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Polymeric glycoconjugates protect and activate macrophages to promote killing of *Bacillus cereus* spores during phagocytosis

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Abstract Diseases caused by *Bacillus* spores might be attenuated if macrophages were able to kill the spores on exposure. Glycoconjugate-bearing polymers, which have been shown to bind to *Bacillus* spores, were tested for modulation of phagocytosis of *B. cereus* spores. Without glycoconjugate activation, murine macrophages were ineffective at killing *Bacillus* spores during phagocytosis. In the presence of glycoconjugates, however, the macrophages efficiently killed the organisms. The glycoconjugates were shown to have a protective influence, sparing macrophages from spore-induced cell death. Very low concentrations of the glycoconjugates prevented macrophage cell death, as shown by lactate dehydrogenase (LDH) release and trypan blue assays. Increased levels of inducible nitric oxide (NO) production by the macrophages in the presence of glycoconjugates suggested that the glycoconjugates provide an activation signal to the macrophages. These results suggest that glycoconjugates promote the killing of *Bacillus* spores by blocking spore-induced macrophage cell death, while increasing their activation level. Polymeric glycoconjugates may suggest novel approaches to improve existing vaccines as well as prevent and treat infections incurred through either *B. cereus* or *B. anthracis* spores.

Keywords *B. cereus* spores · Glycoconjugate · Macrophages · Phagocytosis · Nitric oxide

Abbreviations

Glyc-PAA-flu	glycoconjugate-polyacrylamide-fluorescein polymer
GC1	Galα 1–3GalNAcα-PAA-flu glycoconjugate
GC3	GalNAcα1–3GalNAcβ-PAA-flu glycoconjugate
Gal	galactose
GalNAc	N-acetylgalactosamine
PAA	polyacrylamide
flu	fluorescein
LDH	lactate dehydrogenase
NO	nitric oxide
CFU	colony forming units
MP	macrophages only
OD	optical density

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Introduction

Phagocytosis plays an important role in a variety of cell functions ranging from innate to adaptive immunity, tissue repair and morphogenetic remodeling, and homeostasis [1]. Phagocytic cells including macrophages and neutrophils guard, attack, and destroy microorganisms and other foreign materials.

Bacillus genus species are responsible for food spoilage, and food-borne illness problems [2]. Additionally, *Bacillus*

anthracis strains are potential biological warfare agents and bioterrorism [2, 3]. Under nutrient-poor conditions, *Bacillus* organisms produce spores that are highly resistant to heat, drying, radiation, and antiseptic treatment [2, 3]. Spores typically have exosporium, coat, inner coat, cortex, inner membrane, and a core [2, 3]. Several proteins expressed on *B. anthracis* and *B. cereus* spores are glycosylated [4–8]. *Bacillus* spores enter the body through the skin, lungs, or the gastrointestinal tract and are engulfed by macrophages, which may carry them to local lymph nodes [2–3, 9]. Once *B. anthracis* spores germinate into vegetative cells, the vegetative cells produce powerful toxins that diminish macrophage capacity to kill the bacteria [10–12]. Vegetative cells divide, disseminating through the blood stream and produce toxins such as edema factor (EF) and lethal factor (LF), which along with protective antigen (PA), reduce host resistance to infection [13, 14]. Cellular internalization of EF and LF causes the clinical symptoms of anthrax infection. Diseases caused by *Bacillus* spores might be attenuated if macrophages were able to kill the spores on exposure [10–14].

PA appears to be the main target of all existing anthrax vaccines [15]. Antibodies recognize PA, block the binding and internalization of EF and LF [16, 17]. Only two bacterial strains were used in anthrax vaccine preparation. The U.S. vaccine (AVA Biothrax, also known as AVA or MDPH-PA) is prepared from the virulent *B. anthracis* V770-NP1-R strain [16]. The vaccine developed in the U.K. is prepared from the filtered *B. anthracis* 34F2 Sterne strain [17, 18]. Antibodies induced by both anthrax vaccines only recognize PA [16–18], but not spores. Specificity and selectivity of antibodies depend on the antigen-binding region and antigen structure. Changes of *B. anthracis* antigens can render antibodies ineffective. Further, post-vaccination immunity will develop only on the 15th day of urgent immunization. Anthrax symptoms, however, will usually develop as early as 4–5 days after exposure [3]. Investigators have reported limitations with the current, licensed vaccines [18, 19]. The PA-specific response to the U.K. vaccine peaks 2 weeks post-immunization and declines to pre-boost levels by the 12th week [19]. In addition, the PA-specific antibody response varies widely with host heterogeneity [19]. Although the safety of both vaccines has been established, concerns over side effects still persist [18, 19] (http://video.google.com/videoplay?docid=2346712021702683294&q=anthrax&hl=en). While available anthrax vaccines can prevent classical anthrax, genetic mixing of different resistant *B. anthracis* strains, or even modification of non-virulent bacterial species, can render the vaccine ineffective.

