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Articles

Development and Characterization of Mouse Monoclonal Antibodies Specific for *Clostridiodes (Clostridium) difficile* Lipoteichoic Acid

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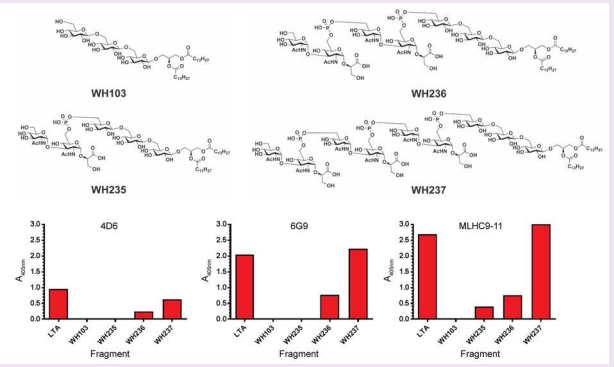
ABSTRACT: *Clostridiodes (Clostridium) difficile* is an anaerobic Gram-positive, spore-forming nosocomial, gastrointestinal pathogen causing *C. difficile*-associated disease with symptoms ranging from mild cases of antibiotic-associated diarrhea to fatal pseudomembranous colitis. We developed murine monoclonal antibodies (mAbs) specific for a conserved cell surface antigen, lipoteichoic acid (LTA)of *C. difficile*. The mAbs were characterized in terms of their thermal stability, solubility, and their binding to LTA by surface plasmon resonance and competitive ELISA. Synthetic LTA molecules were prepared in order to better define the minimum epitope required to mimic the natural antigen, and three repeat units of the polymer were required for optimal recognition. One of the murine mAbs was chimerized with human constant region domains and was found to recognize the target antigen identically to the mouse version. These mAbs may be useful as therapeutics (standalone, in conjunction with known antitoxin approaches, or as delivery vehicles for antibody drug conjugates targeting the bacterium), as diagnostic agents, and in infection control applications.



Clostridiodes (Clostridium) difficile is a Gram-positive bacterium and a common cause of nosocomial infections, certainly following antibiotic treatment.[1] Beyond the development of new antimicrobial agents to treat this infection, vaccine approaches are in development to battle *C. difficile* associated disease (CDAD). Vaccine approaches are mostly focused on the production of antibodies to deactivate the toxins which are the key virulence factors for the bacterium.[2−5] These toxins cause the pathology observed in CDAD which includes diarrhea, intestinal pain, and other serious complications such as pseudomembranous colitis and toxic megacolon. Antibody facilitated neutralization of toxicity will ease clinical symptoms, but anti-toxin methodologies do not impact the pathogen directly and thus will probably not enable the eradication of the bacterium. Recurrent *C. difficile* infection is acknowledged as a serious issue, and as such, it is vital that as well as the neutralization of toxins, actions are employed to guarantee that the organism is cleared from the intestine. Alternate strategies to expedite this process include the advancement of new antibiotics[6] or fecal replacement therapy to refurbish a healthy host gut microbiome and preclude *C. difficile* colonization.[7] The latter, although thought upon as an action of last resort, has shown some success in severe infections.

We are focusing our efforts on utilizing cell-surface carbohydrates as vaccines or as targets for therapeutic interventions. These approaches rely upon the determination of a preserved surface carbohydrate motif for the targeted species. Poxton et al. were the first to study lipocarbohydrate molecules of *C. difficile* and to show that these structures were immunologically conserved among strains, with anti-lipocarbohydrate antisera cross-reacting with only *C. sordellii* and *C. bifermentans*.[8,9] Ganeshapillai et al. found two polysaccharides of *C. difficile* that they designated PS-I and PS-II. PS-I was found on only by a limited subpopulation of *C. difficile* strains; however, PS-II appears to be preserved over the majority of *C. difficile* strains.[10] Several groups have

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attempted to utilize these structures via the development of glycoconjugates based upon the isolated polymer[11] or on synthetic derivatives.[12−15] We found another carbohydrate polymer that *C. difficile* elaborates with the identification and structural determination of a lipoteichoic acid (LTA)-like polymer.[16] The LTA we found in all strains of *C. difficile* was made up of the repeat unit [(-6)-α-D-Glc*p*NAc-(1−3)-[P-6]-α-D-Glc*p*NAc-(1−2)-D-GroA] where GroA is glyceric acid. The polymeric repeats were connected by a phosphodiester bridge between C-6 of the two Glc*p*NAc residues (6-P-6). A minimal component consisted of Glc*p*N-(1−3) instead of Glc*p*NAc-(1−3) in the polymeric repeat. Through a 6−6 phosphodiester bridge, this polysaccharide was linked to (−6)-β-D-Glc*p*-(1−6)-β-D-Glc*p*-(1−6)-β-D-Glc*p*-(1−1)-Gro, with glycerol (Gro) elaborating fatty acids. Martin et al. communicated the synthesis of a disaccharide of the polymeric repeat and, via a glycan array, established that antibodies to LTA were circulating in patients with *C. difficile* infection.[17] In a subsequent study, we described our work in developing glycoconjugates of the LTA, and the establishment of the broad serum cross-reactivity generated following immunization of mice and rabbits with these molecules against a library of *C. difficile* strains confirmed the absolute conservation of this antigen.[18] Several studies from the group of Seeberger have examined synthetic antigens based upon the repeat units of PS-I, PS-II, and LTA (PS-III) as a source of glycan for glycoconjugates and described protective immunity with some of these structures.[19−21] In this study, we describe the development and characterization of monoclonal antibodies (mAbs) to the LTA antigen and describe our efforts to define the minimal synthetic molecule required to mimic the natural antigen.

