Role of chondroitin sulfate C in the action of anthrax toxin

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\textbf{ABSTRACT}

Anthrax toxin is produced by \textit{Bacillus anthracis}, the causative agent of anthrax, and is responsible for the majority of disease symptoms. The toxin consists of 3 proteins, protective antigen (PA), lethal factor (LF), and edema factor (EF), which combine to form lethal and edema toxin. Glycosaminoglycans, which are present on the surface of cells, were investigated with regard to their role in toxicity resulting from anthrax toxin exposure. Lethal toxin-induced cytotoxicity of the RAW 264.7 cells was significantly inhibited by the addition of chondroitin sulfate C as determined by the MTT assay. By contrast, several other glycosaminoglycans, including heparin, heparan sulfate, and dermatan sulfate did not show significant levels of inhibition. Studies utilizing fluorescence-labeled PA demonstrated decreased PA binding to RAW 264.7 cells with the addition of chondroitin sulfate C. Formation of PA oligomers at the surface of cells after binding was also inhibited by chondroitin sulfate C. Interestingly, enzymatic degradation of endogenous chondroitin sulfate C from the cell surface with chondroitinase ABC was accompanied by increased sensitivity to the toxin. These findings were further confirmed by pretreating cells with sodium chloride to reduce the degree of cell surface glycosaminoglycans sulfation. In addition, chondroitin sulfate C effectively inhibits edema toxin-induced cAMP accumulation in cells. Our results indicate that chondroitin sulfate C may play an important role in the toxicity of anthrax toxin.

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1. Introduction

Anthrax infection can occur after ingestion of spores of \textit{Bacillus anthracis}, a toxigenic gram-positive, rod-shaped bacterium (Dixon et al., 1999; Collier and Young, 2003). The recent use of \textit{B. anthracis} as a biological warfare and terrorism agent has prompted the dramatic increase in research for new therapeutics. The inhalation of anthrax spores is fatal if the condition is not properly diagnosed and treated with antibiotics during the early stage of infection. Once \textit{B. anthracis} has released large amounts of toxins, no specific therapy is available and the prognosis is very poor (Mock and Fouet, 2001; Moayeri and Leppla, 2004). Hence, new therapeutic methods are needed to inactivate the toxins released by \textit{B. anthracis}.

Anthrax toxin consists of 3 proteins: a protective antigen (PA) and 2 catalytic moieties, lethal factor (LF) and edema factor (EF). PA is a receptor-binding and pore-forming subunit. LF is a protease that cleaves mitogen-activated protein kinase kinases (MAPKK), while EF is an adenylate cyclase that increases levels of cAMP in cells. Individually, none of the 3 are toxic, but a combination of PA and LF (LeTx) or PA and EF (EdTx) contributes to anthrax toxicity. In order for PA to bind LF and EF, it must be proteolytically activated. After binding to its receptor at the cell surface, native 83-kDa PA (PA83) is cleaved by furin-like protease to generate a 63-kDa fragment (PA63) and a 20-kDa fragment; the smaller being released from the cell. The remaining PA63 spontaneously oligomerizes to the heptamer or octameric prepropeptide. These complexes traffic to low-pH endosomes. The acidic pH conditions trigger protease conversion to form pores within endosomal membranes, enabling LF and EF to enter the cytosol. Once within the cytosol, LF activates cell death, while EF is responsible for the loss of chloride ions and water from cells. This disruption in osmolarity results in severe edema (Chauhan and Bhatnagar, 2002; Kurzchalia, 2003; Collier, 2009; Kintzer et al., 2010).

The actions of anthrax toxin are contingent on the binding of PA to cellular receptors (Young and Collier, 2007). Two cell surface receptors have been characterized: ANTXR1, also known as tumor endothelial marker 8 (TEM 8) and ANTXR2, also known as capillary morphogenesis protein 2 (CMG2) (Bradley et al., 2001; Scobie et al., 2003). Both membrane proteins have an extracellular domain related to von Willebrand factor A, which is critical for binding with PA. Investigations uncovering details about the molecular interactions between PA and receptors are being used to identify new targets for preventing this first step of intoxication and potential therapeutic agents for anthrax (Basha et al., 2006; Sharma et al., 2009).

