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Production and Characterization of Monoclonal Antibodies to E1 Tor Toxin Co-Regulated Pilus of *Vibrio cholerae*

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ABSTRACT

Murine monoclonal antibodies (MAbs) against *Vibrio cholerae* toxin co-regulated pilus (TCP) were generated using conventional hybridoma procedures. Four hybridomas were obtained and two characterized. Hybridomas 10E10E1 and 4D6F9 secreted antibodies of the IgG2a and IgG1 isotypes, respectively, that reacted with a 24-kDa antigen corresponding to the product of the El Tor *tcpA* gene fused to a six Histidine tail. Additionally, MAbs produced by 4D6F9 selectively recognized the major pilin subunit (TcpA) of El Tor and O139 vibrios in western immunoblot, while MAbs from 10E10E1 also cross-reacted with classical TcpA. Furthermore, vibrios expressing TCP on their surface selectively inhibited binding of the antibodies secreted by both hybridomas to TcpA-coated microtiter plates. Thus, the MAbs reported in this work detected the structural subunit of the pilus either denatured or assembled on the bacterial surface.

INTRODUCTION

Cholera is a diarrheal disease caused by the action of cholera toxin, which induces a massive flux of fluid into the intestinal lumen of people infected with *Vibrio cholerae* of serogroups O1 and O139.[1] Colonization of human small intestine is a crucial event in *V. cholerae* pathogenesis. There are several bacterial surface components that have been recognized as potential adhesion or colonization factors, such as the O-antigen of the lipopolysaccharide, outer membrane proteins and pili.[2–6] Among all pili studied from this microorganism, toxin co-regulated pilus is the only one demonstrated as essential for colonization in mice and humans.[7–9] TCP is expressed on the surface of O1 (classical and El Tor biotypes) and O139 vibrios by assembling TcpA, a 20.5-kDa antigen encoded by the *tcpA* gene, which is located within a cluster of type IV pilin genes.[10–12] More recently, TcpA and its variants have been identified in non-O1/non-O139 *V. cholerae* strains.[13–15] In addition to the colonization ability of this pili, TCP is the receptor of cholera toxin phage (CTXϕ),[16] and it has been postulated that the *tcp* locus can, in fact, be mobilized as a single stranded genome within a TcpA coat, forming the *V. cholerae* pathogenicity island phage (VPIϕ).[17] Polyclonal and monoclonal antibodies have been generated to study the characteristics of this molecule,[18–21] but most of them are specific to classical TcpA. As far as we know, no monoclonal antibodies have yet been identified that only recognize the El Tor TcpA. The generation of El Tor specific anti-TCP MAbs should facilitate studying the divergent regions of this molecule, which could offer a better epitope mapping of the protein and potentially provide a method for distinguishing the different pilin types among *V. cholerae* isolates.

We report the generation of hybridomas producing MAbs that recognize El Tor TcpA-coated microtiter plates. Two selected clones recognize a 20.5-kDa protein that assembled in the vibrios surface selectively inhibiting the activity of both MAbs.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Strains used in this work (Table 1) were conserved at −70°C in 10% skim milk (Oxoid, Hampshire, England) containing 20% (v/v) glycerol. The AKI procedure[22] was used to maximize TCP expression, briefly: a *V. cholerae* colony, from a blood agar plate incubated overnight at 37°C, was inoculated in a cylinder

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TABLE 1. STRAINS USED IN THIS STUDY