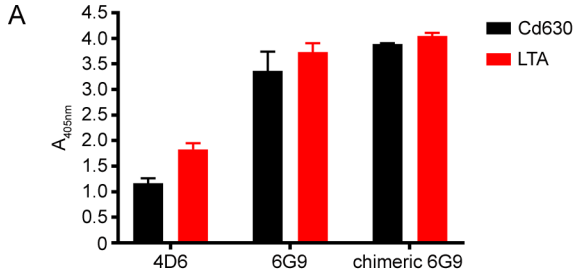
RESULTS AND DISCUSSION

Generation and of anti-*C. difficile* LTA Antibodies. Two LTA-specific mAb-producing hybridomas (4D6 and 6G9) were identified. MAbs 6G9 and 4D6 were of the IgG2b:kappa and IgG3:lambda isotypes, respectively. ELISA analysis revealed that supernatant from both mAbs recognized the purified LTA antigen[18] and the Cd630 strain that the LTA was isolated from (Figure 1A).

Purified mAbs were evaluated for binding to whole cells from our collection of *C. difficile* strains and other species by ELISA (Figure 2A,B). Although not completely specific to *C. difficile*, the mAbs did not recognize all Clostridia species: only *C. butyricum*, *C. bifermentans*, and *C. subterminale* were recognized in addition to *C. difficile*. Some Clostridia species (*C. perfringens*, *C. sporogenes*, and *C. barati*) and non-Clostridia species as well as other Gram-negative and Gram-positive bacterial species were not recognized, highlighting the restricted binding properties of these mAbs.

Characterization of anti-*C. difficile* LTA mAbs. *Antibody Sequencing.* The heavy and light chain variable regions of mAbs 4D6 and 6G9 were sequenced, and their amino acid sequences are shown in Figure 1B.

Measurement of Melting Temperature (T_m) by CD Spectroscopy. The T_m of the mAb 6G9 was determined to be 68.9 ± 0.6 °C (Figure 3A). The T_m of the mAb 4D6 could not be determined, because it aggregated upon heating at approximately 65 °C. Regardless, 4D6 unfolding did not occur until ∼55 °C, and the T_m could be estimated to be at least ∼60 °C.



B

4D6 VL
QAVVTQESALTTSPGETVTLTCRSSNGAVTSRNYANWVQEKPDHLFTGLIGGTNNRA
PGVPARFSGSLIGDKAALSITGAQTEDEAIYFCALWYSNRWVFGGGTKLTVL

4D6 VH
QVQLQQSDAELVKPGASVKISCKASGYTFTDHAIHWVKQKPEQGLEWIGYISPGNDD
IKYNEKFKGKATLTADTSSSTAYMQLNSLTSEDSAVYFCKVLRRFAYWGQGTLVTVSA

6G9 VL
DIVLTQSPASLAVSLGQRATISCRASKSVSTSGYSYMHWYQQKPGQPPKLLIYLASNL
ESGVPARFSGSGSGTDFTLNIHPVEEEDAATYYCQHSRELPRTFGGGTKLEIK

6G9 VH
EVQLQQSGPELVKPGASVKISCKASGYTFTDYNMWVKQSHGKSLEWIGYIYPYNGGT
GYNQKFKSKATLTVDNSSSTAYMELRSLTSEDSAVYYCARNYYGSSWFAYWGQGTLV
TVSA

Figure 1. (A) ELISA analysis of binding of 4D6 and 6G9 mAb-containing hybridoma supernatant (used neat) and chimeric 6G9 mAb (at 1 μg mL^{-1}) to purified LTA antigen and killed whole cells of *C. difficile* strain Cd630. (B) Variable region amino acid sequences of mAbs 4D6 and 6G9.

Size Exclusion Chromatography (SEC). Both mAbs, as well as 6G9 Fab, were primarily monomeric as shown by their elution profiles in SEC (Figure 3B). No evidence of aggregation or degradation was observed.

Surface Plasmon Resonance. Flowing natural purified LTA (multiple concentrations ranging from 5 to 500 nM) over immobilized 4D6 and fitting to a 1:1 binding model yielded an apparent (bivalent) K_D of approximately 30 nM (Figure 3C). The data were also analyzed using a steady state model giving an apparent K_D of approximately 35 nM. LTA bound to 6G9 with an apparent (bivalent) K_D of approximately 11 nM and a good fit to the 1:1 binding model. However, the observed maximal binding response (R_{max}) for both 4D6 and 6G9 were low, corresponding to approximately 4% and 7% of the theoretical R_{max}, possibly due to the amine coupling affecting the activity of the mAb or the quality of the natural LTA (heterogeneous/purity). Attempts to capture the mouse mAbs with an anti-mouse Fc surface resulted in approximately 2.5-fold less mAb on the surface (∼800−1000 RUs of mAb captured compared to ∼2500 RUs of mAb amine coupled), and flowing LTA did not produce a measurable binding response (data not shown). No binding of LTA was observed to an immobilized irrelevant mAb L3B5 used as negative control that is specific for *Neisseria meningitidis* LPS.[22] The binding of the monovalent Fab fragment of 6G9 was also studied and flowed over immobilized HSA−LTA conjugate. This conjugate was prepared via direct reductive amination and elaborates ∼3 LTA molecules, each ∼8 kDa in length per carrier HSA protein. Fitting of these data to a steady state model yielded a monovalent K_D of approximately 3.4 μM (Figure 3D). The 6G9 binding affinities obtained by SPR differed due to analyte valency in the two assay formats. Flowing LTA resulted in a higher (apparent) affinity for the immobilized mAb because LTA possesses multiple binding sites available for avid binding. Conversely, when Fab was