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Glycosaminoglycans (GAGs) are long linear, highly negatively charged, heterogeneous polysaccharide macromolecules possessing a repeating disaccharide unit usually comprising an amino sugar with N-glucosamine or galactosamine and an uronic acid residue of either α-glucuronic acid or iduronic acid. GAGs are usually covalently attached to a protein core through an O-link to serine or an N-link to asparagine residue, and this overall structure is called a proteoglycan. Proteoglycans are expressed by all mammanial cells and are found on the cell surface, intracellular granules, and in the extracellular matrix (Bernfield et al., 1999; Sugahara et al., 2003; Gandhi and Mancrea, 2008).

GAGs have been shown to interact with a plethora of proteins, ranging from proteases, extracellular signaling molecules, lipid-binding and membrane-binding proteins, and cell-surface receptors on viruses (Iozzo and San Antonio, 2001; Patricia, 2004). They dynamically influence cell adhesion, differentiation, and proliferation. In addition, they act directly as receptors and assembly factors, such that many pathogens take advantage of them for localization and entry into cells. Furthermore, extracellular GAGs can potentially sequester proteins and enzymes, and present them to the appropriate compartment for activation (Bandtlow and Zimmerman, 2000; Sasisekharan et al., 2002).

Although the importance of GAGs in cell biology is widely studied, the exact role of GAGs in anthrax toxin-induced toxicity is unknown. In the present study, we specifically demonstrated chondroitin sulfate C may play important role in anthrax toxin-induced toxicity primarily by targeting the PA receptor function.

2. Materials and methods

2.1. Materials

Heparan sulfate, heparin, chondroitinase ABC, heparinase II, chondroitin sulfate A and C, and dermatan sulfate (also known as chondroitin sulfate B) were furnished by Sigma−Aldrich (Saint Louis, USA). MTT was obtained from US Corp. (Cleveland, USA). Anti-PA antibody was polyclonal serum derived from rabbits. PA, LF, and EF were purified as described previously (Kassam et al., 2005).

2.2. Cell culture

Macrophage-like cell line RAW 264.7 was cultured in IMDM (Invitrogen, Carlsbad, USA) with 10% heat-inactivated fetal bovine serum and 1% antibiotics (streptomycin + penicillin). Cells were grown in 24-well plates and incubated at 37 °C with 5% CO₂. The Chinese hamster ovary (CHO) cell line was maintained in RPMI 1640 medium with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin.

2.3. Cytotoxicity assay

RAW 264.7 cells were cultured with or without LeTx (1.0 μg/ml PA and 0.1–1.0 μg/ml LF) in 96-well plates (1 × 10⁴ cells/well) for 4 h, and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was then added at a final concentration of 0.5 mg/ml. After incubating at 37 °C for an additional 1 h, cell culture medium was removed. The insoluble formazan was dissolved overnight in solubilization solution. A microplate reader was used to read the A₅₇₀ of each well and the percentage of viable cells was calculated relative to untreated controls.

2.4. Glycosaminoglycans and glycosaminoglycan lyase treatment

Cells were treated with GAGs and glycosaminoglycan lyases essentially as described as before (Sava et al., 2009). To evaluate the effect of GAG on anthrax toxin toxicity, cells were preincubated with various concentrations of soluble heparin; heparan sulfate; dermatan sulfate; and chondroitin sulfate A, C, or B, followed by treatment LeTx (PA, 0.1 μg/ml and LF, 0.3 μg/ml) for 4 h. Cytotoxicity was assayed as described above. To test the effect of glycosaminoglycan lyases, cells were incubated with heparinase II or chondroitinase ABC for 1 h at concentrations ranging from 0.1 U/ml to 2.0 U/ml. Cells were then washed gently with serum free medium and then LeTx (PA, 1.0 μg/ml and LF, 0.3 μg/ml) was added according to the usual protocol. After incubation at 37 °C, cell toxicity was measured.

