CONCISE COMMUNICATION

Human Antibody Response to Longus Type IV Pilus and Study of Its Prevalence among Enterotoxigenic Escherichia coli in Bangladesh by Using Monoclonal Antibodies

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Mouse monoclonal antibodies (MAbs) were derived against longus (CS20), a type IV pilus expressed by human enterotoxogenic Escherichia coli (ETEC). One MAb (ICA39) detected longus in 56 (8.5%) of 662 ETEC isolates obtained from a routine surveillance of diarrheal stools from children and adults. Five patients with diarrhea from whom longus-positive ETEC were isolated were also recruited. Of these 61 isolates, 50 were positive for other colonization factors (CFs; 61% for CFA/I and 21% for CFA/II), and 11 were negative for any of the other 8 CFs that were tested. They were either positive for the heat-stable enterotoxin (ST; n = 29) or for the heat-labile enterotoxin (LT) and ST (n = 32). All longus-positive ETEC were confirmed by polymerase chain reaction to harbor lngA, the longus structural pilin gene. Sera and/or fecal extracts from the patients reacted with the 22-kDa pilin polypeptide in immunoblots and ELISA. These studies show that longus is prevalent among ETEC in Bangladesh and that longus gives rise to IgA antibody responses in patients.

The colonization factors (CFs) of enterotoxigenic Escherichia coli (ETEC) are known to be important virulence determinants that mediate attachment to the epithelium of the small intestine [1]. A plasmid-encoded type IV pilus antigen (CS20) has been identified in human ETEC isolates [2]. Termed longus because of its characteristic length, it is composed of structural subunits of 22 kDa. It bears similarities in the N-terminal amino acid sequence to other type IV pili, such as the toxin-coregulated pilus of Vibrio cholerae and the bundle-forming pilus of enteropathogenic E. coli [2].

Several epidemiological studies conducted in endemic areas of ETEC infections revealed that immunity against ETEC is achieved through anti-pili or anti–heat labile enterotoxin (LT) IgA antibodies. It has been suggested that a multifimbria-based vaccine could induce protective immunity in diarrhea-endemic areas [1, 3–5]. Thus it is important to identify the most common adhesive factors in areas of the world where ETEC represents a major health problem.

To study the prevalence of longus-producing ETEC in Bangladesh, we have produced specific mouse monoclonal antibodies (MAbs) and used MAb ICA39 to examine the frequency at which longus is expressed in ETEC isolates obtained from stools of patients [6]. We confirmed this by polymerase chain reaction (PCR). The coexpression of longus with other CFs and enterotoxins was also studied. In addition, we investigated whether longus elicits specific IgA antibodies in the course of natural infections.

Methods

Bacterial strains, media, and antigen. Bacterial strains included in this study are listed in table 1. ETEC E9034A (O8:H9) produces longus and was used as a prototype strain. OG140 is an E. coli K12 DH5α that harbors longus structural gene (lngA) in a recombinant plasmid (pSK) that expresses the 22-kDa pilin subunit [7]. As negative controls, DH5α, which carries plasmid pSK, and E9034P, a longus-minus derivative of E9034A, were used. Control ETEC strains known to express 12 other CFs were used to test the specificity of the antibodies (table 1). To study the prevalence of longus-producing ETEC, we analyzed 662 isolates from our collection that were obtained from stools of diarrheal patients enrolled in the 2% systemic routine surveillance system of the International Centre for Diarrhoeal Disease Research, Bangladesh, over a 2-year period from September 1996 to August 1998 [6]. For the detection
of longus, cultures were plated onto trypticase soy agar (Gibco, Grand Island, NY) containing 5% defibrinated sheep blood (TSAB) [2]. For expression of other CFs, strains were cultured on CFA agar [8]. LT and heat-stable enterotoxin (ST) were detected by a GM1-based ELISA. For expression of other CFs, strains were cultured on CFA agar [8]. LT and heat-stable enterotoxin (ST) were detected by a GM1-based ELISA.

Production of specific mouse MAb s to longus. Female BALB/c mice were immunized with 10 μg of purified longus from strain E9034A 4 times at weekly intervals, as described elsewhere [9]. Spleen cells from 2 immunized BALB/c mice were fused with SP2/O myeloma cells. Supernatant fluids were screened for reactivity against the longus antigen by ELISA [9]. To test the specificity of the MAbs, we studied 3 strains each of E. coli, K-12; and Shigella dysenteriae, S. flexneri spp.; Salmonella typhi; enteroinvasive, enteropathogenic, and enteroaggregative E. coli; E. coli K-12; and Neisseria gonorrhoeae. In brief, 2 L of bacterial suspension (corresponding to tube number 9 in the McFarland scale) was applied to strips of nitrocellulose filter paper (Sigma, St. Louis). The strips were probed with MAb ICA39 (culture supernatant at 1 : 10 dilution or ascites at 1 : 1000 dilution). All isolates were also tested for other CFs by using specific MAbs [6]. Both longus-positive (E9034A) and -negative (E9034P) control strains were included (table 1). Purified longus and wholecell lysates of bacteria were also analyzed by using ICA39 for immunoblotting [10].