Strain	Relevant genotype and/or phenotype	Reference
V. cholerae		
N16961	Wild type, O1, El Tor, Inaba, Bangladesh 1975	(28)
C6706	Wild type, O1, El Tor, Inaba, Peru 1991	(29)
KHT52	*str2, ΔtcpA10* derivative of C6706	(8)
C7258	Wild type, O1, El Tor, Ogawa, Peru 1991	(29)
569B	Wild type, O1, Classical, Inaba, Calcutta, 1945	(28)
SG25-1	Wild type, O139, Calcutta, 1993	(30)
E. coli		
XL1-blue	*Tn 10 proA$^+$B$^+$, lacI^q (lacZ), M15/recA1 endA1 gyrA96(Nal^r) thi hsdR17 $r_k^-m_k^+$)supE44 relA1 lac*	(31)
XL-TCP	XL1-Blue with tac-*tcpA* on a plasmid vector	This study

containing 10 mL of sodium chloride 0.5%, bacto peptone 1.5%, and yeast extract 0.4%. Just before inoculation, 0.4 mL of sodium bicarbonate solution (4%) was added to the tube and incubated at 30°C. After 4 h, the culture was transferred to an erlenmeyer and incubated with shaking at 30°C for another 16 h.

Cloning of tcpA gene from V. cholerae

Chromosomal DNA from the *V. cholerae* El Tor, Inaba, N16961 strain was prepared as previously described.[23] Purified DNA was used as template in PCR to amplify the entire *tcpA* coding region flanked by *Bam*HI and *Eco*RI restriction sites. PCR amplification was performed with 1 μL of DNA in a 100-μL reaction volume containing 0.2 mM deoxynucleotide triphosphate, 2 mM $MgSO_4$, 0.5 μM of each sense and antisense primers, and 2 U of Vent DNA polymerase; all reagents for PCR were purchased from New England BioLabs (Bishop's Stortford Herts, U.K.). The PCR profile included 94°C denaturalization for 5 min, followed by 30 cycles of denaturalization at 94°C for 1 min, annealing at 54°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 5 min. The primers used were CNC 6048 sense, 5′ CAG GAT CCT ATG ACA TTA CTC GAA G 3′ and CNC 6050 antisense, 5′ CTT AAG AAT TCG CCC ATT TCC ATG G 3′. The amplified *tcpA* gene was purified using the PCR band purification Kit (Amersham Bioscience, Piscataway, NJ). The extracted DNA was digested *Bam*HI and cloned *Bam*HI-*Sma*I into the pQE-31 vector system (Qiagen Inc., Valencia, CA) to fuse a six histidine-tag coding region to the *N*-terminal portion of *tcpA*. The resultant plasmid (pQETCP8) was introduced into *E. coli* XL1-blue strain by electroporation for recombinant *tcpA* gene expression.

Preparation of purified TCP antigen

The *E. coli* strain transformed with pQETCP8 (XL-TCP) was grown in Luria-Bertani medium supplemented with 100 μg/mL of ampicillin and the expression of TcpA was induced by adding 2 mM of isopropil-β-D-thiogalactopyranoside to the culture that was incubated with shaking at 37°C for 10 h. A crude preparation of El Tor TcpA, fused to a six histidine tail, was obtained from sonicated cells in phosphate-buffered saline (PBS) plus urea 8 M. The soluble proteins were fractionated in a Histidine affinity column HisTrap (Amersham Pharmacia, Uppsala, Sweden), to recover the purified TcpA, following manufacturer's instructions.

Direct ELISA for detection of anti-TcpA antibodies

Polystyrene microtiter ELISA plates (Costar, Cambridge, MA) were coated overnight at 4°C with 100 μL per well of purified recombinant TcpA at a concentration of 5 μg/mL in PBS. The wells were subsequently filled with 2% skim milk solution in PBS and incubated for 1 h at room temperature (RT) to reduce non-specific binding. Samples, consisting of serial dilution of sera or culture supernatants, were added and incubated for 2 h at RT. Plates were washed with 0.05% Tween-20 in PBS (PBS-T) and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co., St. Louis, MO). Color was de-

