Effects of Desialylation on Chemoattractant Induced Chemotaxis

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ABSTRACT

The amount of sialic acid on the surface of the neutrophil influences its ability to interact with other cells. Neutrophil activation with various stimuli mobilizes intracellular sialidase to the plasma membrane where it cleaves sialic acid from cell surfaces. Since enhanced neutrophil adherence, spreading, deformability and motility each are associated with surface desialylation and critical to neutrophil diapedesis, we studied the role of sialic acid on neutrophil chemotaxis with interleukin-8 (IL-8), leukotriene B4 (LTB4), fMet-Leu-Phe (fMLP) and complement 5a in vitro. Migration of NANase-treated neutrophil across 3 μ pore size polycarbonate membranes was decreased in response to IL-8 and LTB4 but not to fMLP and complement 5a. These findings suggest that sialic acid content of receptors have a key role on chemoattractant-receptor binding and may be a novel strategy for limiting the inflammatory response.

Key Words: Sialic acid, Chemotaxis, Desialylation.

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INTRODUCTION

Adhesion of neutrophil (PMN) to vascular endothelial cell (EC) requires a highly orchestrated sequence of cell-cell interactions. The surface of PMN is highly sialytated. Sialic acid glycoconjugates impart a negative surface charge to cells and discourage intimate heterotypic cell-cell interactions. Evidence to support a role for sialic acid in mediating PMN/EC interactions includes:

1. PMN adherence to EC is enhanced by neuraminidase (NANase) treatment of PMNs, in vitro[1].

2. Trauma-enhanced intravascular margination in rabbits is inhibited by sialic acid infusion and[2]

3. Adherence and aggregation of PMNs occurs after mobilization of endogenous sialidase to the cell surface with a release of sialic acid[3].

Endogenous sialidase activity appears to have a regulatory role in adhesion and diapedesis. Interleukin-8 (IL-8) activates PMNs with release of sialic acid into

the medium and sialidase is co-expressed with CD11b/CD18 following either IL-8 or fMet-Leu-Phe (fMLP) treatment[3]. Consequently, we reasoned that the mobilized PMN sialidase might play an important role in diapedesis and chemoattractant-receptor interaction through surface desialylation. To test this hypothesis, we studied the effect of PMN desialylation with bacterial NANase on chemotaxis with various chemoattractants.

MATERIALS and METHODS

Reagents: Recombinant human IL-8 (On-cogene Research Products, Cambridge, MA) with a purity of 98% was reconstituted in sterile PBS, aliquoted, and stored at-20°C fMLP (sigma, St. Louis, MO) and leukotriene B4 (LTB4) (Oxford Biomedical Research, Inc. Oxford, MI) were reconstituted in PBS at 10^{-3} M, aliquoted and stored at-20°C. NANase type V extracted from *Clostridium perfringens* (sigma) with a specific activity of 1.2 U/mg as well as the NANase inhibitors, 2,3-dehydro-2-deoxy-N-acety/neuraminic acid (2-deoxy NANA) (Calbiochem Biochemical, La Jolla, CA) and N-acetyl-neuraminic acid (sigma) each was reconstituted in PBS. N-acetylneuraminly-lactose (sigma) was reconstituted in PBS immediately prior to use. To prepare anti-NANase antibody, New Zealand white rabbits were immunized with highly purified type X *C. perfringens* NANase (sigma). Rabbit IgG (sigma) and 2-keto-3-deoxyoctulosonic acid (KDO) (sigma) were used as negative controls for anti-NANase antibody and 2-deoxy NANA, respectively.

PMN preparation and fluorescent labeling: Whole peripheral blood from healthy human volunteers was collected into acid citrate dextran (sigma) solution and PMNs were isolated by dextran erythrocyte sedimentation and density gradient centrifugation through ficoll-hypaque (sigma) as previously described[3]. PMNs were resuspended in Hanks balanced salt solution without divalent cations ($HBSS^-$) (Life Technologies, Gaithersburg, MD) at 10^7 PMNs/mL and were incubated with 5 µM calcein AM (Molecular Probes, Eugen, OR) for 40 min with gentle agitation in the dark[4]. Calcein AM fluoresces upon cleavage by intracellular esterases[5]. PMNs were washed 3 times with HBSS after which their purity was > 95% and viability > 98% by trypan blue dye exclusion. Calcein AM fluorescence was stable for up to 4 days when stored in the dark at 4°C.