Immunoblotting of longus and other CFs on ETEC. Bacteria were analyzed for production of pili by immunodot blot assay [6], in which 2 L of bacterial suspension (corresponding to tube number 9 in the McFarland scale) was applied to strips of nitrocellulose filter paper (Sigma, St. Louis). The strips were probed with MAb ICA39 (culture supernatant at 1 : 10 dilution or ascites at 1 : 1000 dilution). All isolates were also tested for other CFs by using specific MAbs [6]. Both longus-positive (E9034A) and -negative (E9034P) control strains were included (table 1). Purified longus and wholecell lysates of bacteria were also analyzed by using ICA39 for immunoblotting [10].

PCR for detection of longus. To confirm the reactivities of ICA39, we analyzed ETEC isolates by PCR for the presence of the longus gene lnuA [7] by using oligonucleotide primers JG1 (5′-CGGAATTCCATGAGCCTGCTGGAAGTTATCA-3′) and JG2 (5′-CGGAATTCCCGCTACCTAAAGTAATTGAGT-3′) [11]. The resulting 0.63-kbp amplicon was resolved by 1.5% agarose gel electrophoresis.

Immune response to longus in patients. The presence of anti-longus IgA antibodies was studied by ELISA and by immunoblot in sera and feces obtained from 5 patients at the acute stage and in convalescence (table 2) [12]. In addition, fecal extracts obtained at the acute stage from 27 of the 56 patients from whom longus-positive ETEC were isolated were studied by immunoblot. Sera and fecal samples from 12 healthy children (aged 2–5 years) with no history of diarrhea for the last 3 months were also tested. The cutoff value for a positive response to longus was the geometric mean + 2 SD above the mean (titer >1096). A seroconversion was defined as a ≥2-fold increase from the titer observed at the acute stage. Both sera (1 : 30 dilution) and fecal extracts (1 : 10 dilution) were reacted with purified longus in immunoblots.

Results
Reactivity and specificity of longus MAbs. Five MAbs (ICA39, ICA40, ICA41, ICA43, and ICA47) were specific for longus and gave strong reactions in ELISA and in immunoblots. ICA39 and ICA40 were of the IgG2b isotype, ICA41 and ICA43 were IgG2A isotypes, and ICA47 was an IgG1 isotype. These MAbs reacted in dot blot assays with OG140 and ETEC strains (E9034A, B2C, and M47C4) known to produce longus (table 1) and did not react with strains that did not express longus. No cross-reactions were seen with other enteric pathogens, including other diarrheogenic E. coli or other type IV pili-expressing bacteria. ICA39 was chosen for further studies.

Prevalence of longus in ETEC isolates. Longus was detected in 56 (8.5%) of the 662 isolates studied from the collection of ETEC isolates. In addition, 5 more longus-positive ETEC were isolated from patients who were recruited in the study (table 2). Of the 61 ETEC isolates, 35 (57%) were from children aged 0–5 years (median age, 12 months) and 26 (43%) were from the older age group (aged ≥5–65 years; median age, 25 years). Of the total 61 longus-positive isolates, 50 (82%) were positive for some other CF (table 1). These were isolated from patients with mild (51%), moderate (31%), or severe diarrhea (18%) and in approximately similar frequency from adults as from chil-
of a complete correlation between the ICA39-based immunoblot and PCR. Longus-negative ETEC were also not detected by PCR, confirming the specificity. Thus, ICA39 can be used to screen longus-positive ETEC isolates. This will be useful when equipment or costly reagents to perform DNA amplification or DNA hybridization techniques to screen for lngA are not available. ICA39 can also be used as an accessory test for detection of longus-producing ETEC.