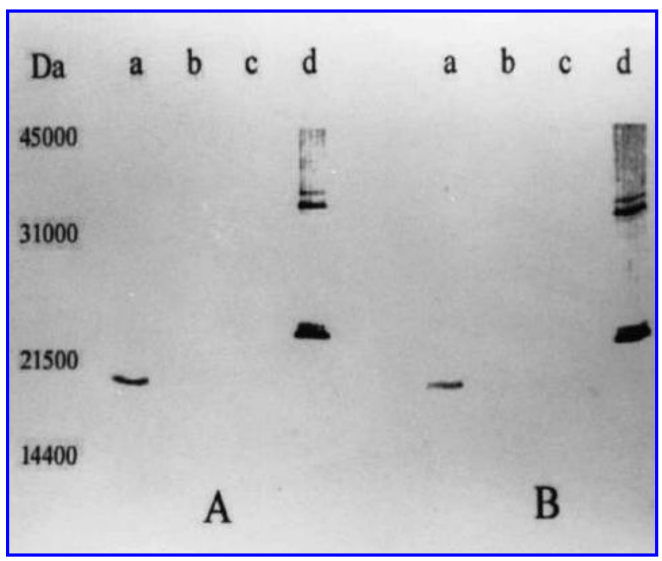

FIG. 1. Immunoblot analysis of *V. cholerae* and *E. coli* cell lysates with MAb 10E10E1 (**A**) and MAb 4D6F9 (**B**). Lane a, C6706; lane b, KHT52; lane c, *E. coli* XL1-blue; lane d, *E. coli* XL-TCP. Samples were boiled in the presence of SDS. A broad range molecular weight standards kit (BioRad, CA) was run together with samples and molecular weights indicated in Daltons (Da).

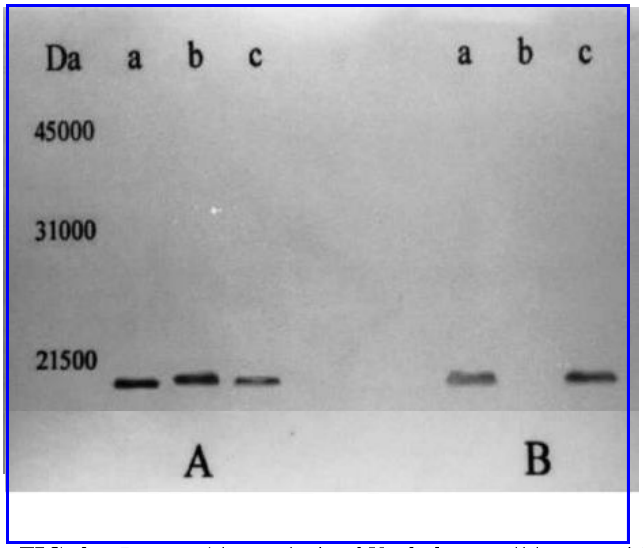

FIG. 2. Immunoblot analysis of *V. cholerae* cell lysates with MAb 10E10E1 **(A)** and MAb 4D6F9 **(B).** Lane a, C7258; Lane b, 569B; Lane c, SG25-1. Samples were boiled in the presence of SDS. A broad range molecular weight standards kit (Bio-Rad, CA) was run together with samples and molecular weights indicated in Daltons (Da).

veloped with *o*-phenylenediamine (0.4 mg/mL) and 0.4% H_2O_2 in 0.1 M sodium citrate buffer, pH 5.0. The reaction was stopped by adding 2.5 N H_2SO_4 and the $O.D._{492nm}$ read with a plus-multiscan microplate reader (Labsystem, Hampshire, U.K.).

