Anthrax Spore Detection by a Luminex Assay Based on Monoclonal Antibodies That Recognize Anthrose-Containing Oligosaccharides

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Anthrax is an acute zoonotic disease caused by the spore-forming bacterium Bacillus anthracis. It affects primarily herbivores in many countries of Southern Europe, South America, Asia, and Africa (24). Endospores are the infecting agent and remarkably resistant to extreme heat, dryness, chemicals, or irradiation, thus ensuring long-term survival. The principal virulence factors are a capsule and two exotoxins produced by the growing vegetative form. The major sources of human anthrax infection are direct or indirect contact with infected animals. A growing vegetative form. The major sources of human anthrax infection are direct or indirect contact with infected animals. The similarity of endospore surface antigens between bacteria of the Bacillus cereus group complicates the development of selective antibody-based anthrax detection systems. The surface of Bacillus anthracis endospores exposes a tetrasaccharide containing the monosaccharide anthrose. Anti-tetrasaccharide monoclonal antibodies (MAbs) and anti-anthrose-rhamnose disaccharide MAbs were produced and tested for their fine specificities in a direct spore enzyme-linked immunosorbent assay (ELISA) with inactivated spores of a broad spectrum of B. anthracis strains and related species of the Bacillus genus. Although the two sets of MAbs had different fine specificities, all of them recognized the tested B. anthracis strains and showed only a limited cross-reactivity with two B. cereus strains. The MAbs were further tested for their ability to be implemented in a highly sensitive and specific bead-based Luminex assay. This assay detected spores from different B. anthracis strains and two cross-reactive B. cereus strains, correlating with the results obtained in direct spore ELISA. The Luminex assay (detection limit 10^3 to 10^4 spores per ml) was much more sensitive than the corresponding sandwich ELISA. Although not strictly specific for B. anthracis spores, the developed Luminex assay represents a useful first-line screening tool for the detection of B. anthracis spores.

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**Materials and Methods**

Generation of anti-anthrose-rhamnose disaccharide MAbs. The anthrose-containing synthetic carbohydrates were prepared as described previously (20, 22, 23). Mice carrying human immunoglobulin Cγ1 heavy and Cκ light chain gene segments (16) were immunized with an anthrose-rhamnose disaccharide conjugated to keyhole limpet hemocyanin (KLH) and formulated in ImmunEase adjuvant (Qüagen AG, Hombrechtikon, Switzerland). Mice received 3 doses of 40 μg conjugate at 3-week intervals. Three days before cell fusion, a mouse received an intravenous booster injection with 40 μg of conjugate in phosphate-buffered saline (PBS). From the sacrificed mouse, the spleen was aseptically removed, and a spleen cell suspension in Iscove’s modified Dulbecco’s medium (IMDM) was mixed with PAI mouse myeloma cells as a fusion partner. Spleen and myeloma cells in a ratio of 1:1 were centrifuged; after the supernatant was discarded, the pellet was mixed with 1 ml prewarmed polyethylene glycol 1500 sterile solution. After 60 s, 10 μl of culture medium was added. After 10 min, cells were suspended in IMDM containing hypoxanthine, aminopterin, thymidine, and 20% fetal bovine serum and cultured in 96-well plates. Cells secreting disaccharide-specific IgG were selected using disaccharide-bovine serum albu-
B. pumilus
ATCC 14884 Bp
ATCC 4525 Bsp
ATCC 9885 Bm1
ATCC 12759 B1
B. licheniformis
ATCC 61 Bcir ND ND 33
ATCC 9372 Bat
B. atrophaeus
Kurstaki SP09 BT1
B. thuringiensis
B. subtilis
ATCC 6051 Bs1
ATCC 6633 Bs2
ATCC 11774 Bs3
Biocontrol AG Bs4
B. cereus
ATCC 10876 Be1
ATCC 13061 Be2
ATCC 14579 Be3
ATCC 33019 Be4
ATCC 11778 Be5
B. anthracis
Ames Ba0 pX01 + pX02 +
Ba0 pX01 + pX02 −
B1 pX01 + pX02 −
ATCC 14580 Bl2
ATCC 10792 Bl1
ATCC 33019 Bl4
ATCC 11778 Bl5
B. anthracis
Spore production and inactivation. Strains of Bacillus spp. (Table 1) were cultured on tryptone soya agar (Oxoid, Basel, Switzerland) at 37°C for 1 to 2 days. Then, the culture plates were kept in the dark at room temperature for 4 weeks. Colony material was suspended in sterile water, and spores were collected by centrifugation at 5,000 g for 30 min at 4°C. To remove vegetative cells, spores were treated with 65% isopropanol for 1 h at room temperature and subsequently washed with sterile water until the supernatant appeared clear. The washed spores were stored in sterile water at 4°C, and the concentrations were determined by using a Thoma counting chamber. B. anthracis spores were inactivated by suspending 10^8 spores in 1 ml 10% paraformaldehyde for 1 h, subsequently washed with PBS, and recounted. For Yersinia pestis (ICM 1/41) and Brucella melitensis (NCTC 10094, biotype 1), inactivation was essentially done as described above using 3% formaldehyde. For both inactivation methods, sterility was verified by cultivation. Cultivation and inactivation of risk group 3 bacteria were done in a BSL-3 laboratory.