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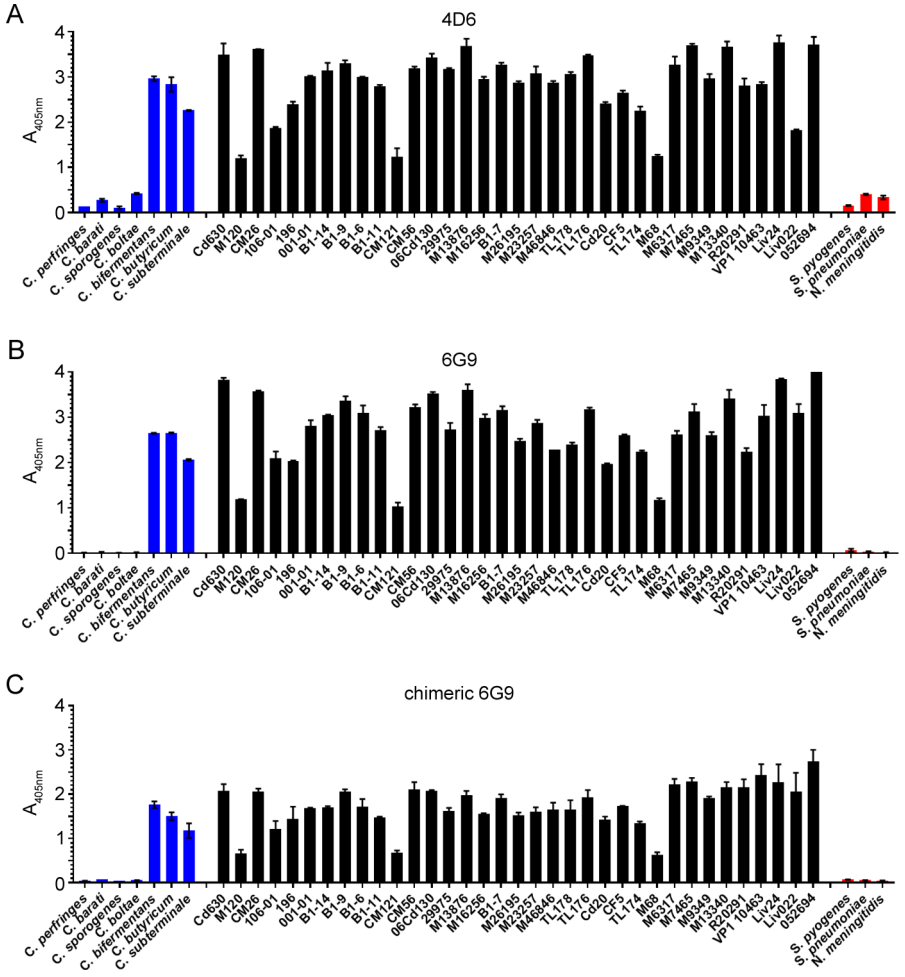

Figure 2. ELISA analysis of binding of mouse mAbs (A) 4D6 (38.4 μg mL^{-1}); (B) 6G9 (200 ng mL^{-1}); and (C) chimeric mAb 6G9 (200 ng mL^{-1}) to killed whole cells of various strains as indicated.

flowed over the LTA-HSA surface, binding was monovalent and resulted in a weaker affinity.

By the above methods, the mAbs were characterized, and it was highlighted that they were thermostable up to around 60 °C, were soluble, and had apparent bivalent affinities for LTA in the 11−35 nM range. The monovalent affinity of 6G9 Fab for an HSA−LTA conjugate was approximately 3.4 μM. Thus, similar to most other anti-carbohydrate antibodies, these mAbs showed relatively weak monovalent binding to LTA that was compensated by avidity in bivalent format.[23,24]

Epitope Mapping of anti-*C. difficile* LTA mAbs. *LTA Inhibition Whole Cell ELISA.* Plates were coated with killed whole cells of *C. difficile* 630 (Cd630). MAbs 4D6 and 6G9 were preincubated with a range of concentrations of natural purified LTA and these antibody−LTA mixtures were applied to the ELISA plate in order to examine the ability of the soluble LTA to bind to the antibody and restrict its availability to bind to the killed whole cells. Reduction of binding was observed for mAbs 6G9 and 4D6 although with lower overall binding for mAb 4D6 (Figure 4A), illustrating the specificity of the antibodies for the LTA antigen.

mAb Competition ELISA. Plates were coated with purified LTA or killed whole cells of Cd630. mAb 6G9 was titrated and incubated on the plate first. After washing, the second mAb, 4D6 was added at a constant concentration. A secondary mAb specific for 4D6, anti-mouse IgG3-AP (alkaline phosphatase), was added to the plate, and after incubation, the absorbance at 405 nm was measured (Figure 4B). If the competing mAbs were specific for the same epitope, the absorbance for the second antibody would increase as the concentration of the first, titered, antibody decreased. However, a constant absorbance was observed, indicating that the mAbs had distinct epitopes on LTA.

In the reverse assay, plates were similarly coated with purified LTA or killed whole cells of Cd630. This time, mAb 4D6 was titered and incubated on the plate first. After washing, the second mAb, 6G9, was added at a constant dilution. A secondary mAb specific for 6G9, anti-mouse IgG2b-AP, was added to the plate, and after incubation, the absorbance at 405 nm was measured (Figure 4B). As before, a constant absorbance was observed, indicating that the mAbs were specific for separate LTA epitopes.

Therefore, it was readily determined that the two mAbs had unique epitopes on the LTA molecule, as these competition experiments established that neither mAb binding interfered with the access of the other to the antigen.