2.5. Biochemical assay of PA binding

PA binding assay was performed as previously described (David et al., 2005). The purified PA was labeled with AnaTan™ 5-FITC Microscale Protein Labeling Kit (AnaSpec, Fremont, USA), according to the manufacturer’s manual. For the surface binding assay, RAW 264.7 cells (1 × 10⁵ cells/ml) were harvested and washed 3 times, followed by chondroitin sulfate C treatment for 1 h at 25 °C. Chondroitin sulfate C treated cells were washed with PBS containing 1% BBS, followed by incubation with labeled PA (2 μg/ml) for 2 h at 4 °C in ice-cold growth medium containing 10% FBS. Cells were washed 3 times with ice cold PBS containing 10% FBS, and fixed in 4% paraformaldehyde for 15 min at 25 °C. Cells were then sorted and assessed by fluorescence-activated cell sorting (FACS) analysis using a Becton Dickinson FACSCalibur (Franklin Lakes, USA).

2.6. Detection of SDS-resistant PA oligomers

Cells were treated for 1 h at 4 °C with 1.0 μg/ml PA in the presence of chondroitin sulfate C, washed with PBS, and then pre-warmed culture media was added. After incubation at 37 °C for 1 h, cells were washed with cold PBS solution 5 times and lysed in lysis buffer containing a mixture of protease inhibitors. Cell lysates were incubated for 1 h in 5% normal dry milk in TBS (10 mM Tris–HCl, pH 7.5; 150 mM NaCl, and 0.1% Tween 20) and probed for 1 h with primary rabbit anti-PA polyclonal antibodies diluted in TBS/5% normal dry milk (1:8000). Membranes were then washed with TBS 3 times for 20 min and incubated for 1 h with anti-rabbit-horseradish peroxidase-labeled secondary antibodies in TBS. After an additional 4 washes with TBS, the bound antibody was visualized using an X-ray film. Chemiluminescence of bands and their relative intensities were quantified using a VersaDoc instrument (Bio-Rad, Hercules, USA).

2.7. Adenylate cyclase activity of EdTx in CHO cells

To determine the cAMP response generated by EdTx in cells, CHO cells treated with various concentrations of EdTx in RPMI 1640 medium at 37 °C. After incubation for 2 h, the media was removed and cells were lysed with lysis buffer. The cell lysate was clarified by centrifugation. The supernatant was collected for determination of intracellular cAMP concentrations by using the cAMP EIA Kit (Cayman, MI, USA).

2.8. Sodium chloride treatment of cells

Sodium chloride was used to inhibit sulfated adenylyltransferase activity, thus preventing sulfate donation to newly synthesized GAG chains (Greve et al., 1988). Sodium chloride was added to the cells 48 h prior to experiments, at a concentration of 30 mM as described previously (Greve et al., 1988).

2.9. Statistical analysis

Multiple group comparisons were performed using one-way ANOVA followed by Dunnett’s test, using GraphPad Prism software. The criterion of significance was “p < 0.05” and “p < 0.01.”

3. Results

3.1. Cytotoxicity of anthrax toxin in a macrophage cell line

To evaluate the influence of GAGs on LeTx-induced cytotoxicity in the murine alveolar macrophage cell line RAW 264.7, we first established the 50% effective concentration (EC₅₀) dose of toxin for this cell line. We then treated cells with various GAGs in combination with this dose. Cell viability was estimated using the MTT assay, which measures the reduction of a tetrazolium salt to formazan by metabolically active cells. As shown in Fig. 1, a concentration of 1.0 μg of PA per ml in the presence of 0.3 μg of LF per ml was required for lysis of half of the macrophages after 4 h incubation at 37 °C with LeTx (at EC₅₀). Further increase in LF exposure resulted in a sharp increase in cytotoxicity.

3.2. Effect of exogenous GAGs on LeTx-induced cytotoxicity

Initial experiments were performed to determine if treating the cells with exogenous GAGs would have any effect on LeTx-induced cytotoxicity. In these experiments, cells were treated with various GAG such as heparin, heparan sulfate, chondroitin sulfate A, dermatan sulfate (chondroitin sulfate B), and chondroitin sulfate C. Fig. 2 displays the change in cytotoxicity with LeTx only (untreated control) or LeTx in combination with GAGs. Specifically,
the addition of chondroitin sulfate C was associated with a significant decrease in cytotoxicity (Fig. 2A). Treatment with 80 μg/ml of chondroitin sulfate C increased survival to approximately 85%, a 35% improvement compared to cells treated with LeTx alone (EC50). This level of protection was statistically significant compared to that of the group of cells treated with LeTx alone (p < 0.05). In contrast, the addition of other GAGs: chondroitin sulfate A, dermatan sulfate, heparan sulfate, and heparin showed less ability to protect cells from LeTx-induced cytotoxicity as compared to chondroitin sulfate C.