Enzyme-linked immunosorbent assay (ELISA). For the detection of saccharide-binding antibodies, Immulon 4 microtiter plates (Dynex Technologies Inc., Chantilly, VA) were coated at 4°C overnight with 50 μl of a 10-μg/ml solution of saccharide-BSA conjugate in PBS, pH 7.2. Wells were then blocked with 5% milk powder in PBS for 1 h at room temperature followed by three washings with PBS containing 0.05% Tween 20. After being washed, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (γ-chain-specific) antibodies (Sigma, St. Louis, MO) for 1 h at room temperature and then washed. Phosphatase substrate (1 mg/ml p-nitrophenyl phosphate) in buffer (0.14% Na_2CO_3, 0.3% NaHCO_3, 0.02% MgCl_2 [pH 9.6]) was added and incubated at room temperature. The optical density (OD) of the reaction product was recorded after an appropriate time at 405 nm using a microplate reader (Sunrise [Tecan Trading AG, Switzerland]).

For the detection of spore-binding antibodies, Maxisorp microtiter plates (Nunc/Thermo Fisher Scientific, Wohlen, Switzerland) were coated at 4°C overnight with 100 μl of a spore suspension in 0.05 M carbonate-bicarbonate buffer (pH 9.6). Wells were then blocked with 3% BSA in PBS and washed with PBS containing 0.05% Tween 20. Wells were then incubated with MAbs at a concentration of 1 μg/ml for 1 h. After being washed, the plates were incubated with peroxidase-conjugated goat anti-mouse IgG (γ-chain-specific) antibodies (KPL Inc., Rockville, MD).
beads were resuspended in 125 l of a streptavidin-R phycoerythrin (ProZyme Inc., Hayward, CA) solution was of 50 l/H9262 were added to each bead-containing well and incubated for 2 h on a shaker at 37°C. After being washed with PBS containing 0.05% Tween 20, 50 l of biotinylated detection antibody MTD6 diluted in blocking buffer was added to each well and incubated for 1 h. Biotinylation was performed using the EZ-Link sulfo-NHS-biotin labeling kit (Pierce/Thermo Fisher Scientific Inc., Rockford, MA) according to the manufacturer’s instructions. After repeated washing, streptavidin-peroxidase polymer conjugate (1 g/ml) (Sigma, St. Louis, MO) was added and developed with the ABTS substrate.

FIG. 1. Reactivity of anti-disaccharide MAbs with carbohydrate-BSA conjugates bound to ELISA microtiter plates. Shown are response patterns of individual anthrose-rhamnose disaccharide-specific MAbs (MTD1 to MTD6) and of a tetrasaccharide-specific MAb (MTA1) with anthrose-BSA (A), disaccharide-BSA (B), and tetrasaccharide-BSA (C). Structure of the synthetic anthrose (D), anthrose-rhamnose disaccharide (E), and tetrasaccharide (F).