Production of MAbs

Ten BALB/c mice were immunized by SC injection of recombinant TcpA (20 μg) emulsified in Freund's adjuvant (Sigma Chemical Co.). Each animal received four injections administrated at 2-week intervals. Mice were bled by their tail veins 7–10 days after the final injection and their serum tested for anti-TcpA antibodies in the direct ELISA described above. At 3–4 days before cell fusion, the appropriate mouse received a final injection of antigen (10 μg) in PBS. Splenocytes were fused with the SP_2/O myeloma cell line using polyethylene glycol 1300 Hybri-Max (Sigma Chemical Co.) as described by Campbell.[24] The hybrid cells were screened for their ability to secrete antibodies binding El Tor TcpA in the direct ELISA. Four hybrid-secreting reactive antibodies were subcloned by limiting dilution and stabilized. Two clones, coded 4D6F9 and 10E10E1, were further characterized. The selected hybridoma cells were grown as ascites in the peritoneal cavity of pristane-primed BALB/c mice. Ascites fluid was tapped from the peritoneal cavity and rendered cell-free by centrifugation at $\times$1,000 g for 15 min at 4°C. The MAbs were purified from ascites fluid using protein A affinity chromatography.[25]

Isotyping

The classes and subclasses of MAbs secreted by 4D6F9 and 10E10E1 were determined with an ImmunoType Kit (Sigma Chemical Co.) following manufacturer's instructions.

Immunoblot analysis of MAbs

Lysates of pelleted *V. cholerae* cells (2.4 O.D. $_{600nm}$ units) were fractionated in 13.5% SDS-PAGE according to Laemmli[26] and transferred to nitrocellulose paper as described by Towbin.[27] After blocking with 5% skim milk in PBS, the blot was reacted with a suitable dilution of MAbs 4D6F9 and 10E10E1. The membranes were washed with PBS-T and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co.). Color was developed with 4-

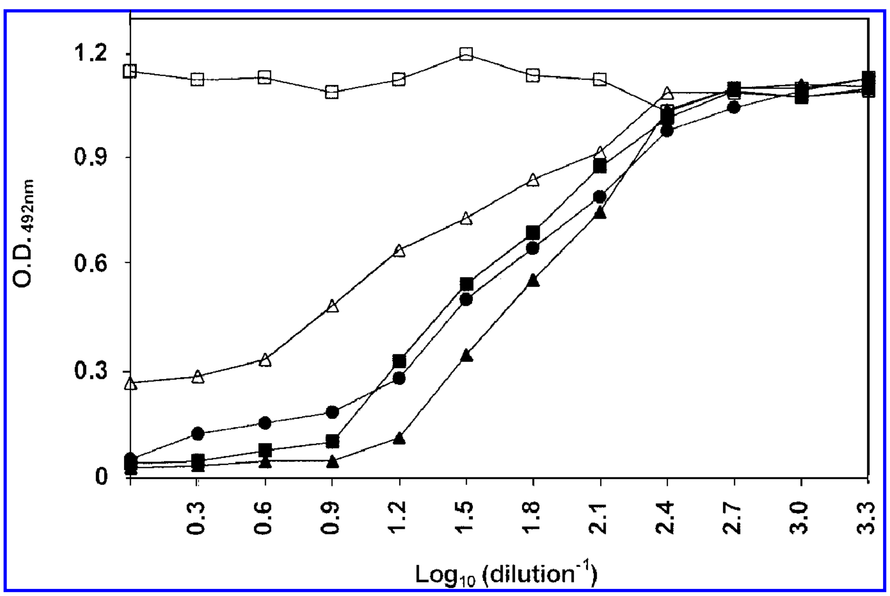

FIG. 3. Inhibition of MAb 10E10E1 binding by a recombinant TcpA preparation (r-TcpA) and classical, El Tor or O139 vibrios. The following preparations were tested for inhibition: r-TcpA (●), strain C6706 cells (■), 569B cells (▲), KHT52 cells (□), SG25-1 cells (Δ). Each experimental point plotted is the average of three determinations.

chloro-1-naphthol (0.2 mg/mL) (Sigma Chemical Co.) and 0.4% H_2O_2 in tris-buffered saline, pH 8.0.