C5a fragment preparation: Fresh serum was incubated overnight at 4°C with 1 M epsilon amino caproic acid. The following day, serum compartment was activated with baker's yeast to generate the low molecular weight chemotactic C5 fragment according to Vallota et al[6]. This preparation was subsequently passed through PM30 membrane (Amicon corp., Lexington, Mass.) which exclude molecules with a molecular weight of > 30.000 and allowed passage of chemotactic C5 fragment. For optimum chemotaxis, titrated in the chemotaxis assay.

Preparing assay (Boyden) chambers: Polycarbonate filters (13 mm diameter and 3 µm pore size) (Nucleopore, Pleasanton, CA) were treated 0.5% aqueous solution of acetic acid at 50°C x 20 min, with constant stirring, washed in distilled H_2O and immersed in boiling pigskin gelatin (Fisher Scientific, Pittsburgh, PA) solution (10 mg/L distilled H_2O) x 60 min. The filters were then dried, glued to polysterne chemotactic chambers (ADAPS, Dedham, MA), and inserted in 24 well plates and gas sterilized with ethylene oxide.

PMN chemotaxis thrugh 3µ pore size filters: Calcein-labeled PMNs (5 x 10^5 cells/well) were introduced into the upper compartments of assay chambers, incubated for 2h at 37°C, after which time each lower compartment of well was sampled and fluorometrically assayed. After migration through naked filters > 99% of fluorescence remained PMN-associated (data not shown). A standard curve was established for each experiment from which PMN numbers could be interpolated from fluorescence units and PMN migration was expressed as % migration.

Assay of NANase activity: PMN lysate sialidase was measured by the Warren assay as previously described, and *C. perfringens* NANase activity were used for positive control[3]. PMN lysates were prepared as previously described[3]. Samples to be assayed for NANase activity were incubated (10 min, 37°C) with the artificial substrate, N-acetylneuraminyl-lactose in 100 mM sodium acetate, 2 mM $CaCI_2$, pH 5.0 and the liberated sialic acid measured by the thiobarbituric acid assay. For each assay, increasing concentrations of N-acetylneurominic acid NANA standard solution were used to generate a standard curve from which µ moles of NANA liberated from N-acetylneuraminyl-lactose was determined.

Statistical methods: Analysis of variance (ANOVA) was used to compare the mean responses among experimental and control groups. A p-value of < 0.05 was considered significant. Linear regression analyses of standard curves were used to calculate cell number.

RESULTS

PMN chemotaxis: The dose requirements for chemoattractants established in our chemotaxis system. A fixed concentration of labeled PMNs (5×10^5 PMNs in 0.5 media) in the upper compartment were incubated in the presence of increasing concentrations of either human IL-8, FMLP, LTB4 or C5a in the lower compartment of assay chambers, and maximum migration was occurred with 100 ng/mL, 10^{-9} M, 10^{-9} M and 1/60 dilution respectively (data not shown).

Specificity of NANase activity: NANase (40 µg/mL) activity was measured as the release of sialic acid from an artificial substrate neuraminly-lactose (Table 1). Anti-NANase IgG (40 µg/mL), 2-deoxy NANA (200 µg/mL) or boiling (1.5 h) each decreased NANase activity compared to the untreated NANase, whereas the preimmune IgG did not. The amount of sialidase activity in Iysate of 2×10^8 PMNs was equivalent to approximately 5 µg/mL of clostridial enzyme (Table 2). Anti-NANase (40 µg/mL) and 2-deoxy NANA (200 µg/mL) each decreased PMN lysate sialidase activity, whereas pretreatment with preimmune IgG did not. Thus, human PMN sialidase activity, like that of the bacterial homologues, was inhibited by either antibody or pharmacologic blockade. Also, PMN lysate sialidase activity decreased with incubation for 1 h, at 37°C (5.48 mU/mL), compared to its activity when maintained at 4°C (8.78 mU/mL). This is consistent with the known thermal instability of lysosomal sialidase.