The preferred drugs for anthrax treatment are penicillin, ciprofloxacin, and doxycycline [3]. However, antibiotic resistance due to a high bacterial mutation rate has potential life-threatening consequences [20].

There is a need for new effective vaccines and drugs and/or immunomodulators that decrease morbidity and mortality associated with infections caused by spores.

The potential use of synthetic glycoconjugates for biomedical and pharmaceutical applications [21, 22] is due to the fact that glycoconjugates can be designed to meet specific, physicochemical requirements [21] and have a long shelf-life and low toxicity [21–25]. Glycoconjugates are involved in cell recognition and signaling processes intrinsic to biochemical functions in cells. In addition, carbohydrates are recognized as differentiation markers of cells and antigenic determinants [26, 27]. Carbohydrates play a central role in cell-to-cell adhesion and in subsequent recognition and receptor activation [28–30].

Among potential cellular targets by glycoconjugates, macrophages are considered ideal, since they play a central role in inflammation and innate immunity to microorganisms involved with infectious diseases [9–12, 14, 17].

Our group previously reported that glycoconjugate-bearing polymers [carbohydrate moiety (Glyc)-polyacrylamide (PAA)-fluorescein (flu)] contribute to recognition [7] and inhibition of *B. cereus* spores [31, 32]. It is apparent that i) glycoconjugates predominantly bind to *B. cereus* compared to *B. thuringiensis*, *B. pumilus,* and *B. subtilis* [7]; and ii) glycoconjugates scarcely bind to *B. subtilis* yielding in low optical density (OD) values compared to other spores [7]. Previously obtained data indicate that several glycoconjugates, namely GalNAcα1–3GalNAcβ-PAA-flu and Galα1–3GalNAcα-PAA-flu, have a marked inhibition affinity for *B. cereus* [31, 32]. Both glycoconjugates contribute to a more efficient extracellular inhibition of *B. cereus* compared to *B. subtilis* [31, 32]. This conclusion is based on the dilution effect of glycoconjugates on extracellular inhibition of *Bacillus* spores on trypticase soy agar [31, 32]. Changes in glycoconjugate concentrations did not potentially hinder binding [7] and inhibition efficacy [31, 32]. Even highly diluted glycoconjugate solutions enabled binding [7] and inhibited *B. cereus* on trypticase soy agar [31, 32].

In the present study, we evaluated the efficacy of selected GalNAcα1–3GalNAcβ-PAA-flu and Galα1–3 GalNAcα-PAA-flu [31, 32] glycoconjugates in phagocytosis of *B. cereus* spores using murine macrophages.

The Glyc-PAA-flu polymers are synthetic ligands in which carbohydrate units are bound to a polyacrylamide matrix thereby creating a fluoresceinated 30kd multivalent polymer in a ratio of 20:1. Glyc-PAA-flu binds non-specifically to cellular components [30]. Both the fluorescent label and the polymer backbone (Fig. 1), affect this interaction minimally [30]. PAA-flu by itself is hydrophilic and shows low binding affinity and flexibility. In addition, its structure allows shorter ligands to bind [30].

B. cereus was selected as a model for *B. anthracis* spores due to the close genetic relationship between *B. cereus* and

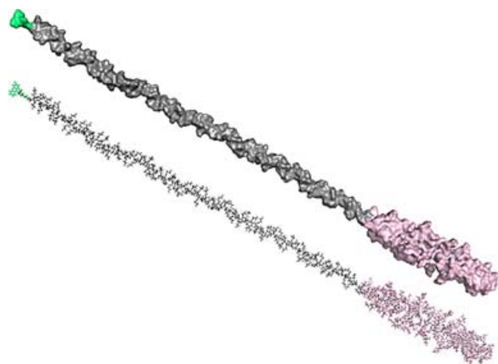

Fig. 1 Wireframe (*lower part*) and surface mapped model (*upper part*) of a glycoconjugate molecule. The fluorescein group is shown in *green* (*far left*), the backbone in *gray*, and reactive carbohydrate moieties are shown in *pink* (*lower right*)