Longus was only associated with bacteria expressing CFA/I and CFA/II, although not all ETEC expressing these CFs produced longus. We have shown that CFA/I and coli surface antigens of CFA/II (CS2 and CS3), together with coli surface antigens of CFA/IV (CS4, CS5, and CS6), are the most prevalent in Dhaka, Bangladesh [6]. When ETEC isolates from different geographic regions were tested for the presence of lngA gene by DNA hybridization techniques, longus was found to be mainly associated with ETEC producing CFA/I, followed by ETEC producing CFA/I and CFA/IV [13]. However, none

### Table 2. IgA antibody responses to longus among patients, as determined by ELISA and immunoblot.

<table>
<thead>
<tr>
<th>Patient (age)a</th>
<th>ETECb isolate (CF and toxin)</th>
<th>ELISA (serum)c</th>
<th>Immunoblot (serum)d</th>
<th>Immunoblot (fecal extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Acute</td>
<td>Early</td>
<td>Late</td>
</tr>
<tr>
<td>Patient 1 (40 years)</td>
<td>CS1 + CS3 (LT/ST)</td>
<td>+ (8061)</td>
<td>+ (80145)*</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 2 (6 months)</td>
<td>CS1 + CS3 (LT/ST)</td>
<td>+ (640)</td>
<td>+ (1969)*</td>
<td>+ (5741)</td>
</tr>
<tr>
<td>Patient 3 (1 year)</td>
<td>CFA/I (ST)</td>
<td>+ (2671)</td>
<td>+ (2454)</td>
<td>+ (1378)</td>
</tr>
<tr>
<td>Patient 4 (16 months)</td>
<td>CFA/I (ST)</td>
<td>+ (5546)</td>
<td>+ (5616)</td>
<td>ND</td>
</tr>
<tr>
<td>Patient 5 (5 months)</td>
<td>CFA/I (ST)</td>
<td>+ (2036)</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

**NOTE.** ETEC, enterotoxigenic *Escherichia coli*; CF, colonization factor; LT, heat-labile enterotoxin; ST, heat-stable enterotoxin. *Positive IgA response to longus (>geometric mean + 2 SD of values [titer >1096]) determined in healthy children; ND, not determined because patient did not return for follow-up visits and samples were not collected; ++ and, relative intensity of the longus band detected by immunoblot analysis. *Seroconversion at convalescence.

a Patients infected with longus-positive ETEC.

b ETEC isolates were positive for longus and some other CFs. The toxin pattern is also shown.

c Samples from patients were collected after diagnosis and recruitment in the study (acute stage, day 2 or 3 after hospitalization) and at follow-up visits 7–9 days (early convalescence) and 30 days after hospitalization.
of the 114 CFA/IV+ isolates tested here expressed longus [6]. We did not detect longus on isolates that expressed the less prevalent CFs, such as CS8 and CS12, although isolates with these phenotypes from other geographic locations have been shown to harbor the lngA gene [13]. Longus was also not detected on those isolates that expressed other CFs, such as CS7, CS14, or CS17.

We have recently shown that 44% (n = 292) of the ETEC isolates from a systematic collection did not produce any of the known CFs [6]. Of these isolates, 11 expressed longus alone, bringing the prevalence of CF-positive ETEC from 56% to 58%. It was more common to find longus expressed together with other CFs. These data support the view that longus may be encoded alone or together with other CF antigens, suggesting an important role as an adhesive determinant or as an accessory CF contributing to the adhesive attributes of ETEC [13].

Several studies have demonstrated a strong association between the presence of CFs and toxin types [14]. Longus was detected in ETEC with ST and LT/ST phenotype, as shown elsewhere [13]. We tested 168 ETEC isolates that were only positive for LT, and none expressed longus. The strong association between ST and longus suggests that they are encoded on the same plasmid or, alternatively, that the plasmids harboring these genes may be compatible with each other and thus may coexist.

The longus-positive ETEC were isolated from patients with mild, moderate, or severe diarrhea and from adults and children, showing that both are susceptible to infections with longus-positive ETEC. However, other CFs were mostly coexpressed on the bacteria; therefore, the susceptibility to the infection cannot be correlated with the expression of longus alone. Studies of ETEC infections, as well as studies of experimental infections, have shown that protective antibodies against CFs and LT subunit B are elicited after the onset of diarrhea [4, 5]. In addition, the presence of antibodies against virulence determinants in diarrheogenic E. coli may be considered a marker of the production of the antigen in vivo [15]. All 5 patients who shed longus-positive ETEC showed α-longus IgA antibodies in the local and systemic circulation. Specimens from patients at the acute stage of the disease detected longus, which is understandable because these specimens were collected ~6–7 days after onset of diarrhea. In addition, not all patients showed a seroconversion to longus, because the response at the acute stage was already elevated. The longus antigen was also detected in specimens from healthy children, suggesting an asymptomatic infection in the recent past. The stimulation of an IgA antibody response in the blood and in the intestine suggests that longus is capable of generating a specific immune response in disease. However, whether it is protective against further infections is an issue that needs to be addressed.

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References