycoconjugates with Fuc or Neu5Ac (H_{di}, $SiaLe^a$, and $SiaLe^x$) at the terminal branch were common inhibitors of FMLP- and VAA-induced responses and failed to affect those induced by the digitonin. 2,3-Sialylated glycocompounds are rather poor inhibitors of VAA binding (Galanina et al., 1997).

To disclose whether binding of neoglycoconjugates to PMNs and their inhibitory potency, with respect to H_2O_2 generation, is correlated, the multiple regression analysis was performed. A negative correlation with quantitative differences is observed between the extent of binding sites for neoglycoconjugates and digitonin-, FMLP-, or VAA-induced generation of H_2O_2 (data not presented). It is known that digitonin, FMLP, and VAA trigger NADPH-oxidase (or H_2O_2-generating) activity of PMNs by different molecular mechanisms. Our results of multiple regression analysis appear to reflect this disparity, note also the impact of the probes on lectin binding.

The data on sialylated Lewis tetrasaccharides $SiaLe^a$, and $SiaLe^x$ and their effects on digitonin- and FMLP/VAA-dependent responses deserve a further comment. These carbohydrate determinants serve as ligands for selectins (Gabius, 1997; Weitz-Schmidt et al., 1996). The inhibition of FMLP/VAA-induced generation of H_2O_2 by $SiaLe^a$ and $SiaLe^x$ supports the notion for an activity of these determinants in PMNs activation. In line with this reasoning is the activity of monoclonal antibodies against L-selectin priming generation of superoxide and H_2O_2 by human neutrophils (Bengtsson, Grenegard, Olsson, Sjögren, Stenolahl & Zalavary, 1996; Crockett-Torabi, Sulenbarger, Smith & Fantone, 1995; Waddel, Fialkow, Chan, Kishimoto & Downey, 1994). Since the neoglycoconjugates can mimic natural ligand display, it will be informative to extend this line of research, using this class of synthetic polymers as tools.

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