To optimize the protective potential of chondroitin sulfate C against LeTx-induced cytotoxicity, we treated cells with a range of chondroitin sulfate C concentrations. This dose–response experiment showed a positive correlation between the dose of chondroitin sulfate C and the increase in cell viability. As little as 20 μg/ml of chondroitin sulfate C treatment was effective in improving survival, with an approximate 20% increase. Maximal inhibition of LeTx-induced cytotoxicity was achieved with a final concentration of 80 μg/ml chondroitin C (Fig. 2B). None of the GAG concentrations used in our experiments caused any cytotoxic effects or changes in the cell morphology (data not shown).

3.3. Enzymatic digestion of cell surface GAGs with glycosaminoglycan lyases

The role of GAGs in LeTx-induced cytotoxicity was further investigated by treating cells with glycosaminoglycan lyases. RAW 264.7 cells were subsequently treated with chondroitin ABC (which degrades chondroitin sulfate) and heparinase II (which cleaves heparin and heparan sulfate), as a frequently used means to remove highly sulfated GAGs from cells (Akula et al., 2001; Leistner et al., 2008). As indicated in Table 1, treatment of RAW 264.7 cells with increasing concentrations of chondroitin ABC, from 0.1 to 2.0 U/ml, significantly increased LeTx-induced cytotoxicity. The highest chondroitin ABC concentration (2.0 U/ml) also resulted in the highest toxicity to LeTx-treated RAW 264.7 cells. In contrast, treatment of cells with heparinase II had no significant effect on cytotoxicity. Thus, increasing cytotoxicity with chondroitin ABC treatment but not heparinase II revealed that chondroitin sulfate may play an important role in LeTx-induced RAW 264.7 cell toxicity.

Table 1

<table>
<thead>
<tr>
<th>GAG lyases</th>
<th>Concentration (U/ml)</th>
<th>Cell viability (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>–</td>
<td>100</td>
</tr>
<tr>
<td>LeTx alone</td>
<td>–</td>
<td>51 ± 2</td>
</tr>
<tr>
<td>Chondroitinase ABC</td>
<td>0.1</td>
<td>50 ± 3</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>45 ± 1</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>41 ± 2*</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>39 ± 1*</td>
</tr>
<tr>
<td>Heparinase II</td>
<td>0.5</td>
<td>51 ± 7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>52 ± 5</td>
</tr>
</tbody>
</table>

RAW 264.7 cells were treated with indicated dose of chondroitinase ABC and heparinase II for 1 h at 37°C in culture media. Cells were washed 3 times with serum-free culture media and then LeTx (PA 1.0 μg/ml and LF 0.3 μg/ml) was added according to the usual protocol. After incubation for 4 h at 37°C, cell toxicity was measured. Numbers present average of triplicate experiments ± S.D. expressed as a percent control of maximum count without toxin treatment of cells (*p < 0.05 versus LeTx alone).

3.4. Chondroitin sulfate C inhibits the binding of PA to the cell surface receptor

Since chondroitin sulfate C clearly inhibited LeTx-induced cytotoxicity in previous experiments, we examined whether this effect...
is due to a decrease of PA binding to cell surface receptors. To test this hypothesis RAW 264.7 cells were treated with chondroitin sulfate C and PA. After incubation at 4°C for 2 h, unbound PA was removed by washing with ice-cold PBS containing 10% FBS. Under these conditions, the majority of PA remains bound to the cell surface (Milne et al., 1994). Fluorescence-labeled PA was used to further quantify binding to RAW 264.7 cells in the presence or absence of chondroitin sulfate C. Cells were characterized for levels of the FITC-labeled PA binding by FACS analysis. Fig. 3 shows that fluorescence-labeled PA binding to cells is much less in the presence of chondroitin sulfate C. There was an approximate 40% decrease in cell-bound PA in the presence of 80 μg/ml chondroitin sulfate C compared to PA alone. These results suggest chondroitin sulfate C interferes with PA binding to its receptor.