B. anthracis, both classified within the *B. cereus* group [34–37].

Materials and methods

Materials

Fluoresceinated disaccharide glycoconjugates Galα1–3 GalNAcα-PAA-flu and GalNAcα1–3GalNAcβ-PAA-flu were obtained from GlycoTech, Inc. (Rockville, MD). *B. cereus* ATCC 11778 was purchased from Raven Biological Laboratories Inc. (Omaha, NE). Trypan blue and neutral red were obtained from Sigma Chemical Co. (St. Louis, MO). The CytoTox 96® kit was obtained from Promega, Inc. (Madison, WI). C57BL/6 mice were purchased from Harlan Sprague Dawley, Inc. (Indianapolis, IN) and maintained in an AALAC-approved vivarium at the University of Arkansas for Medical Sciences (Little Rock, AR).

Cell Culture

C57BL/6 mice, 6 to 8 weeks old, were injected intraperitoneally with 1.0 ml of 3% thioglycollate broth. Four days after injection, mice were euthanized and peritoneal exudate cells were collected by lavage with 5.0 ml RPMI 1640. Macrophages were plated in 6-well plates at a concentration of $1.0\times1.2\times10^6$ macrophages per culture. After 1 h of incubation at 37°C (95% air, 5% CO_2), nonadherent macrophages were removed by washing using RPMI 1640 containing 10% fetal calf serum, 50 nM 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin, thus resulting in a macrophage concentration of $6.0\times8.0\times10^5$ macrophages per culture.

Spore inhibition

Studied glycoconjugates (1 mg/ml or 10^0 mg/ml) were serially diluted (10^{-1}–10^{-12} mg/ml) prior to use in phagocytosis studies. To evaluate intracellular spore destruction by macrophages, 0.5 μl of *B. cereus* spores [2.4×10^6 colony forming units (CFU)/0.1 ml] were treated with 5.0 μl of each serially diluted glycoconjugate (10^0–10^{-12}) and incubated for 1 h at room temperature. Untreated spores were used as a control (2.4×10^6 CFU/0.1 ml). Macrophage cultures ($6.0\times8.0\times10^5$/culture) were infected with glycoconjugate-treated or untreated spores (2.4×10^6 CFU) at a ratio of 3–4 spores/macrophage and incubated for 24 h at 37°C (95% air, 5% CO_2). Macrophages were then lysed in order to release spores for viability studies (Fig. 2). The resulting pellets were washed three times with 1 ml of sterile, distilled water and centrifuged using an accuSpin Micro centrifuge R (Walham, MA) for 10 min at 5,000 rpm. Pellets were serially diluted (10^{-1}–10^{-6}) and 10 μl of each dilution was plated onto trypticase soy agar petri dishes. Plates were incubated overnight at 37°C and resulting colony forming units (CFU) were counted.