RESULTS

Generation and characterization of anti-anthrose-rhamnose disaccharide MAbs. Anthrose-rhamnose disaccharide-specific MAbs were generated basically in the same way as the previously described tetrasaccharide-specific MAbs MTA1 to MTA3 (20, 21). Chemically synthesized disaccharide (Fig. 1E) was covalently attached to the keyhole limpet hemocyanin (KLH) carrier protein by reductive amination. After repeated immunizations of mice with the disaccharide conjugate delivered with a CpG-based adjuvant (ImmunEasy, Qiagen), six anti-disaccharide MAbs (named MTD1 to MTD6) were generated that reacted with a synthetic disaccharide-BSA conjugate in ELISA (Fig. 1B). Analyses of the IgG subclass profiles of the induced disaccharide-specific MAbs showed a predominance of the mouse IgG1(1) isotype; only MTD6 was of the IgG2b(1) isotype.

While the binding patterns of disaccharide- and tetrasaccharide-specific MAbs differed, MAbs generated against the same antigen exhibited similar fine specificities. All anti-disaccharide MAbs showed cross-reactivity with the tetrasaccharide (Fig. 1C) and the anthrose monosaccharide (Fig. 1A), demonstrating that rhamnose moieties were not crucial structural elements of their epitopes. MAbs MTD1 and MTD3 showed lower affinities for the synthetic antigens than the other MAbs and were therefore not selected for the further assay development. The failure of MAbs from tetrasaccharide-immunized mice to bind to anthrose (Fig. 1A) and to the disaccharide (Fig. 1B) indicated that rhamnose sugars attached to anthrose were essential for recognition.

Cross-reactivity of the disaccharide-specific MAbs with endogenous tetrasaccharide expressed by B. anthracis strain Ba4 was established by indirect immunofluorescence assay (Fig. 2).
and with immunoblotted *B. anthracis* endospore lysates (not shown).

Binding of the newly generated anti-disaccharide MAbs as well as of the anti-tetrasaccharide MAbs to spores of a broad spectrum of different *Bacillus* spp. was analyzed in a direct ELISA using plates coated with a spore suspension. Irrespective of their different fine specificities, both sets of MAbs recognized spores of the tested *B. anthracis* strains but also showed cross-reactivity with the *B. cereus* strains Bc1 and Bc4. Spores of none of the other *Bacillus* spp. were reactive with the MAbs. All tested MAbs showed uniform reactivity patterns, and representative results with the anti-tetrasaccharide MAb MTA1 and the anti-disaccharide MAb MTD6 are shown (Table 1).

**Development of a Luminex assay for rapid detection of anthrax spores.** To develop a highly sensitive and specific assay for the detection of anthrax spores from complex samples, the anti-carbohydrate MAbs were used for the development of a Luminex sandwich assay. The Luminex technology is based on fluorescent beads that are color coded. Each bead subset can be coated with a reagent specific for a particular analyte, allowing the capture and detection of this analyte from a complex sample. Within the BioPlex analyzer, lasers excite the internal dyes that identify each bead particle and also any reporter dye captured during the assay. Here, the anti-tetrasaccharide MAb MTA1 was coupled to magnetic beads and used as the capture antibody, and the biotinylated anti-disaccharide MAb MTD6 was used as the detection antibody. The Luminex assay detected the different *B. anthracis* strains and the *B. cereus* strains Bc1 and Bc4, correlating with the results obtained in direct spore ELISA (Table 1). For the *B. cereus* strains Bc2, Bc3, and Bc5, fluorescence background signals were weak at very high spore concentrations (≥1 × 10⁶ spores/ml) and were absent within one lower log stage of spores (not shown). Other antibody combinations were not adapted for the Luminex assay, since all MAbs showed similar reactivity patterns in direct spore ELISA.

The sensitivity of the developed bead-based assay was determined by analyzing a serial dilution of the *B. anthracis* spores. The limit of detection (LOD) was defined by the spore concentration yielding a signal two times as high as the mean fluorescence intensity of the blank (dashed lines in Fig. 3). Depending on the anthrax strain tested, the assay was able to detect 50 to 500 spores in a sample volume of 50 μl. The sensitivity of the Luminex assay was 10- to 100-fold higher than that of a corresponding antigen capture ELISA (Fig. 3), where at least 5 × 10³ spores per 50 μl sample volume were required for an accurate detection. The developed Luminex assay for anthrax spore detection was further evaluated in mixed samples combining three inactivated bacterial species. In these complex samples, the anthrax spores were accurately detected, and no cross-reactivities were observed (Fig. 4).