Competition ELISA for detection of assembled TcpA

Several *V. cholerae* strains (10^9 cell/mL) or recombinant TcpA (25 μg/mL) were serially (twofold) diluted in PBS-T containing a suitable dilution of MAbs 4D6F9 or 10E10E1. After 1-h incubation at RT, the mixtures were transferred to recombinant TcpA-coated, skim milk blocked, microtiter plates and incubated for another 2 h. Plates were washed with PBS-T and incubated with peroxidase-conjugated anti-mouse IgG (whole molecule) (Sigma Chemical Co.). Color was developed as in direct ELISA.

RESULTS

Production of MAbs

Hybrids producing anti-TcpA antibodies were screened by a direct ELISA and four clones selected on the basis of their highest reactivity against recombinant El Tor TcpA. Two hybridomas and their MAbs were coded 10E10E1 and 4D6F9, which secreted antibodies of the IgG2a and IgG1 isotypes, respectively. These MAbs were purified from ascites fluid by protein A affinity chromatography.[25] The approximate quantities of MAbs secreted by 10E10E1 and 4D6F9 hybridomas as estimated from the yield of the purification process were 4.3 and 2.2 mg/mL, respectively.

Immunoblot analysis of MAbs

The specificity of anti-TcpA MAbs was investigated by immunoblot analysis of *E. coli* and *V. cholerae* cell lysates. Figures 1 and 2 show that MAb 10E10E1 recognized a 20.5-kDa antigen in all wild-type strains of *V. cholerae* examined (Fig. 1A, lane a; Fig. 2A, lanes a–c), and MAb 4D6F9 reacted with the same protein of El Tor biotype and O139 serotype vibrios (Fig. 1B, lane a; Fig. 2B, lanes a and c), but not with the classical strain of *V. cholerae* analyzed (Fig. 2B, lane b). No immunoreactive material could be detected with any of the MAbs in mutant KHT52, which contains an in-frame deletion in *tcpA*,[8] the structural gene of the major pilin subunit (Fig. 1A, lane b; Fig. 1B, lane b) or in *E. coli* XL1-blue strain (Fig. 1A, lane c; Fig. 1B, lane c). In strain XL-TCP, the *tcpA* gene is expressed from the strong tac promoter on a plasmid vector in *E. coli* XL1-blue. Upon induction of XL-TCP, both MAbs recognized a strong protein band of somewhat higher molecular weight (Fig. 1A, lane d; Fig. 1B, lane d).

Competition ELISA for detection of assembled TcpA

We have developed a competitive ELISA test based on inhibition of binding of anti-TcpA MAbs to an immobilized antigen by intact TCP expressing vibrios. Purified El Tor recombinant TcpA inhibited binding of MAbs 10E10E1 and 4D6F9 (Figs. 3 and 4). Strains positive by western blot analysis (Figs. 1 and 2) were also inhibitory for both MAbs in the competition ELISA (Figs. 3 and 4). No inhibition was observed (Figs. 3 and 4) by strains diagnosed as negative in the immunoblot test for each MAb (Figs. 1 and 2).

DISCUSSION

We have produced four MAbs against TcpA and characterized two of them. Western blot analysis (Fig. 1) showed that MAbs 10E10E1 and 4D6F9 recognized a 20.5-kDa antigen, produced by El Tor strain that closely corresponds to the molecular mass predicted for the mature product of the *tcpA*