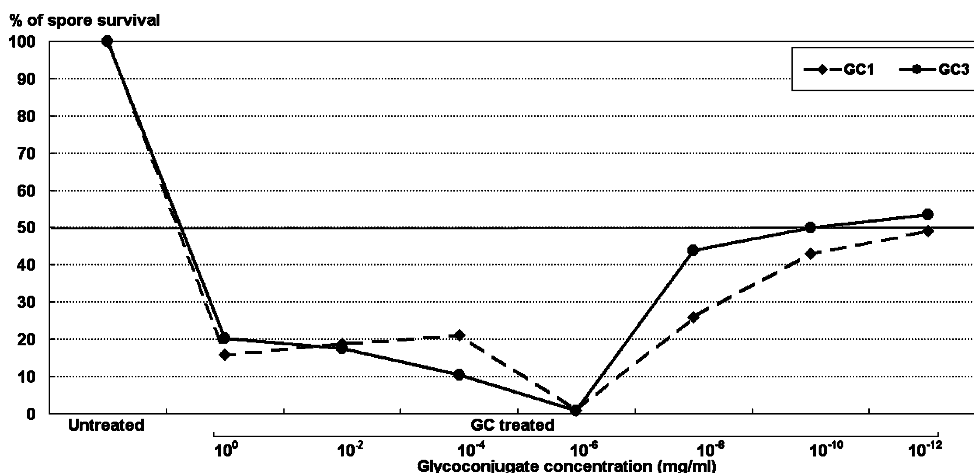

Fig. 2 Glycoconjugates Galα1–3GalNAcα-PAA-flu (*GC1*) or GalNAcα1–3GalNAc β-PAA-flu (*GC3*) (here and hereafter) facilitate spore destruction by macrophages, $p<0.01$. These results were expressed as mean percent spore killing of untreated and glycoconjugate treated spores. These results are representative of triplicate experiments. Note: a spore survival rate of x% inversely indicates that 100-x% of spores were killed (or have perished) during phagocytosis

Macrophage studies

Spore-induced macrophage damage was measured by cell morphology, trypan blue, lactate dehydrogenase (LDH) release, and nitric oxide production. To determine cell viability, the culture supernatants were replaced with 500 µl of 0.4% trypan blue solution and examined microscopically using a Nikon Eclipse E400 POL fluorescence microscope at a magnification of ×400. Digital micrographs were acquired in real time (Fig. 4). Percent viability was determined by counting individual live/dead macrophages. Ten determinations were made for each culture well (Fig. 4).

Macrophage integrity was analyzed using CytoTox 96®kit (Promega, Inc., Madison, WI). LDH is a stable cytosolic enzyme released upon cell lysis with conversion of tetrazolium salt to a red formazan product. The amount of color was proportional to the number of lysed cells. The absorbance was measured at 490 nm using a Bio-Tek Ex800 plate reader.

For nitric oxide (NO) production, macrophages were plated at 1.0×10^6 cells/culture in 6-well flat-bottomed tissue culture plates and incubated with glycoconjugate-treated or untreated spores. Cells were incubated at 37°C in 5% CO_2 for 24 h. Supernatants (100 µl) were then assayed for NO. The nitrite ion (NO_2^-) concentration, indicative of NO, was determined using $NaNO_2$ as a standard [38]. Briefly, 100 µl of cell culture supernatant was mixed with an equal volume of Griess reagent [0.1% (w/v) *N*-(1 naphthyl)ethylenediamine dihydrochloride and 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid]. The samples were incubated at room temperature for 20 min and absorbance was measured at 490 nm using a Bio-Tek Ex800 plate reader.

Statistics

Results were considered statistically significant at *p*-values <0.01 using ANOVA. A Tukey test was performed for post-ANOVA.

Results

In this study, selected Glyc-PAA-flu polymeric glycoconjugates namely Galα1–3GalNAcα-PAA-flu and GalNAcα1–3 GalNAcβ-PAA-flu were evaluated for the ability to modulate phagocytosis of *Bacillus* spores using murine macrophages (Fig. 2).

The addition of glycoconjugate Galα1–3GalNAcα-PAA-flu resulted in almost total destruction of the spores (Fig. 2). As shown in Fig. 2, glycoconjugate Galα1–3GalNAcα-PAA-flu facilitated the destruction of 89.4% ($p<0.01$) of spores, while glycoconjugate GalNAcα1–3GalNAcβ-PAA-flu facilitated the destruction of 86.4% of spores ($p<0.01$) compared to untreated spores. To judge the efficacy of glycoconjugates, these were diluted to 10^0–10^{-12} prior to addition to spores. Results showed that 50% of the spores were killed at a dose of only 0.1 pg/ml GalNAcα1–3GalNAcβ-PAA-flu glycoconjugate or 0.001 pg/ml Galα1–3GalNAcα-PAA-flu glycoconjugate (Fig. 2).

To further characterize the glycoconjugates, their effects on spore-induced damage to macrophages were examined. One day after macrophage exposure to treated or untreated spores, LDH release was determined (Fig. 3). LDH analysis showed that macrophages exposed to untreated spores released 85% of LDH (Fig. 3). Glycoconjugate GalNAcα1–3GalNAcβ-PAA-flu induced 11.9% LHD production, whereas 8.9% LDH was observed with glycoconjugate Galα1–3GalNAcα -PAA-flu ($p<0.01$).