Effect of NANase on PMN chemotaxis: NANase pretreatment of PMNs decreased their migration in a dose-and time-depen-dent manner across naked filters in response to IL-8 (Figure 1A and B). After treatment for 0.5 h, NANase at ≥ 10 mU/mL decreased PMN migration (Figure 1A). NANase treatments (10 mU/mL) as brief as 10 min decreased PMN migration with further time-dependent decrements through 90 min. (Figure 1B). To determine whether this NANase-induced decrease in PMN migration was specific for the IL-8 stimulus, migration of NANase-treated PMNs across naked filters in response to IL-8 (100 ng/mL), fMLP (10^{-9} M), LTB4 (10^{-9} M) and C5a fragment (1/60) were simultaneously studied (Figure 2). Migration of NANase-treated PMNs was decreased in response to IL-8 (39.9% to 26.4%) and LTB4 (40.26% to 23.09%) but not in response to fMLP (38.69% to 34.10%) and C5a (42.39% to 40.23%). These results suggest that NANase does not blunt chemotactic responsiveness through a generalized PMN surface desialylation but rather through desialylation of a specific glycoconjugates, possibly the receptors.

DISCUSSION

These studies demonstrate that modulation of sialic acid has a profound effect on PMN migration. NANase treatment of PMN was selectively decreased in response to IL-8 and LTB4 but not to fMLP and C5a frag-

Table 1. Inhibition of clostridial NANase activity by anti-NANase antibody, 2-deoxy NANA and boiling

		NANase activity (mU/mL)	
Treatment	n	Mean	SEM
NANase	5	23.61	0.65
NANase + anti-NANase antibody*	5	3.83	0.6
NANase + pre-immune IgG	5	21.23	0.56
NANase + 2-deoxy NANA	4	2.84	0.41*
2-deoxy NANA	3	2.11	0.39
Boiled NANase	4	1.45	0.78*

NANase (40 µg/mL) boiled (1h at 100°C) NANase, or NANase preincubated with anti-NANase IgG (40 µg/mL), pre-immune rabbit IgG (40 µg/mL), or 2-deoxy NANA (200 µg/mL) were each measured for NANase activity in the colorometric assay. The number of times tested, "n", for each determinant is indicated.

* Indicates significantly decreased compared to the fresh, intact NANase control at $p < 0.001$.

Table 2. Inhibition of PMN lysate sialidase activity by anti-NANase antibody and 2-deoxy NANA

		NANase activity (mU/mL)	
Treatment	n	Mean	SEM
PMN Iysate	3	5.32	0.47
PMN Iysate + anti-NANase antibody	3	1.90	0.47*,**
PMN Iysate + pre-immune IgG	3	4.51	0.67
PMN Iysate + 2-deoxy NANA	2	1.62	0.6*

PMN Iysate (200 x 10^6 PMN/mL), PMN Iysate preincubated with anti-NANase IgG (40 µg/mL), pre-immune rabbit IgG (40 µg/mL), or 2-deoxy NANA (200 µg/mL) were each measured for NANase activity in the colorometric assay. The "n" for each determinant is indicated.

* Indicates significantly decreased compared to PMN Iysate activity at $p< 0.05$.

** Indicates significantly decreased compared to preimmune IgG-treated PMN Iysate activity at $p< 0.05$.

ment. Although the reasons for these differences remain unclear, the effect of NANase treatment does not appear to be due to a generalized, nonspecific desialylation of the cell surface.

Sialic acid is a family of aminosugars that are present on all living cells as glycoconjugates, which impart a negative electric charge to the cell surface. Within the bone marrow, myeloid precursors are characterized, in part, by a high neuraminidase-susceptible sialic acid content, negative net surface charge, a high degree of cellular rigidity, hypoadhesiveness, as well as impaired pseudopod extension and phagocytic activity[7, 8]. As these myeloid cells mature and leave the bone marrow, sialic acid is progressively lost from their surface[7]. This desialylation is associated with increases in cellular deformability, motility, adhesiveness and phagocytic potential[7,9]. Once in the peripheral circulation, activation of PMNs is associated with an additional loss of cell surface sialic acid and a negative surface charge[3,10]. Moreover sialic acid is an important determinant of cell behavior and the activation state of immune competent cells. The degree of si-alylation influences the likelihood of contact inhibition among cells (which may in turn be relevant to morphogenesis) as well as the metastatic potential of malignant cells[11]. Numerous reports document that the interaction between T and B lymphocytes, and antigen presenting cells and T cells are associated with modifications of sialic acid[12-16]. Activated cells lose sialic acid residues from the surface major histocompatibility complex (MHC)[17]. The glycosylation of ligands or their cellular receptors may influence the ability of hormones (such as human chorionic gonadotrophin and erythropoietin), cytokines (such as MIF), immunoglobulins or toxins to interact with their cellular targets[18-22]. The- refore, surface sialic acid expression appears to be an important determinant for complex receptor-ligand and cell-to-cell interactions.