LTA Subunit ELISA. Synthetic fragments of the LTA antigen (fragment WH103: lipid + core region; fragment WH235: lipid + core region + 1 repeat unit; fragment WH236: lipid + core region + 2 repeat units; fragment WH237: lipid + core region + 3 repeat units) (Figure 4C) were examined for their recognition by the purified mAbs compared with natural LTA

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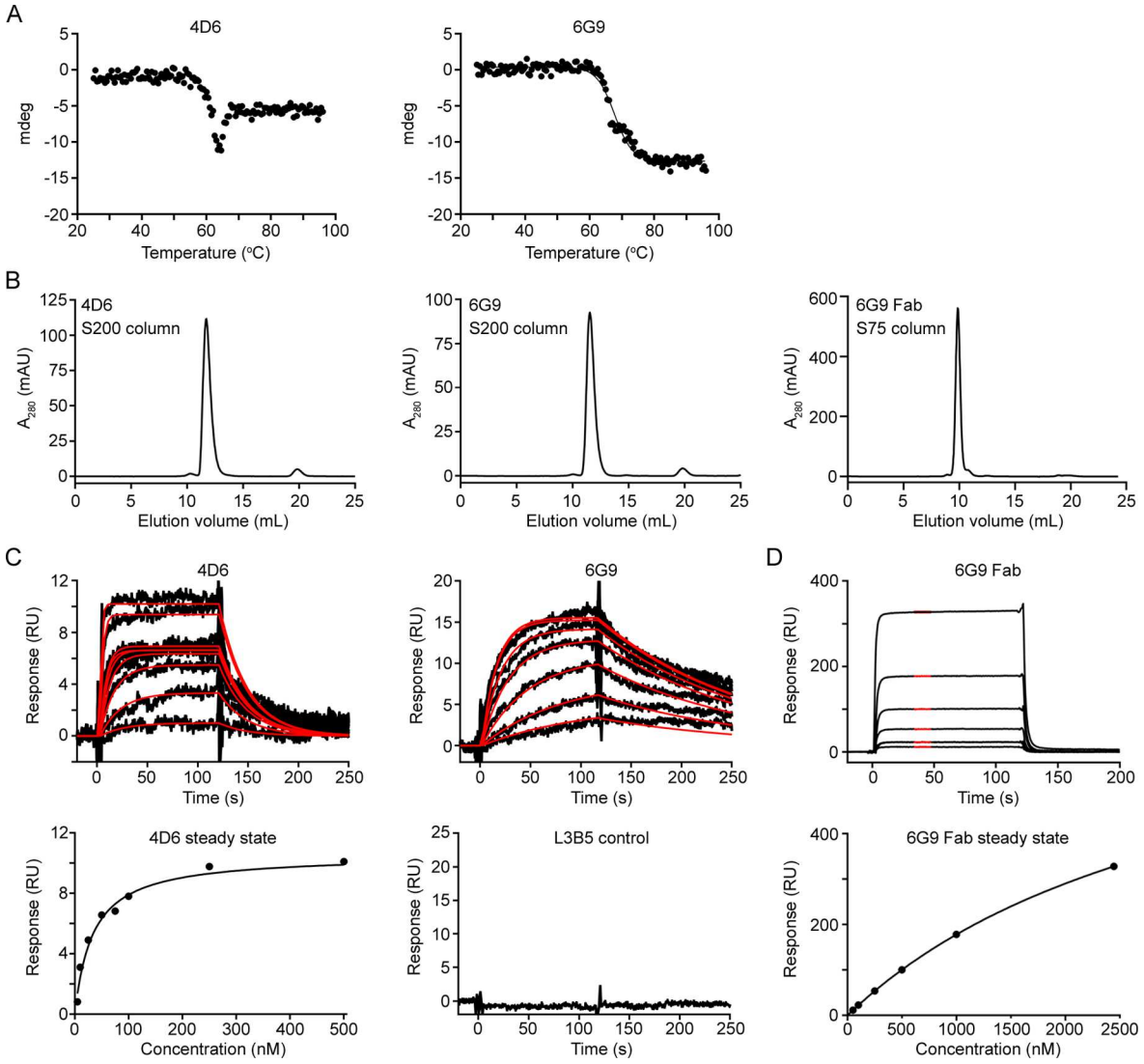

Figure 3. (A) Thermal denaturation curves for mAbs 4D6 and 6G9 at 200 μg mL^{-1}. A representative result of three repeat measurements for each is shown. (B) Size exclusion chromatography profiles of mAbs 4D6 and 6G9 (S200 column) as well as 6G9 Fab (S75 column). (C) SPR sensorgrams and steady-state plots for binding of LTA (5−500 nM) to immobilized 4D6, 6G9, and control mAb L3B5. (D) SPR sensorgram and steady-state plot of 6G9 Fab (50−2400 nM) binding to immobilized HSA−LTA conjugate.

(Figure 4D). It was evident that a minimum of two repeat units of the LTA polymer were needed for any recognition of the synthetic molecule, and for recognition comparable with that of the natural LTA molecule, three repeat units were required. This recognition pattern was also observed for polyclonal antisera from mice (MLHC 9−11) that had been immunized with natural LTA alone (10 μg dose) (Figure 4D).

Synthetic molecules were prepared in order to interrogate the minimal epitope requirements for recognition of the natural LTA molecule. It was felt that since the natural molecule elaborated on a core unit and lipid region, the synthetic molecules should also contain these segments in order to not impact the conformation of the polymeric region's repeat units. Synthetic strategies were utilized to achieve target compounds identical to the isolated LTAs via a modular approach. A building block consisting of the fully protected repeating unit was attached to the lipid core. Regioselective removal of the terminal protective group followed by chain extension allowed the synthesis of LTAs with specific degree of oligomerization. Deprotection and purification gave the substrates used in this study.[25]

Utilizing the synthetic molecules, we were able to identify the minimal epitope required for recognition of the natural LTA antigen as a molecule including three units of the phosphorylated, glycerated, *N*-acetyl glucosamine disaccharide repeat attached to the core and lipid units. This requirement for recognition was consistent whether the antibody source was the purified mAbs or polyclonal mouse sera raised by immunization with the purified natural LTA molecule. Two repeat unit-containing molecules were recognized by both the mAbs and the polyclonal sera, but clearly not at levels seen with the three repeat unit synthetic molecule nor the natural LTA molecule. Furthermore, one repeat unit was not recognized at all by either of the mAbs or the polyclonal sera. This observation is consistent with elements of the publication from the Seeberger group[20] that identified that secretory IgA in fecal sera taken from patients with *C. difficile* infection (CDI) could not recognize the disaccharide or tetrasaccharide structures. Similarly, serum IgG binding to the