### 3.5. Chondroitin sulfate C effects on PA oligomer formation

Once bound to the cell receptor, PA83 undergoes protease cleavage resulting in the active PA63 subunit. This further assembles spontaneously into PA oligomers (Milne et al., 1994). Therefore, we evaluated the potential effects of chondroitin sulfate C on PA processing. To determine the influence of exogenous chondroitin sulfate C on the formation of PA oligomers, lysates from RAW 264.7 and CHO cells that had been treated with PA or PA plus chondroitin sulfate C were analyzed by Western blotting using anti-PA antibody. As expected, formation of the SDS-resistant PA oligomers was inhibited by the addition of chondroitin sulfate C. Fig. 4 shows the results for both RAW 264.7 and CHO cells. Densitometry analysis indicated approximately 30% less detection of PA-oligomers in chondroitin sulfate C-treated cells as compared to control cells. These results indicate that protection from cytotoxicity by chondroitin sulfate C treatment is due to the reduced binding of PA to cell surface receptors, leading to less accumulation of PA oligomers.

### 3.6. Effects of chondroitin sulfate C on EF-induced cAMP accumulation

Encouraged by the results from the PA binding assay, we tested whether chondroitin sulfate C also mitigates the biological effects on edema toxin (EdTx). EF also enters host cells via formation with PA, in a complex known as edema toxin (Young and Collier, 2007). Previously, the cAMP response generated by edema toxin was analyzed in CHO cells (Leppla, 1982). In similar experiments, we have found the addition of chondroitin sulfate C can effectively inhibit EdTx-induced cAMP accumulation in CHO cells. As shown in Fig. 5, cells treated with chondroitin sulfate C exhibited nearly 60% inhibition of cAMP accumulation compared to control, EdTx-treated cells. These results suggest chondroitin sulfate C offers protection from both lethal and edema toxins, through its general effect on inhibiting the binding of PA to cell receptors.

### 3.7. Effect of sodium chloride as an inhibitor of sulfation for chondroitin sulfate C

To further investigate the role of chondroitin sulfate C in anthrax toxin-induced toxicity, we examined the effects of endogenously synthesized GAGs on anthrax toxin-induced toxicity. We utilized sodium chloride, a potent inhibitor of sulfatidyltransferase, to inhibit sulfation of all types of GAG chains (Greve et al., 1988). CHO cells were cultured for 48 h in the presence of 30 mM sodium chloride to decrease sulfation of GAG chains. Under these conditions, a significant increase in cAMP accumulation occurred with sodium chloride in the presence of EdTx compared with EdTx alone (Fig. 6).

To further determine whether inhibiting endogenous GAG formation also affected PA oligomer formation, PA was added to the sodium chloride-pretreated CHO cells. Formation of SDS-resistant PA oligomers was increased by sodium chloride pretreatment. As shown in Fig. 7 the band was darker when PA was incubated in the
presence of sodium chloride on CHO cells. PA oligomer formation increased approximately 24% in the presence of sodium chloride as compared to PA alone, as determined by densitometry (Fig. 7). These observations indicated that the presence of sodium chloride to reduce chondroitin sulfate C synthesis is associated with increasing PA-mediated toxicity to cells.

4. Discussion

PA, a key molecule in the tripartite anthrax toxin, functions by binding to cell surface receptors. This binding of PA to the receptor is a critical step in the intoxication process, and the sensitivity of cells to anthrax toxin is primarily determined by the expression of susceptible receptors. GAG polysaccharides occurring either in their free form or as components of proteoglycans, participate in a wide variety of biologic processes, especially in the regulation of binding of many ligands, such as chemokines and growth factors as well as larger proteins. In the present report, we studied the influence of GAGs on PA binding and subsequent anthrax toxin sensitivity. Competitive binding assays, enzymatic digestion, and reduction of sulfation of the GAG chains indicated that GAGs and specifically chondroitin sulfate C play an important role in toxicity from anthrax toxin.