NANases are enzymes that cleave sialyl residues from cell surface glycoconjugates. NANases have been reported in a variety of microorganisms such as viruses, bacteria, and protozoa as well as in mammalian cells (where they are referred to as sialidase). Each NANase possesses a conserved structural motif which has been proposed as the operative catalytic domain for the cleavage of specific sialic acid residues[23]. Cleavage of dissimilar sialic acid glycosidic linkages requires specific forms of NANase. In addition, each sialidase may differ in its tissue and subcellular distribution as well as in its biochemical properties (e.g. molecular weight, thermal stability). Mammalian sialidases have been cloned from the cytosol of rat skeletal muscle, the Chinese hamster ovary cell, and from the MHC of human EBV-transformed lymphoblastoid cells[24-26]. However, their role in the physiologic function of these cells has not been well-characterized.

The membrane-bound sialyltransferases (STs) catalyze the rapid transfer of free sialic acid (present in tissues as cytidine monophosphate-N-acetyl neuraminic acid, [CMP-NANA] onto glycoconjugate backbones[27]. Although localized predomenantly in the Golgi apparatus, glycosyltransferases also have been demonstrated on cell surfaces[28]. This family of enzymes, specific for different glycosidic linkages of sialic acid, restores to glycoconjugates the negative electric charge and mask-like function of sialic acid, thereby returning the cell to its resting state.