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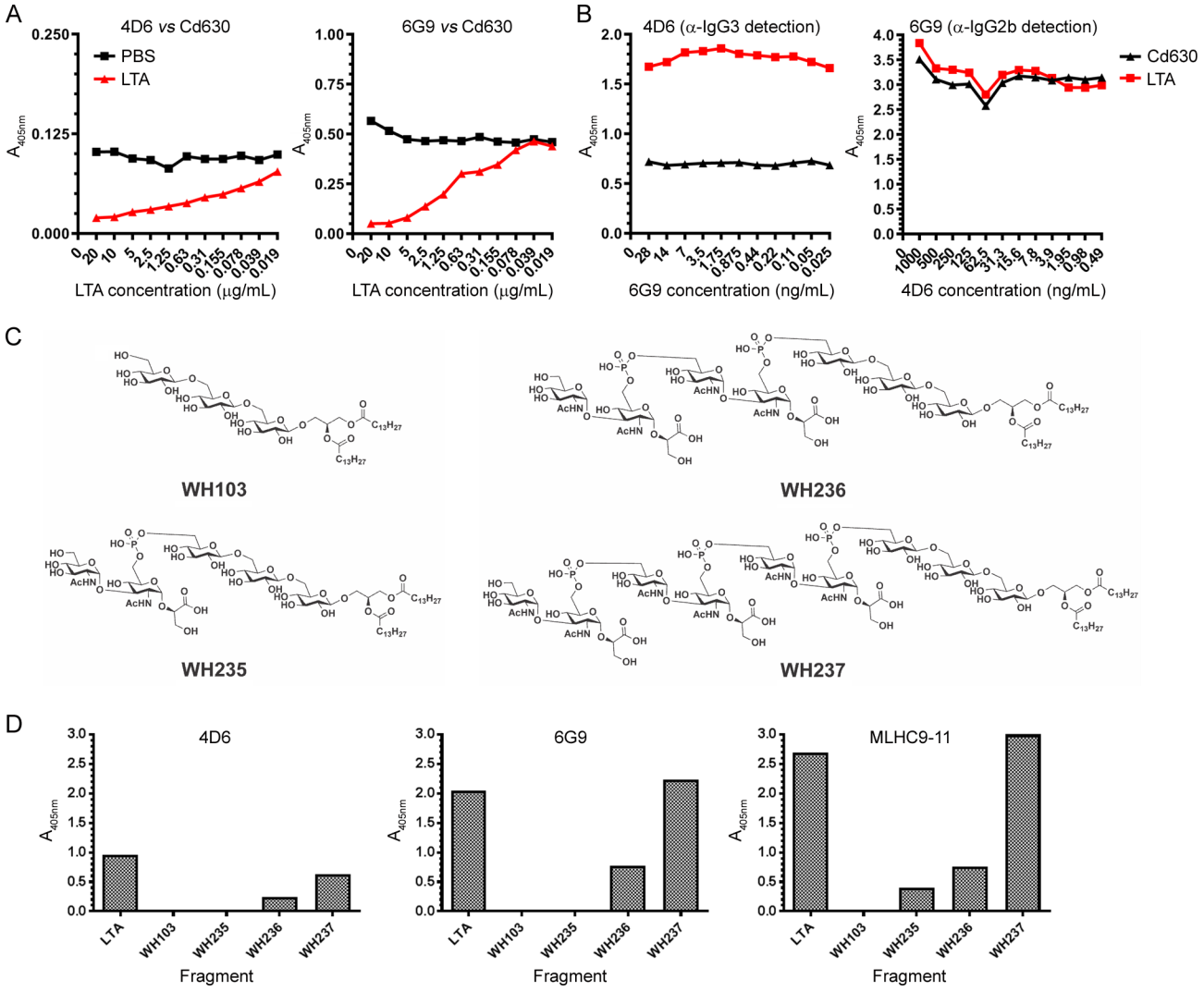

Figure 4. (A) LTA inhibition ELISA between mAbs 4D6 and 6G9 with PBS or LTA in solution and plated killed whole cells of *C. difficile* strain 630. Left: mAb 4D6 (500 μg mL^{-1}) was preincubated with PBS or increasing concentrations of LTA as shown prior to ELISA. Right: mAb 6G9 (14 μg mL^{-1}) was preincubated with PBS or concentrations of LTA as shown prior to ELISA. (B) Competition ELISA between mAbs 4D6 and 6G9 for purified LTA or killed whole cells of *C. difficile* strain 630 as indicated. Right: mAb 4D6 is titered at concentrations shown on the *x*-axis onto ELISA plate with coated LTA or Cd630 cells. Plate is washed and mAb 6G9 is added at a constant concentration (28 ng mL^{-1}). Secondary mAb specific for mAb 6G9 is added and absorbance measured after color reagent added. Left: mAb 6G9 is titered at concentrations shown on the *x*-axis onto an ELISA plate with coated LTA or Cd630 cells. Plate is washed and mAb 4D6 is added at a constant concentration (1 μg mL^{-1}). Secondary mAb specific for mAb 4D6 is added and absorbance measured after color reagent was added. (C) Structures of the synthetic LTA molecules: WH103: Lipid + core region; WH235: Lipid + core region + 1 repeat unit; WH236: Lipid + core region + 2 repeat units; WH237: Lipid + core region + 3 repeat units). (D) ELISA analysis of binding of mAbs 6G9 (14 μg mL^{-1}) and 4D6 (80 μg mL^{-1}) and pooled mouse polyclonal sera (MLHC 9−11 raised to purified natural LTA; 1:40 dilution) to synthetic LTA molecules as indicated.

synthetic disaccharide and tetrasaccharide was slightly higher in recovered CDI patients compared with that from patients without a history of CDI, although this difference did not reach statistical significance. Taken together, a possible explanation for these data are that the disaccharide and tetrasaccharide without a core unit or lipid region do not effectively mimic the epitopes of the natural LTA molecule.