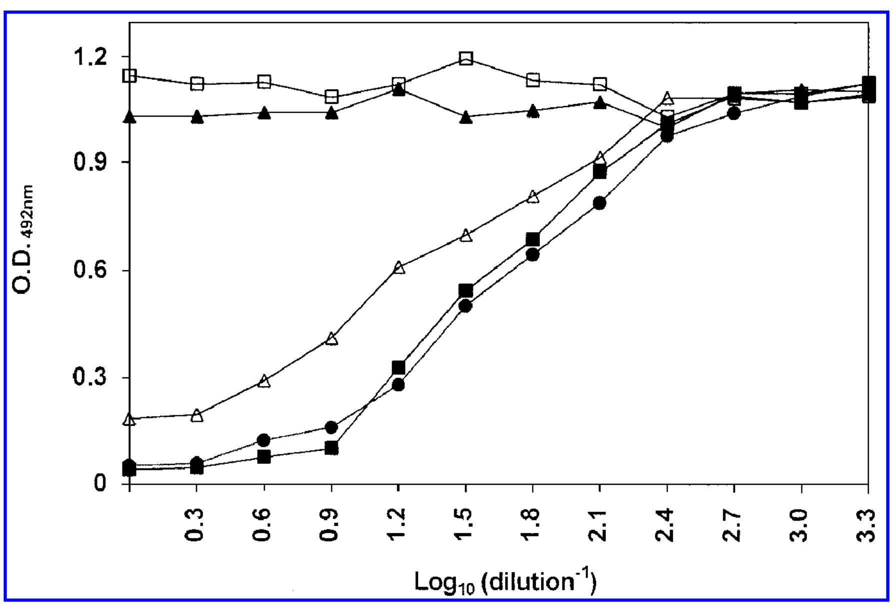

FIG. 4. Inhibition of MAb 4D6F9 binding by a recombinant TcpA preparation (r-TcpA) and classical, El Tor or O139 vibrios. The following preparations were tested for inhibition: r-TcpA (●), strain C6706 cells (■), 569B cells (▲), KHT52 cells (□), SG25-1 cells (Δ). Each experimental point plotted is the average of three determinations.

gene.[10] The antibodies did not cross-react with mutant KHT52, an isogenic derivative strain of C6706 with an in-frame deletion in the *tcpA* gene[8] nor with the *E. coli* strain XL1-blue. Expression of the *tcpA* gene product from the tac promoter in *E. coli* XL-TCP produced an antigen that reacted with both MAbs. This protein was larger than the mature antigen detected in *V. cholerae* lysates, with an estimated molecular weight of 24 kDa. The higher molecular weight of recombinant protein (r-TcpA) is due to a six-histidine tail added to the TcpA mature protein. MAbs 10E10E1 and 4D6F9 varied in their reactivities with the TcpA subunit of El Tor and classical vibrios, as tested in immunoblot analyses; MAb 10E10E1 reacted with the same strength with both classical and El Tor TcpA, and also reacted well with O139 TcpA (Fig. 2A); MAb 4D6F9, on the other hand, did not react with TcpA of classical vibrios and thus seems to be El Tor biotype specific (Fig. 2B). The *tcpA* sequence from El Tor and O139 vibrios are identical but show significant deviation from the classical-biotype gene, especially in the portion encoding the C-terminal region of the pilin,[12] where epitope recognized by monoclonal antibody 4D6F9 should map.

We have designed a competitive ELISA based on inhibition of binding of anti-TCP MAb to TcpA-coated plates. Four *V. cholerae* strains were evaluated for their ability to inhibit binding of MAbs to TcpA-coated plates. Strains positive in western blot analysis (Figs. 1 and 2) selectively inhibited binding of both MAbs at a concentration of 10^9 cells/mL (Figs. 3 and 4). Strain KHT52 failed to inhibit the activity of both MAbs (Figs. 3 and 4), while the classical strain 569B did not reduce binding of 4D6F9 (Fig. 4). Thus, the MAbs reported in this work detected the structural subunit of the pilus either denatured (Figs. 1 and 2) or assembled on the bacterial surface (Figs. 3 and 4). These results suggest that MAbs 10E10E1 and 4D6F9 might recognize lineal rather than conformational epitopes. This is the first report of a Mab specific to El Tor TcpA, which efficiently recognizes the TCP pilus. The production of new MAbs facilitates studying the structure and function of this molecule in greater detail. The El Tor–specific Mab generated in this work should provide a valuable tool to study the epitope differences among classical and El Tor TCP, and will facilitate the characterization of clinical isolates.

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