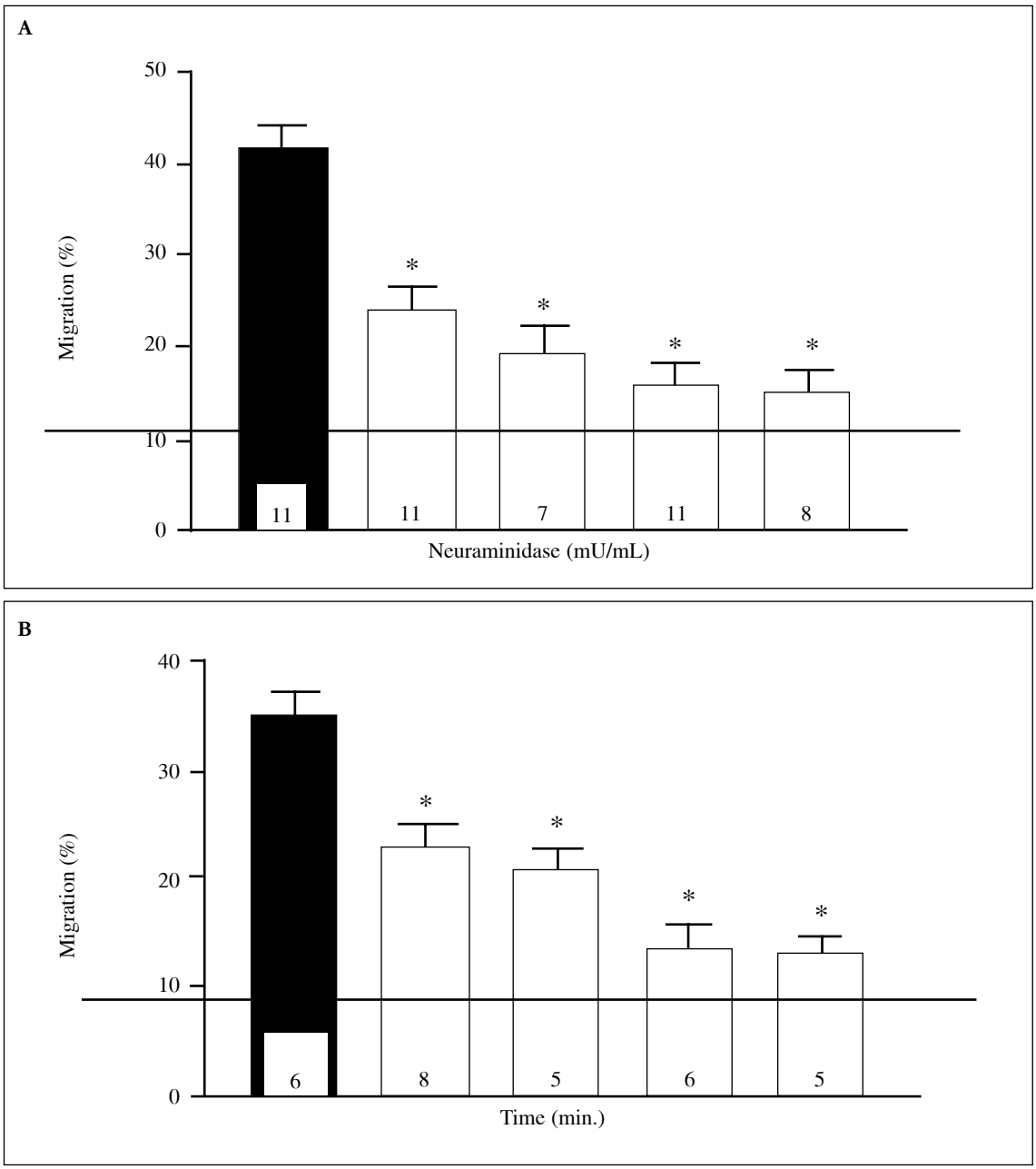

Figure 1. Effect of NANase on PMN chemotaxis. Each bar represents mean (± SE) percent migration of PMNs treated for 0.5 h with increasing concentrations of NANase (open bars) or media alone (closed bar) across naked filters in response to IL-8 (100 ng/mL) (A), PMNs treated for increasing times with NANase (10 mU/mL) (open bars) or media alone (closed bar) (B). The "n" for each group is indicated in each bar.
* Significantly decreased compared to the simultaneous media controls at $p < 0.001$.

Endogenous sialidase activity has been demonstrated in PMNs[29]. It has been previously shown that in human PMNs, sialidase exists in a pre-formed pool within the specific granules. Upon activation by various stimuli, the intracellular sialidase is mobilized to the plasma membrane where it becomes an integral