Anthrax lethal toxin (LeTx) induces rapid cell death of RAW 264.7 macrophages. It triggers a cascade of physiological events in macrophages, including loss of membrane potential, drop of ATP levels and morphological changes, leading to cell death (Lin et al., 1996; Ha et al., 2007). Previous studies have used purified GAGs as competitive inhibitors of ligands to study the role of these molecules in the ligand–receptor interaction (Shishido et al., 1995; Ghiabe et al., 2002). In their experiments, 10–100 μg/ml range of GAG concentrations demonstrated significant influence on ligand–receptor interaction. We exogenously applied similar concentrations of chondroitin sulfate, heparan sulfate, and heparin and examined their effects on cell toxicity. Specific treatment of RAW 264.7 cells with chondroitin sulfate C could inhibit LeTx-induced cytotoxicity. Other GAGs, namely, chondroitin sulfate A, dermatan sulfate, heparan sulfate, and heparin, could not effectively inhibit the cytotoxicity.

Next, we examined the cytotoxicity of cells after enzymatic removal of cell surface GAGs with chondroitinase ABC or heparinase II digestion. Treatment with chondroitinase ABC significantly compounded the cytotoxicity of LeTx, further supporting that chondroitin sulfates may contribute to LeTx-induced cytotoxicity. These competitive inhibition assay and enzymatic digestion study implied that chondroitin sulfate C at the cell surface is able to modify the cellular response to anthrax toxin. It is noteworthy that heparin-like molecules on the cell surface are a third factor influencing binding of diphteria toxin to cell receptors (Shishido et al., 1995), indicating that GAGs likely play a larger role in pathogen-toxin pathways.

The chondroitin sulfate chains are linear polymers constructed from 40 to more than 100 repeating disaccharide units comprising N-acetyl-galactosamine (GalNAC) and hexuronic acid that are linked together by β-glycosidic (β 1,4 or β 1,3) linkages. However, heparin and heparan sulfate contain glucosamine as a repeating disaccharide unit (Bernfield et al., 1999; Sugahara et al., 2003). Currently, we cannot clearly explain why chondroitin sulfate C showed better ability to protect cells from anthrax toxin-induced cytotoxicity as compared to other GAGs. The different structures of various GAGs might explain our results. Chondroitin sulfate A contains
GalNAc-4 sulfated linked to glucuronic acid. Chondroitin sulfate C is distinguished from chondroitin sulfate A by a 6-sulfation amino sugar moiety. Chondroitin sulfate B (dermatan sulfate) is similar in sulfation to chondroitin sulfate A but uronic acid is predominantly iduronic acid (Bernfield et al., 1999). Thus, we speculate that not only specific disaccharide structure and sulfation pattern, but also charge of chondroitin sulfate chains may play the important role in the protective activity against anthrax toxin. Difference in binding to specific GAGs and cell-specific expression profiles of GAGs may account for selective toxicity of anthrax toxin in different cell lines. Previously, negative charge density and the disaccharide structure of GAG have been proposed to be essential players of cell–protein interaction (Sasisekharan et al., 2002; Gandhi and Mancera, 2008).

The anthrax toxin belongs to a binary toxin family. Each binary toxin consists of 2 nontoxic proteins that combine at the surface of a receptor-bearing cell. One of these proteins is LF or EF (the A moiety) and the other is a receptor-binding and pore-forming protein, PA83 (the B moiety). Toxin activity begins when PA83 binds to receptors. Following receptor binding, PA83 is cleaved by a furin-containing protease of cellular origin, leaving a receptor-bound PA63. The PA63 can bind up to 7 or 8 molecules of receptor and form an oligomer preface structure.