Our observation in this study, that the three repeat unit is the minimal structure required to mimic the natural molecule, contrasts with the studies of Seeberger's group that initially identified that immunization with a glycoconjugate of the tetrasaccharide (two repeat units) without core or lipid units could limit bacterial colonization in a mouse model of CDI[20] and most recently suggested that only a glycoconjugate of the disaccharide (one repeat unit again without core or lipid units) was sufficient to prevent colonic colonization and colitis in a mouse model.[21] Immunizing with Seeberger's well-defined conjugates likely leads to the generation of antibodies that are capable of recognizing a relatively small number of epitopes, of which many copies are presented in the polymeric natural LTA. The antibodies will probably have different binding characteristics than the mAbs generated in this study against naturally sourced LTA as used by the authors. Of course, it may be that a conjugate vaccine generated from the longer fragment performs better than the minimal epitope vaccines described by Seeberger, but this was beyond the scope of the current study. A successful subunit vaccine needs to sufficiently mimic the natural epitopes elaborated by the targeted pathogen, and thus our studies would suggest that further examination of glycoconjugate vaccine-derived sera and

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synthetic antigens is required in order to unequivocally identify the protective epitope and inform intelligent vaccine design.

Recognition of LTA and Whole Cells by Chimeric mAb 6G9. With a view to possible therapeutic applications, one of the mAbs was chimerized by the substitution of the mouse IgG3 and κ constant domains with human IgG1 and κ constant domains. The ability of the chimeric mAb to recognize LTA and whole cells of *C. difficile* was tested using ELISA, initially against purified naturally isolated LTA and killed whole cells from strain Cd630 from which the LTA was isolated (Figure 1A), and subsequently against all *C. difficile* and other species killed whole cells in our collection (Figure 2C). The chimeric 6G9 recognized LTA and whole cells of *C. difficile* just as well as the native mAb, confirming that the sequences of the mAb 6G9 variable domains (V_H and V_L) encode for recognition of the LTA antigen, and most importantly confirming that the switch to human constant region domains had not negatively affected the binding properties of the antibody.

Conclusion. In our previous studies, we identified a novel lipoteichoic acid like polymer on the surface of a range of *C. difficile* cells[16] and demonstrated that the LTA antigen is immunogenic in the context of the bacterial cell, and this molecule is visible and accessible on the cell surface.[18] In this study, we extended those observations with the development and characterization of mouse mAbs specific for the LTA antigen and attempted to define the minimal epitope required for recognition. The therapeutic potential of passive delivery of mAbs is worthy of some consideration as an alternate treatment for CDI. The specificity and lack of collateral damage and thus maintenance of the normal flora is a key advantage of species specific therapies. Recent studies by Seeberger highlighted that a mAb specific for an alternate *C. difficile* surface carbohydrate, PS-I, could reduce colonization in a mouse model.[21] Studies investigating the utility of the LTA mAbs developed in this study in this regard are underway.

METHODS

Production and Purification of LTA. *C. difficile* strain 630 (Cd630) was grown and LTA isolated as described previously.[18]

Generation of anti-*C. difficile* LTA Antibodies. To produce antibodies that target *C. difficile* LTA, mice were immunized with the purified LTA. Hybridomas were prepared, and binding of the purified mAb to the LTA was evaluated.

Immunizations. 6-week-old BALB/c mice (Charles River) were bled (preimmune serum) and immunized i.p. with 10 μg of purified LTA in PBS-citrate and Sigma Adjuvant System (SAS; Sigma-Aldrich). Two weeks later, a second injection of 10 μg of purified LTA in PBS-citrate and SAS was done. After 7−10 days, mice were bled and the serum titer to LTA and phenol-killed Cd630 cells was measured by ELISA. This same boost was repeated 3 weeks later, and once again, the serum titer was measured by ELISA. Three months later, a final i.v. injection using 10 μg of purified LTA in PBS-citrate was done 4 days prior to the fusion experiment.

Fusion of the Harvested Spleen Cells. All manipulations were done under sterile conditions. Spleen cells were harvested from LTA immunized mice and fused to Sp2/0 myeloma cells using polyethylene glycol (PEG). Briefly, spleen cells and myeloma cells were washed in serum-free Dulbecco's modified Eagle's medium (DMEM; Invitrogen), erythrocytes were lysed using red blood cell lysing buffer (Sigma), and splenocytes were counted. Myeloma cells and splenocytes were mixed at a 10:1 ratio. Pelleted cells were fused by adding 1 mL of a 33% solution of PEG (50% Hybrimax, Sigma) in serum-free DMEM preheated at 37 °C dropwise over 1 min, and incubated at 37 °C for an additional 90 s. The reaction was stopped by addition of 30 mL of DMEM at 37 °C over 12 min. Fused cells were centrifuged at 200*g* for 7 min. Cells were resuspended in serum-free DMEM and allowed to rest for 1 h. DMEM supplemented with 20% heat inactivated fetal bovine serum (FBS; Sigma) was added until a concentration of 2×10^5 input myeloma cells per mL was reached. The cells were plated in 96-well plates and incubated at 37 °C, 5% CO_2. After 24 h resting, 100 μL of HAT selection medium [DMEM containing L-glutamine, 20% heat inactivated FBS, gentamicin (Gibco), and HAT media supplement (Sigma)] were added and the cells were again incubated at 37 °C, 5% CO_2. The HAT selection media was changed three times over the next 7 days.

Hybridoma Selection. Hybridoma supernatants were screened for LTA reactivity by ELISA. Two LTA-specific mAb-producing hybridomas were identified. The supernatants from these clones were collected and evaluated for binding to LTA and phenol-killed *C. difficile* whole cells by ELISA. Supernatant was also evaluated for immunoglobulin subtyping. Hybridoma clones were further subcloned by limiting dilution to ensure monoclonality. Briefly, cloned cells were counted and diluted to a density of 1×10^3 cells mL^{-1}. From this stock, 100 μL was plated in each well of a 96-well plate (100 cells $well^{-1}$). A further 1:10 dilution was done and 100 μL was plated in each well of a 96-well plate (10 cells $well^{-1}$). This dilution scheme was done twice more to obtain plates at 1 cells $well^{-1}$ and 0.1 cells $well^{-1}$. Positive subclones were selected from the 0.1 cells $well^{-1}$ dilutions.