As shown in Fig. 4, dilution of the glycoconjugates as much as a million fold did not attenuate the protective effect for macrophages. This was consistent with a dramatic loss of macrophage viability following exposure to untreated *B. cereus* spores (Fig. 4a). In contrast, the glycoconjugates protected the macrophages from spore-induced loss of viability (Fig. 4b,c). The presence of either GalNAcα1–3 GalNAcβ-PAA-flu or Galα1–3GalNAcα-PAA-flu glycoconjugates induces macrophage viability up to 87 and

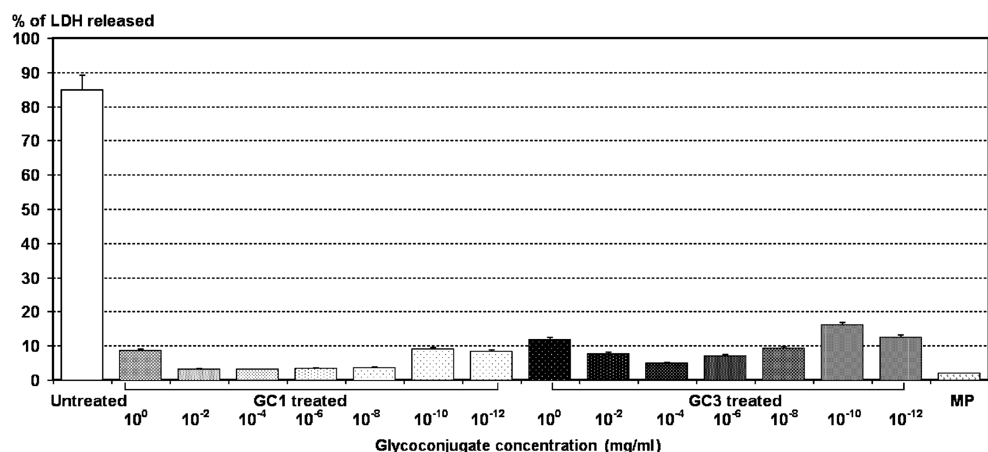

Fig. 3 Glycoconjugates protected macrophages from spore-induced damage, $p<0.01$. Macrophage cultures were exposed to untreated and treated spores. After 24 h, macrophage LDH was assayed in macrophages exposed to untreated spores or to treated spores or to macrophages only (*MP*). These results are representative of triplicate experiments