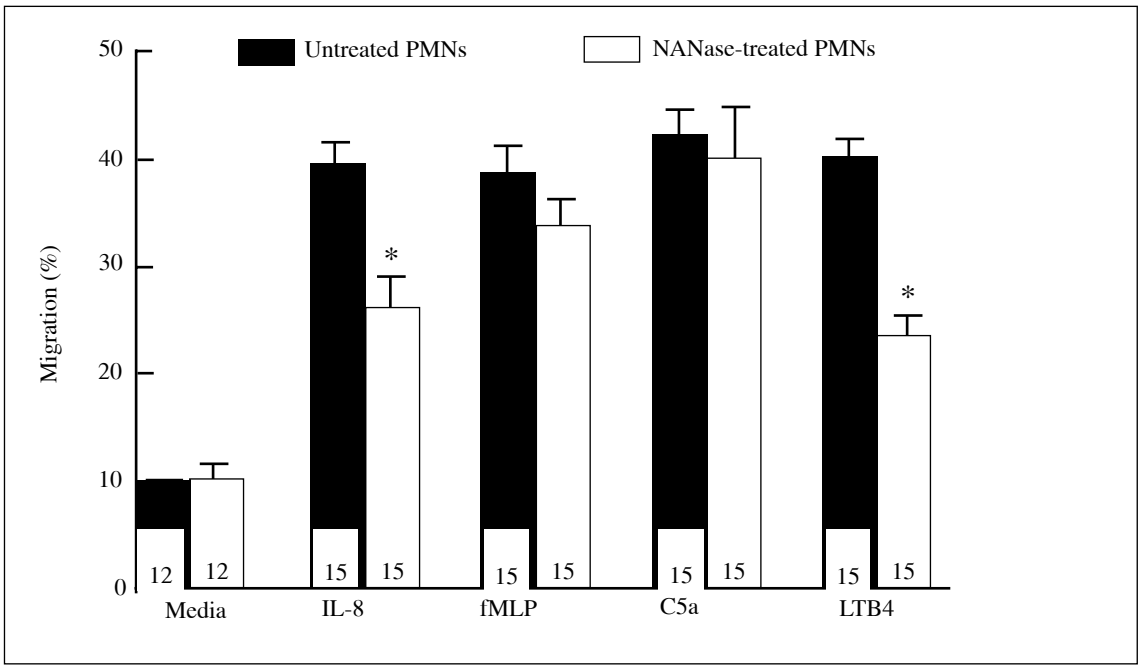

Figure 2. Effect of NANase on PMN chemotaxis with various chemoattractants. Each bar represents mean (± SE) percent migration o PMNs treated for 0.5 h with either NANase (10 mU/mL) (closed bars) or media alone (open bars) across naked filters in response to IL-8 (100 ng/mL), fMLP (10^{-9} M), C5a (1/60 dilution), LTB4 (10^{-9} M) and media alone. The "n" for each group is indicated in each bar.
* Significantly decreased compared to the simultaneous media controls at $p< 0.001$.

membrane protein (i.e. is not secreted). This translocated sialidase then removes sialic acid from glycoconjugates on its own and adjacent PMN surfaces[3]. One functional consequence of this desialylation is an enhanced PMN capacity for aggregation and adherence to substrata. This suggests that following activation-induced desialylation, there is a restoration of sialic acid residues to the cell surface. ST-modulated resialylation therefore may be a key counter-regulatory mechanisms(s) for surface charge homeostasis. Thus, the sialic acid content of mammalian cells may be regulated by the coordinated activity of endogenous sialidases, which remove, and STs, which add sialyl residues to the cell surface. We propose that the dynamic interaction of these enzyme families may play a critical role in the highly localized, rapidly reversible regulation of the inflammatory response.

Alteration in PMN or lymphocyte function is seen after desialylation of ≥ 30% surface sialic acid[3,30]. Whether exogenous NANase or endogenous sialidase activity alters PMN responses through either generalized desialylation and decreased net surface charge, and/or through the selective removal of sialyl residues from specific glycoconjugates remains unknown.

PMN diapedesis is complex but highly orchestrated series of events, and a number of specialized ligands and counter-ligands may be potential target surface structures for sialidase. Several of these surface structures are rich in sialic acids (e.g. Gly CAM-1, Mad CAM-1, PSGL-1 and CD34) whereas others are known to have a number of potential glycosylation sites whose carbohydrate moieties have not been well-characterized (e.g. L-selectin, E-selectin, P-selectin, ICAM-1, ICAM-2, VCAM-1, PCAM-1, cadherins, and the IL-8, the fMLP, LTB4 and the C5a receptors)[31-42].

fMLP, C5a, LTB4 and IL-8 are well known chemoattractants, which also activate leukocytes. FMLP, C5a, and LTB4 attract PMNs and monocytes with equal potency. Whereas IL-8 attract PMNs but not monocytes[43]. Upon activation, specific granule content of

PMNs are exocytosed to the cell exterior. Importantly, specific granules contain functional membrane proteins such as, CD11b/CD18, fMLP receptor, complement receptor, sialidase and cytochrome b.[3,29]. On the other hand 95% of PMN-associated sialic acid is located on the cell surface[44]. Cross, et al. have been shown that translocation of sialidase from intercellular store to the plasma membrane following PMN activation, and this was accompanied by a decrease in cell-associated sialic acid[3]. The translocated sialidase became and integral plasma membrane protein that desialylated glucoconjugates not only on the activated PMN surface but also on the surface of adjacent nonactivated cells. This mobilization of sialic acid may play a key role in cell-cell or receptor-ligand interaction.

Although all these chemoattractant receptors are superfamily of G-protein linked receptors, none of these chemoattractants compete to bind each others receptor[43]. These receptors have different amount of potent N-linked glycosylated sites. Interestingly IL-8 and LTB4 have 5 and 7, and fMLP and C5a have 2 and 1 N-linked glycosylated sites respectively[31,41,45,46]. These sites highly related with receptors carbohydrate moiety and may potential targets for sialidase and mobilization of sialic acid may decrease receptor-chemoattractant binding.

In summary, mobilization of sialic acid by exogenous NANase have critical role on receptor-chemoattractant interaction. Therapeutic interentions that target endogenous sialidase may provide novel strategies for limiting the inflammatory response. We speculate that by changing sialic acid content, modulation of sialidases and perhaps STs may alter other pathophysiological processes where cell-cell interactions are central, including tumor cell metastasis, cell differentiation, morphogenesis and angiogenesis. This remains to be demonstrated, however.

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