Growth and Purification of High Density mAb Supernatant. For high density culture, selected clones at a density of 1.5×10^6 cells mL^{-1} in 15 mL of fresh antibiotic-free DMEM containing 20% FBS were transferred to the cell chamber of a CELLine 1000 Bioreactor (Wheaton Industries Inc.). Briefly, 8−12 mL of mixed cell suspension was removed from the cell chamber, and fresh prewarmed media was added to bring the volume of the cell chamber back up to 15 mL. The harvested cell suspension was centrifuged at 20,000*g* for 8 min. The supernatant was removed, filtered with a 0.22 μM filter, and stored at −20 °C until purification. Half of the 1000 mL nutrient compartment medium was replaced with fresh medium once a week. The supernatant produced in the CELLine bioreactor was diluted 1:1 with binding buffer (0.1 M Tris-HCl, pH 7.5, containing 0.15 M NaCl), and 10 mg IgG at a time was applied to the column and allowed to run through. After each application, the column was washed with binding buffer until the absorbance at 280 nm was <0.02. Bound IgGs were then eluted from the column using dissociation buffer (0.1 M glycine-HCl, pH 2.5−3.5). The absorbance at 280 nm was monitored and the protein peak was collected. The column eluate was neutralized with 1 M Tris. MAbs were dialyzed twice against PBS and concentrated using an Amicon filter (cutoff MW 100,000 Da). The final concentration of the antibodies was determined using a standard BCA kit.

Characterization of anti-*C. difficile* LTA mAbs. *Antibody Variable Region Sequencing.* The DNA sequences encoding the rearranged variable heavy chain (V_H) and variable light chain (V_L) domains of the two LTA mAbs (4D6 and 6G9) were determined by Illumina MiSeq amplicon sequencing. Briefly, total RNA was extracted from 10^7 hybridoma cells using a PureLink RNA Mini Kit (Invitrogen). Approximately 2.5 μg of RNA was reverse transcribed into cDNA in 20 μL reactions using qScript cDNA supermix (Quantabio) according to the manufacturer's instructions. V_H and V_L amplicons were prepared by multiplex PCR using published framework region 1- and framework 4-specific primers[26] followed by a second "tagging" PCR to add MiSeq adapter and barcode sequences.[27] The resulting four barcoded amplicons were pooled and purified using a QIAquick Gel Extraction Kit (QIAGEN) followed by solid phase reversible immobilization using AMPure XP beads (Beckman Coulter). The amplicons were sequenced on a MiSeq instrument using a 500-cycle MiSeq Reagent Kit V2 (Illumina) with a 5% PhiX genomic DNA spike. Approximately 10^5 raw paired-end reads were generated for each amplicon. Following merging and quality filtering,[27] the most abundant unique nucleotide sequence present for each amplicon was taken as the rearranged sequence of the relevant hybridoma V_H or V_L domain.

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Assessment of Thermostability by Circular Dichroism (CD) Spectroscopy. A Jasco J-815 spectropolarimeter equipped with a Peltier thermoelectric type temperature control system (Jasco) was used to carry out CD experiments. A CD cuvette with a path length of 1 mm was used. IgGs were diluted to 200 μg mL^{-1} and also to 50 μg mL^{-1} in 10 mM phosphate buffer, pH 7.4. Both concentrations were used for melting temperature (T_m) determination, and each sample was run in triplicate. IgG unfolding was followed at a wavelength of 206.5 nm with a scanning speed of 100 nm min^{-1}, digital integration time of 0.25 s, bandwidth of 1 nm, data pitch of 0.2 nm, and an integration time of 1 s. To measure T_m,[28,29] CD spectra were recorded over a temperature range of 25 to 96 °C with a heating rate of 0.5 °C min^{-1} and a resolution of 0.2 °C. All IgG CD spectra were subtracted from a blank buffer spectrum. T_m's were obtained from the midpoint of the unfolding curve (raw mdeg values plotted versus temperature) produced from a nonlinear regression curve fit (Boltzman sigmoidal equation) using GraphPad Prism (version 4.02 for Windows).

Surface Plasmon Resonance (SPR). Immediately prior to SPR analysis, all antibodies were subjected to size exclusion chromatography (SEC) purification to remove potential aggregates and impurities. Approximately 200 μg of each mAb was passed over a Superdex 200 10/300 GL column (GE Healthcare) at a flow rate of 0.5 mL min^{-1} in PBS running buffer. Fractions of 1 mL were collected, pooled, and the absorbance measured at 280 nm to determine final mAb concentrations. SPR experiments were carried out by immobilizing the mAb on research grade CM5 sensor chips (GE Healthcare). Approximately 2500 resonance units (RUs) of the LTA-specific mAbs 4D6 and 6G9 or an irrelevant mAb, L3B5, specific for *Neisseria meningitidis* inner core LPS[27] (all at 25 μg mL^{-1}), were immobilized by amine coupling in 10 mM sodium acetate buffer, pH 4.5 (GE Healthcare). The remaining binding sites were blocked with a 7 min injection of 1 M ethanolamine at pH 8.5. All binding experiments were carried out on a Biacore 3000 instrument at 25 °C in PBS buffer at a flow rate of 20 μL min^{-1}. Surfaces were regenerated by washing with PBS buffer. All data were evaluated by fitting to 1:1 binding or steady state models using BIAevaluation 4.1 software (GE Healthcare).