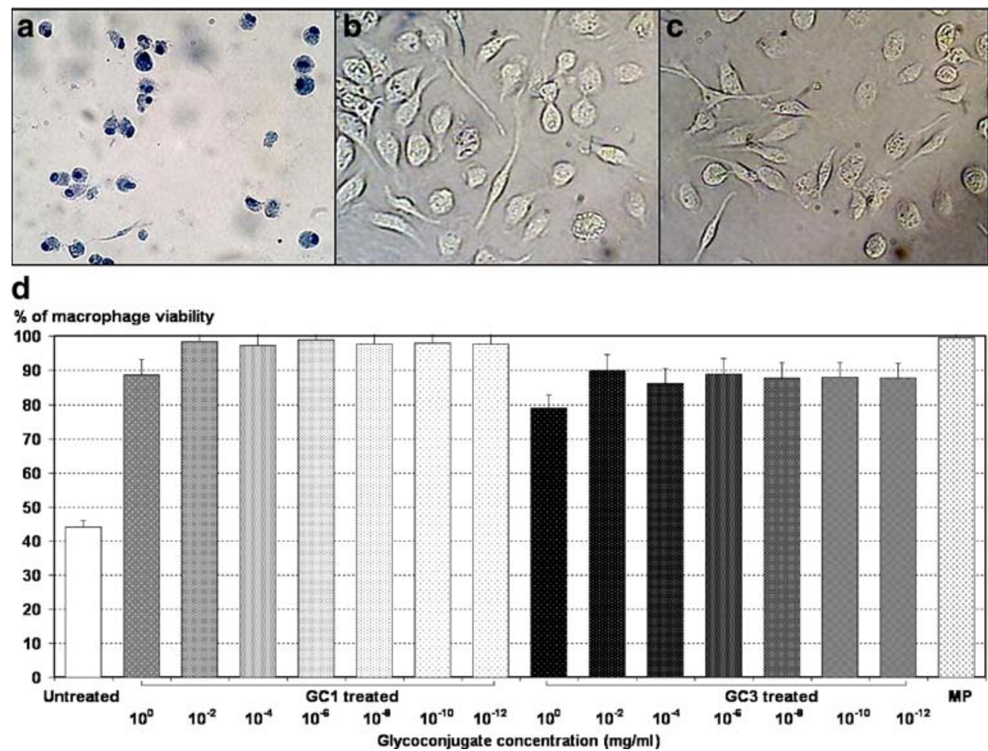

Fig. 4 Glycoconjugates protected macrophage viability after exposure to *Bacillus* spores, $p<0.01$. Macrophages were exposed to untreated (**a**) and Galα1–3GalNAcα-PAA-flu (GC1) (**b**) or GalNAcα1–3GalNAcβ-PAA-flu (GC3) (**c**) treated spores (**b**) and stained for viability with trypan blue 24 h later. The viability for macrophages exposed to untreated spores, treated spores, and macrophages only (MP) are shown (**d**). These results are representative of triplicate experiments. Note: a macrophage viability x% inversely indicates that 100-x% of macrophages did not survive during phagocytosis

97.8%, respectively (Fig. 4d). Viability of macrophages exposed to untreated spores was only 44% (Fig. 4d).

Since macrophage NO production is important in the killing of phagocytized bacteria [43] and a marker of macrophage activation [38–42], the effects of the glycoconjugates on macrophage NO production were examined (Fig. 5).

Macrophages were exposed to spores, but were not otherwise activated. In the absence of glycoconjugates, *B. cereus* spores killed most of the macrophages without inducing more than background NO production (Fig. 5). The presence of either Galα1–3GalNAcα-PAA-flu or GalNAcα1–3GalNAcβ-PAA-flu glycoconjugate induced macrophage NO production ($p<0.0001$). Glycoconjugate Galα1–3GalNAcα-PAA-flu doubled macrophage NO production, while glycoconjugate GalNAcα1–3GalNAcβ-PAA-flu was less stimulatory. In general, NO production decreased with increasing dilution of the glycoconjugates (Fig. 5). It was reported that a high level of NO can promote apoptosis cells [38–42]. Our results have shown that the less NO is produced, the more macrophages survive (Fig. 4) and consequently the more spores are killed (Fig. 2).

Discussion

Our main aim is to identify alternative synthetic glycoconjugate ligands (Fig. 1) that would mimic antibodies and can selectively recognize and kill spores on exposure prior to their germination and production of deadly toxins.

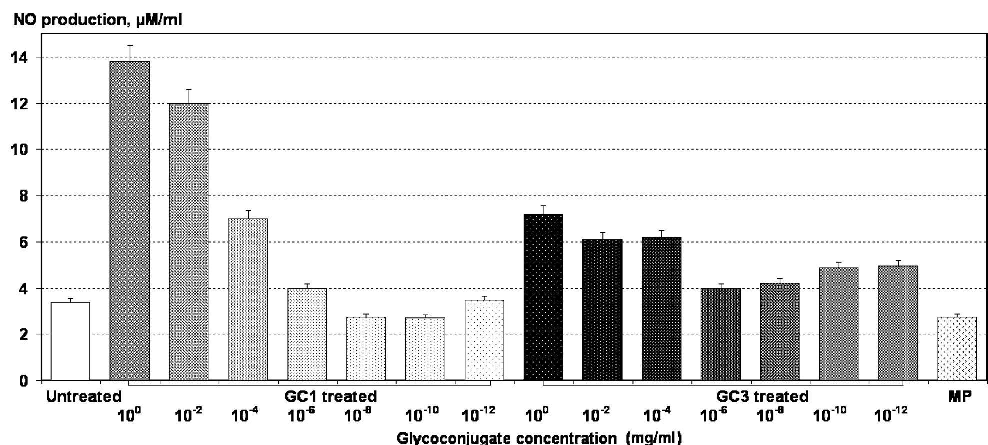

Fig. 5 Glycoconjugates stimulated macrophage nitric oxide (*NO*) production, $p<0.0001$. Macrophage cultures were exposed to untreated and treated spores and to macrophages (*MP*) only. After 24 h, macrophage NO production was measured by the Griess assay. These results are representative of triplicate experiments

Our research was inspired by the fact that current antibody-based vaccines only recognize PA toxin produced by vegetative bacterial cells [15–17]. In addition, current vaccines will only recognize PA of *B. anthracis* strains used in vaccine production. In addition, numerous problems correlated with antibodies have been reported, *i.e.* A) antibodies have a short shelf life, and B) they are chemically unstable characterized by a high degradability rate. Glycoconjugates, however, provide numerous advantages over antibodies [21–25, 27, 46, 47, 49, 50]. Glycoconjugates have a longer shelf-life and exhibit low toxicity. They can be easily modified using organic or enzymatic approaches [21–25, 27, 46, 47, 49, 50].