**DISCUSSION**

Antibodies can provide the basis for specific and sensitive immunoassays for the diagnosis of infectious diseases (2, 3). The development of immunochemical assays specific for *B. anthracis* endospores has been hampered by the presence of cross-reactive antigens in closely related spores, in particular in *B. cereus*. Since the anthrose monosaccharide was not found in spores of the *B. cereus* T strain and a *B. thuringiensis* subsp. *kurstaki* strain (6), it has been considered a potential target antigen for the detection of *B. anthracis* spores. After the first chemical synthesis of the anthrose-containing tetrasaccharide (23), several synthetic approaches of the tetrasaccharide or corresponding sequences have been reported (1, 4, 9, 15, 17–19). Covalent attachment of the synthetic tetrasaccharide to a
carrier protein produced a carbohydrate-protein conjugate that was immunogenic in mice (21). Cross-reactivity of anti-tetrasaccharide MAbs with native B. anthracis endospores in immunofluorescence analysis confirmed the structural analysis of the tetrasaccharide and its expression on the endospore surface (21). Screening of anti-tetrasaccharide MAbs for cross-reactivity with immunoblotted spore lysates of panels of B. anthracis and B. cereus strains demonstrated the presence of anthrose in all B. anthracis strains tested and in some B. cereus strains (20). A recent genomic analysis demonstrated the presence of the anthrose biosynthetic operon in B. cereus strains (7). Additionally, structures similar to anthrose were found in the capsular polysaccharide of Shewanella spp. MR-4 and on flagella of Pseudomonas syringae (13).

Anti-tetrasaccharide MAbs (21) and anti-anthrose-ramnosyl disaccharide MAbs described in this study were tested for their specificities in a direct ELISA using plates coated with spores of B. anthracis strains and related species of the Bacillus genus. Both types of MAbs recognized the tested B. anthracis strains but also showed cross-reactivity with two B. cereus strains, confirming the previously observed cross-reactivity with immunoblotted spore lysates of the same B. cereus strains (20). Kuehn and colleagues (14) generated one polyclonal antibody against the anthrose-containing tetrasaccharide that showed no cross-reactivities with other Bacillus strains in capture ELISA. Interestingly, the synthetic anthrose monosaccharide included the already-essential structural motifs required for binding of the anti-disaccharide MAbs. In contrast, the anti-tetrasaccharide MAbs recognized more complex epitopes that probably comprised all four sugar residues of the tetrasaccharide. Therefore, the target epitope might be better accessible for the anti-disaccharide MAbs. Biosafety containment requires an inactivation of probes containing B. anthracis spores. Inactivation by paraformaldehyde treatment did not deplete the epitopes recognized by the carbohydrate-specific antibodies, as also observed previously (14). Moreover, the sugar was also conserved on irradiated spores. Antigen conservation is not self-evident, since it was demonstrated that inactivation methods can affect the sensitivity of nucleic acid- and antibody-based assays for the detection of B. anthracis endospores (5).

Even though the MAbs generated against anthrose-containing structures were not strictly specific for B. anthracis, they still may represent a basis for the development of useful first-line screening platforms. Therefore, MAbs MTA1 and MTD6 were further tested as components of a highly sensitive immunodetection assay based on the Luminex technology platform. Use of the assay to detect individual spores of a broad range of Bacillus spp. strains correlated with the results obtained in direct spore ELISA, demonstrating the suitability of the bead-based platform to capture and detect spore particles. The sensitivity of spore detection by the Luminex assay was substantially increased compared to the corresponding capture ELISA, with the detection of 10^3 to 10^4 spores per ml. Currently available immunological detection systems offer higher detection limits (11, 12, 14). The platform can be also used in multiplex assays, and we are currently evaluating the simultaneous detection of different biothreat bacteria in mixed samples. In addition, we are testing the generated anti-carbohydrate monoclonal antibodies with field samples from different countries where anthrax is endemic to incorporate a broader genetic diversity of strains within the B. cereus group.

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