To determine the monovalent binding affinity and kinetics of 6G9, a Fab fragment was prepared by enzymatic digestion using standard methods (kindly provided by Dr. Kenneth Ng, University of Calgary) and purified by SEC, as above, with the exception of approximately 400 μg were loaded onto a Superdex 75 Increase 10/300 GL column (GE Healthcare). Approximately 1250 RUs of LTA-human serum albumin (HSA) conjugate[18] were amine coupled to a CM5 sensor chip (10 μg mL^{-1} in 10 mM sodium acetate buffer, pH 4.0). An ethanolamine blocked surface and a surface with 1450 RUs of HSA served as references. Various concentrations of 6G9 Fab (50 nM − 2.4 μM) were then flowed over the surfaces at a flow rate of 40 μL min^{-1} for 120 s followed by 120 s of dissociation. The reference subtracted sensorgram was evaluated using a steady state binding model (BIAevaluation 4.1 software).

ELISA. The binding of anti-*C. difficile* LTA mAbs to LTA and to *C. difficile* was evaluated by ELISA.

LTA and Synthetic LTA ELISA. Wells of Nunc Maxisorp EIA plates were coated with 1 μg of LTA or LTA synthetic fragments in PBS overnight at 4 °C and then brought to RT before use. Plates were blocked with 1% bovine serum albumin (BSA)−PBS for 1 h at RT. Wells were washed three times with PBS−0.05% Tween 20 (PBS-T), and incubated with supernatant or purified mAb for 1 h at RT. Following washing with PBS-T, alkaline phosphatase (AP)-labeled goat anti-mouse IgG (Cedarlane Laboratories) diluted 1:2500 in 1% BSA−PBS was added for 1 h at RT. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min, absorbance was measured at 405 nm using a microtiter plate reader.

Whole Cell ELISA. Wells of Nunc Maxisorp EIA plates were coated with 100 μL of phenol-killed bacteria (absorbance at 620 nm of 0.080) in dH_2O overnight in a 37 °C drying oven and then brought to RT before use. Plates were blocked with 1% BSA−PBS for 1 h at RT. Wells were washed three times with PBS-T, and incubated with supernatant or purified mAb for 3 h at RT. Following washing with PBS-T, AP-labeled goat anti-mouse IgG (Cedarlane Laboratories) diluted 1:2500 in 1% BSA-PBS was added for 1 h at RT. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min, absorbance was measured at 405 nm using a microtiter plate reader.

Epitope Mapping. *LTA Inhibition Whole Cell ELISA.* Wells of Nunc Maxisorp EIA plates were coated with 100 μL of phenol-killed bacteria (absorbance at 620 nm of 0.080) in dH_2O overnight in a 37 °C drying oven and then brought to RT before use. Plates were blocked with 1% BSA−PBS for 1 h at RT. Wells were washed with PBS-T. MAbs were preincubated with PBS or various concentrations of purified natural LTA for 1 h before being added to the plates and incubated for 3 h at RT. Following washing with PBS-T, AP-labeled goat anti-mouse IgG (Cedarlane Laboratories) diluted 1:2500 in 1% BSA−PBS was added for 1 h at RT. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min, absorbance was measured at 405 nm using a microtiter plate reader.

mAb Competition ELISA. Wells of Nunc Maxisorp EIA plates were coated with 1 μg of LTA in PBS overnight at 4 °C or with 100 μL of phenol-killed bacteria (absorbance at 620 nm of 0.080) in dH_2O overnight in a 37 °C drying oven and then brought to RT before use. Plates were blocked with 1% BSA−PBS for 1 h at RT, and then wells were washed with PBS-T. One mAb was titrated and added to the plate for 1 h at RT (for LTA antigen or 3 h for whole cells). Following washing with PBS-T, the second mAb was added at a predetermined concentration and allowed to incubate for 1 h at RT (for LTA antigen or 3 h for whole cells). Following washing with PBS-T, AP-labeled goat anti-mouse IgG3 or IgG2b (Southern Biotech) diluted 1:250 in 1% BSA−PBS was added for 1 h at RT. The plates were then washed and developed with Phosphatase Substrate System (Kirkegaard and Perry Laboratories). After 60 min, absorbance was measured at 405 nm using a microtiter plate reader.

Synthesis of LTA Molecules. Synthetic LTA molecules used in this study were prepared as described previously.[25]

Synthesis of HSA−LTA Conjugate. The HSA−LTA conjugate was prepared via standard direct reductive amination chemistry. Briefly, LTA was deacylated as described previously.[18] The deacylated LTA (45 mg) was dissolved into 0.05 M sodium acetate buffer at pH 7.5 at ∼10 mg mL^{-1} and 4.5 mL of 50 mM sodium metaperiodate was added, and after pH was adjusted to 5.6 with 1 M NaOH, it was left for 30 min at RT in the dark. Three milliliters of ethylene glycol was added and incubated in the dark for a further 45 min. The oxidized polymer was purified on a Sephadex G-25 size exclusion column obtaining 40 mg.

Conjugation. The oxidized polymer (40 mg) and HSA (10 mg) were mixed and left at RT before lyophilizing. The lyophilized material was dissolved in 10 mL of 100 mM $NaPO_4$ and $NaCNBH_3$ (5 mg mL^{-1}) was added and left at RT for 16 h. The conjugate was purified on a 30 kDa molecular weight cutoff spin column in PBS with 10 mM sodium citrate, and the supernatant was assayed for protein and filter sterilized and frozen at −20 °C. An aliquot was examined by MALDI-MS as described previously.[18]

Production of Chimeric mAb 6G9. The 6G9 mAb was cloned and expressed as a chimeric antibody. Briefly, the variable domains (V_L and V_H) of the 6G9 murine mAb were fused to the constant domains of human κ light chain and human IgG1 heavy chain, respectively. The resulting DNA sequences were synthesized and cloned into the pTT5 mammalian expression vector by GeneArt (Thermo Fisher), expressed in HEK293-6E cells, and purified via protein A affinity chromatography as previously described.[30]

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Notes

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