Following exposure to *Bacillus* spores, they will be rapidly taken up by macrophages [9, 14]. The ability of macrophages to kill the organisms will determine whether the exposure will progress to a possibly serious infection. Exposure of murine macrophages to *B. cereus* spores at a ratio of 3–4 spores/macrophage resulted in macrophage death 24 h later. This was demonstrated by LDH and trypan blue assays.

The present study primarily focused on glycoconjugates and their protective role in macrophages exposed to *B. cereus* spores. The presence of glycoconjugates prevented the widespread death of macrophages exposed to the spores (Fig. 4). Moreover, the macrophages were able to efficiently kill the organisms (Fig. 2). Glycoconjugates have been reported to interrupt spore germination [31, 32], which could contribute to macrophage efficiency. Carbohydrates on the spore coat could serve as potential receptors for interactions [7] with the monosaccharide units of the glycoconjugates thus, leading to adhesion [31, 32]. Alteration of the receptors by glycoconjugates might impair spore germination [7, 31, 32]. The glycoconjugates bound to the spores might also act as opsonins, promoting phagocytosis [43]. Even when highly diluted, glycoconjugates were very effective at protecting the macrophages from cell death and promoting killing of the *Bacillus* spores (Figs. 2 and 4).

There is some evidence that glycoconjugates can serve as chemoattractants and/or immunostimulators for macrophages [53] or "skilled" phagocytic cells [44]. Indeed, the glycoconjugates increased macrophage production of inducible NO, which is important in intracellular killing [43, 45] of *B. cereus* spores (Fig. 5) [53].

Specific carbohydrate structures expressed on pathogens are believed to be recognized by complementary molecules expressed on the surface of interacting cells [28, 29, 46–51]. Previous studies showed that the spore exterior layers are, in fact, glycosylated [4–8]. *B. cereus* spore's exterior [7, 31, 32] are likely involved in interactions with galactose (Gal) bearing glycoconjugates [7]. We have previously reported that Gal containing glycoconjugates increase binding to *B. cereus* spores [7, 31, 32]. Prospective bioinformatics studies on receptor-glycoconjugate interactions will clarify this issue. Differences in carbohydrates [4–8, 21] on bacterial spores could lead to differences in glycoconjugate effectiveness (Figs. 2, 3, 4, 5) [7, 31, 32, 44].

Previous reports suggest that complex carbohydrates are involved in recognition processes, including adhesion between cells, adhesion of cells to the extracellular matrix, and specific recognition of cells by one another [28, 29, 46, 47, 50]. Carbohydrate–carbohydrate interactions play an important role in the complimentary binding of glycosphingolipids [48, 51, 52]. Recognition [7] and inhibition [31, 32] of bacterial spores is based on multivalent carbohydrate–carbohydrate interactions [7, 27, 46, 48–50] between disaccharide-bearing glycoconjugates acting as ligands [7, 31, 32, 53] and carbohydrates and/or glycosylated proteins expressed on spores [4–8, 31, 32, 53]. Different glycoconjugates may exhibit different activities and this may be reflected by the differences observed in inducible NO production by macrophages. Furthermore, it was shown that a high level of NO can promote apoptosis in some cells, whereas lower NO levels inhibit apoptosis in others [39–42]. Our results show that lower dilution promotes viability of macrophages (Fig. 4), lower LDH (Fig. 3), and NO production (Fig. 5).

Presumably, binding of glycoconjugates led to alteration of receptors that play an essential role in spore germination [31, 32]. CFU of undigested spores was a direct indicative of glycoconjugate efficacy (Fig. 2). Even after being bound to spores on one side, glycoconjugates serve as chemoattractants for macrophages or other phagocytic cells [44, 53] on the other side. Macrophages may become more prone to adhere to glycoconjugate-coated spores, resulting in increased phagocytosis and NO production (Fig. 5) and killing of *B. cereus* spores (Fig. 2).

The present studies could lead to an improved selection of glycoconjugate ligands having immunomodulating properties and could suggest new targets for vaccine and/or immunomodulator against *B. cereus* or *B. anthracis* spores and/or for the improvement of current vaccines.

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