LF is a Zn\(^{2+}\)-dependent metalloproteinase. Once within the cytosol, LF cleaves the N-terminus of MAPKK family members, disrupting a protein–protein interaction site (Collier, 2009). Treatment of macrophage with PA and LF results in a rapid cell lysis. LF gains access to the cytosol of cells by first binding to PA oligomers. The PA oligomer binds up to three molecules of LF with high affinity (Young and Collier, 2007). By using fluorescence-labeled PA we demonstrated that chondroitin sulfate C was an effective inhibitor of PA binding to the cell surface. Furthermore, these results indicate that impairment of LeTx-induced cytotoxicity by chondroitin sulfate C treatment is due to the inhibition of proper binding of PA to the receptors in the first step of the intoxication process. This was further supported by the observations that addition of chondroitin sulfate C inhibited formation of PA oligomers in both RAW 264.7 and CHO cells. Taken together our results suggest that chondroitin sulfate C affects PA binding, thereby preventing oligomerization of PA and subsequent binding and translocation of LF, finally blocking LeTx-induced cytotoxicity.

The other toxic component from anthrax is edema factor (EF), which also enters host cells via a complex with PA to form the edema toxin. This catalyzes the formation of cAMP resulting in cell signaling disruptions and tissue ion fluxes, leading to failure of various organs (Drum et al., 2000; Firoved et al., 2005). The entry of the EF in different cell types has been studied by monitoring EF-induced changes in intracellular cAMP. Previously, in the CHO cells, EdTx causes a detectable increase of cAMP after 1 h of incubation, which increased 200-fold above normal after 2 h of exposure (Leplla, 1982). We discovered that treatment of CHO cells with chondroitin sulfate C inhibited cAMP production by EdTx. Accordingly, these data indicate that parallel inhibitory activities of chondroitin sulfate C against the anthrax toxins (LF and EF), both of which utilize PA for translocation into the cytosol, support PA binding as its primary target of influence.

To study the biological significance of endogenous GAG chains, sodium chloride has been employed to alter or inhibit their synthesis (Shishido et al., 1995; Sava et al., 2009). In the sodium chloride-pretreated cells, GAG chains are not sulfated and therefore have altered charge and structure characteristics. Studies by Greve et al. (1988) have demonstrated that culture with mM amounts of sodium chloride cause a reduction in sulfate incorporation into chondroitin sulfate proteoglycan by up to 95% in human fibroblast cells. We observed that when CHO cells were pretreated with sodium chloride their cAMP accumulation and PA oligomer formation by anthrax toxin was significantly increased, demonstrating that the expression of sulfated GAGs are important for anthrax toxin-induced toxicity. These results are in agreement with our enzymatic digestion study demonstrating that chondroitinase ABC treatment led to increased LeTx-induced cytotoxicity of RAW 264.7.

In summary, the results of this study demonstrate an important role of GAGs in anthrax toxin-induced toxicity. Our results show that chondroitin sulfate C is capable of modulating interactions between PA and their receptors. However, the precise mechanism of how chondroitin sulfate C can inhibit the action of anthrax toxin remains unknown, but possibility can be considered. In competitive binding assays, chondroitin sulfate C reduced binding of PA to its receptors, while chondroitin sulfate C that had been digested with chondroitinase ABC produced more cytotoxicity, suggesting that the presence of chondroitin sulfate C prevents delivery of PA to the receptors. These findings indicate that chondroitin sulfate C may exert its inhibitory effect by binding to the PA, thus sequestration of PA away from its receptor on cell surface and thereby disrupting PA-receptor interactions. To the best of our knowledge, this is the first time that the interaction of PA with chondroitin sulfate C has been demonstrated, improving our understanding of the potential molecular mechanisms involved in anthrax toxin-induced toxicity. However, it remains to be determined how important this proposed interaction is during actual anthrax toxin exposure in vivo. It may prove evidence that anthrax toxin activity may depend on the GAG species expressed at the membrane of specific cell types.

Although the present study is lacking details regarding the specific structure of chondroitin sulfate C in the interaction with PA, the potential of chondroitin sulfate C as an inhibitor for anthrax toxin provides impetus for further study, particularly, since few methods are presently available to treat anthrax toxin exposure. Without treatment, the mortality rate from inhalation of anthrax is >90%, but even with the intensive treatment provided to the patients of the 2001 bioterrorism attacks, the mortality rate was 45% (Inglesby et al., 2002). Thus, an inhibitor that targets the toxin would be a helpful addition to the current antibiotic therapy.

Conflict of interest

The authors declare no conflict of interest.

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